Mycobacterium ulcerans infections cause progressive muscle atrophy and dysfunction and mycolactone impairs satellite cell proliferation

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Short running head
Buruli ulcer and skeletal muscle atrophy

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Abstract

Clinical observations from Buruli ulcer (BU) patients in West Africa suggest that severe *Mycobacterium ulcerans* (*M. ulcerans*) infections can cause skeletal muscle contracture and atrophy leading to significant impairment in function. In the present study, male mice C57BL/6 were subcutaneously injected with *M. ulcerans* in proximity to the right biceps muscle, avoiding direct physical contact between the infectious agent and the skeletal muscle. The histological, morphological, and functional properties of the muscles were assessed at different times after the injection. On day 42 post-injection, the isometric tetanic force and the cross-sectional area of the myofibers were reduced by 31% and 29%, respectively, in the proximate-infected muscles relative to the control muscles. The necrotic areas of the proximate-infected muscles had spread to 7% of the total area by day 42 post-injection. However, the number of central nucleated fibers and myogenic regulatory factors (MyoD and myogenin) remained stable and low. Furthermore, Pax-7 expression did not increase significantly in mycolactone-injected muscles, indicating that the satellite cell proliferation is abrogated by the toxin. In addition, the fibrotic area increased progressively during the infection. Lastly, muscle-specific RING finger protein 1 (MuRF-1) and atrogin-1/muscle atrophy F-box protein (atrogin-1/MAFbx), two muscle-specific E3 ubiquitin ligases, were upregulated in the presence of *M. ulcerans*. These findings confirmed that skeletal muscle is affected in our model of subcutaneous infection with *M. ulcerans*, and that a better understanding of muscle contractures and weakness is essential to develop a therapy to minimize loss of function and promote the autonomy of BU patients.

**Keywords:** *Mycobacterium ulcerans*, skeletal muscle force, degeneration, edema, atrophy, fibrosis, muscle regeneration
Introduction

Buruli ulcer (BU) is an infectious disease caused by *Mycobacterium ulcerans* (*M. ulcerans*). BU is currently the third most common mycobacterial disease in the world, after tuberculosis and leprosy. This neglected infectious disease, which affects mostly the poorest people in developing countries, can cause very severe chronic skin lesions if diagnosis and treatment are delayed. In West Africa, most BU patients experience disabilities resulting from cutaneous fibrosis and soft tissue contracture, which limit the range of motion of the affected joint (7, 25). While skeletal muscles play a key role in joint mobility and are located just under the necrotic skin infected by *M. ulcerans*, they have received little attention in BU studies. Moreover, clinical investigations of BU patients in West Africa have revealed that over half of the patients suffer from range of motion limitations and functional restrictions (7). Furthermore, anecdotal and empirical observations suggest that BU patients generally suffer from skeletal muscle necrosis, atrophy, and contracture. In our mice model of BU, the subcutaneous injection of *M. ulcerans* has been shown to cause necrosis, chronic inflammatory response, fibrosis, and skeletal muscle stiffness (17). Clinical and experimental findings thus indicate that *M. ulcerans* infections play a major role in the loss of skeletal muscle integrity.

Repair and maintenance of skeletal muscle is attributed to the satellite cells. In response to several stimuli including muscle injury, quiescent satellite cells activate, proliferate and differentiate to repair damaged skeletal muscle (4). Pax-7 is expressed and transcriptionally active in quiescent satellite cells (26). Following activation, satellite cells proliferate and co-express Pax-7 and MyoD (26). Pax-7 is also implicated in the regulation of satellite cell self-renewal and pax-7 null mice present impairment in muscle regeneration combined with a progressive loss in satellite cell number due to cell cycle defect and increased apoptosis (19).

Muscle mass is maintained by a fragile balance between protein synthesis and degradation, and muscle atrophy can occur when this balance is disturbed. The selective loss of contractile proteins relative to cellular proteins generally results in muscles with smaller myofibers (3). Chronic systemic inflammation is one of the potential conditions that can lead to significant muscle atrophy (21). For example, sepsis is characterized by chronic, systemic inflammation associated with both a reduction in protein synthesis and a breakdown of muscle
proteins, in particular the contractile proteins actin and myosin (12, 13). The atrophying muscles display increased rates of protein degradation, mainly caused by the activation of the ubiquitin proteasome system (2). In addition, the muscle-specific E3 ubiquitin ligase muscle-specific RING finger protein 1 (MuRF-1) and atrogin-1/muscle atrophy F-box proteins (atrogin-1/MAFbx) are upregulated while myogenic regulatory factors such as MyoD and myogenin are usually downregulated (20, 35). Since proinflammatory cytokines possess a strong potential for mediating sepsis-induced proteolysis of myofibrillar proteins in skeletal muscles (9, 12, 21) and since a number of these cytokines are upregulated during *M. ulcerans* infections (17), we verified the impact of *M. ulcerans* infections on biceps muscle at the histological, morphological, and functional levels. We demonstrated that the presence of *M. ulcerans* and mycolactone reduces muscle force production, upregulates MuRF-1 and atrogin-1 and impairs satellite cell proliferation, all of which are associated with fibrosis and muscle atrophy and dysfunction.
Materials and methods

Animal care and feeding

Male C57BL/6 mice weighing 20-22 g (Charles River Laboratories, Saint-Constant, QC, Canada) were housed two per cage in pathogen-free conditions. All animal care and handling procedures were approved by the Université Laval Animal Protection Committee. The mice were maintained on a 12 h light-dark cycle, and food and water were provided ad libitum. No differences in food and water consumption or physical activity were observed between groups.

Experimental design

A previously developed protocol was used to evaluate the histological, biochemical, and functional impacts of M. ulcerans infections on skeletal muscles (17). In the present study, the contractile properties, atrophy, fibrosis, and regeneration of biceps muscles were assessed in 72 mice randomly assigned to three groups: 1) no treatment (control, CTR, n = 24), 2) subcutaneous injection near the right biceps muscle with 30 µL of phosphate buffer saline (PBS) containing culture media (Sham, n = 24), and 3) subcutaneous injection with M. ulcerans suspended in PBS (10^5 acid-fast bacilli/30 µL; AFB) near the right biceps muscle (proximate-infected biceps, PIB, n = 24). The biceps muscle was chosen because it is a superficial and easy muscle to dissect which possesses two distinct tendons at his extremities. This superficial muscle allows us to perform subcutaneous injection mimicking somehow the mode of M. ulcerans infection. All mice from each group were sacrificed on days 7 (n = 6), 21 (n = 9), and 42 post-injection (n = 9). The infectious process did not progress extensively and no apparent changes in physical activities were observed in infected mice.

M. ulcerans injection protocol and surgical procedure

The injection and surgical procedures were performed as described previously (15). Briefly, M. ulcerans strain 957-05 isolated from a BU patient in 2005 and identified by PCR using the insertion sequence IS2404 was grown for 6-8 weeks on Lowenstein-Jensen medium at 30°C in a reduced oxygen environment (5% CO₂). The mycobacteria were collected and suspended in PBS. A homogeneous aliquot (10 µL) was stained using the Ziehl-Neelsen method, and the concentration of AFB was determined by manual counting using a microscope equipped
with an immersion objective (1000x magnification). The concentration was adjusted to $10^5$ AFB/30 µL. The mice were anesthetized using 2.5% isoflurane in O$_2$ at a rate of 600 mL/min. The AFB suspension (30 µL) or PBS with medium was injected subcutaneously into the right front limb, avoiding direct contact with the biceps muscle. On days 7, 21, and 42 post-injection, the CTR, Sham and PIB mice were injected with buprenorphine (0.1 mg/kg) and anesthetized 15 min later with pentobarbital (40 mg/kg). An additional dose of pentobarbital was given as needed to maintain profound anesthesia throughout the surgical procedure. The CTR, Sham, and PIB muscles were carefully removed for in vitro contractile, histological, and biochemical analyses. Mice were then euthanized by cervical dislocation.

*Measurements of contractile properties in vitro*

To examine muscle force and function, each tendon of CTR, Sham, and PIB muscles was attached using 3-0 silk sutures. The distal tendon was tied to a rigid support between two platinum electrodes submerged in Krebs-Ringer buffer supplemented with 2 mg/mL of glucose as described previously (17). The muscle was aligned vertically and the proximal tendon was fixed to the arm of a dual-mode servomotor (305B-LR, Aurora Scientific, Aurora, ON, Canada) controlled by Dynamic Muscle Control (DMC) software. Carbogen (95% O$_2$:5% CO$_2$) was constantly bubbled through the solution, which was kept at 25°C. Twitch tension and isometric contractions were performed, and twitch peak tension ($P_t$, mN) and maximal tetanic force ($P_0$, mN; specific $P_0$, N/cm$^2$) values were recorded, and force-frequency curves were generated as described previously (14).

*Assessment of fiber area, regeneration, edema, and fibrosis of skeletal muscles*

Because the histological and contractile results indicated that there was no difference between Sham and CTR muscles, muscle atrophy, repair, and fibrosis were assessed on CTR and PIB muscles only. Following the evaluation of the functional properties, the CTR, and PIB muscles were trimmed of visible fat, tendons, and other connective tissues. The biceps were then weighed and stretched to near resting length, embedded in tissue freezing medium (Triangle Biomedical Sciences, Durham, NC, USA), and frozen in isopentane that was cooled in liquid nitrogen. Muscle tissue sections (10 µm thick) were stained with hematoxylin/eosin and images were acquired at 200x magnification. To measure muscle atrophy, the CSA of 200 randomly
selected myofibers per muscle section were determined using ImageJ software. To assess the necrosis and repairing processes of skeletal muscles, the necrotic area and the number of central nucleated myofibers was determined and expressed per CSA. The necrotic area was estimated in each muscle section by subtracting the summed area of normal and regenerating fibers from the total area of each field. (18). Other tissue sections from CTR and PIB muscles were stained with Masson’s trichrome to color collagen blue. The surface areas of blue-stained tissues were measured and expressed as percentages of the total CSAs of the muscles. In another set of experiments, CTR, Sham, and PIB muscle samples were weighed and dehydrated for 18 h in a SpeedVac concentrator. Wet and dry muscle masses were subtracted to assess water content and edema.

**Protein sample preparation and Western blotting**

CTR, Sham, and PIB muscles were homogenized in 200 µL of lysis buffer (20 mM Tris, pH 7.5, 140 mM NaCl, 10 µM MgCl₂, 1 µM CaCl₂, 2 µM NaF, 8.3 µM Na₃VO₄, 2 µM phenylmethylsulfonyl fluoride (PMSF), 1 mL glycerol, 100 µL Ig epal, and 10 µL of protease inhibitors in a final volume of 10 mL). The homogenate was centrifuged at 10 000 g for 5 min and the pellet was discarded. The protein content of the homogenates was measured using BCA Protein Assay Kits (EMD Chemical, Darmstadt, Germany). The protein concentrations were compared with a standard curve and determined at 562 nm using a Perkin-Elmer plate reader (6). The protein suspensions (50 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) and incubated overnight at 4°C with primary antibodies (atrogin-1 rabbit polyclonal (ECM Biosciences), MuRF-1 goat polyclonal (Novus Biological), MyoD rabbit polyclonal (Santa Cruz Biotechnology), and myogenin rabbit polyclonal (Santa Cruz Biotechnology)) diluted 1:1000 in 5% dry milk. α-tubulin (Santa Cruz Biotechnology) was used as an endogenous reference to monitor protein loading. Protein bands were detected using horseradish peroxidase (HRP)-linked goat anti-rabbit or bovine anti-goat secondary antibodies. The bands were detected using an enhanced chemiluminescence detection substrate (Western Lightning Plus ECL, Perkin Elmer). The chemiluminescent signal was acquired on Kodak film (X-OMAT; LS Kodak scientific imaging film) and relative signal bands were quantified from
scans (TIFF images) by densitometric analysis of integrated gray levels corrected for local background using Quantity One software (Bio-Rad).

Pax-7 expression in injured skeletal muscles

To evaluate myogenic potential in various conditions, skeletal muscles were injected and chemically damaged with 10 μL of PBS (Sham), 70% ethanol (Sham), mycolactone (25 μg) or bupivacaine (50 μg). Bupivacaine is a very well known myotoxin molecule that produces very reproducible induction of muscle damage and repair. Because bupivacaine destroys myofibers but spares satellite cells, leaving the basal laminae and microcirculation intact, it can be used as a positive control for muscle regeneration and recovery. After 7 days post-injury, control and experimental muscles were then dissected, homogenized and immunolabeled with Pax-7 (Abcam) on Western blot as previously described to assess satellite cell survival and proliferation.

Statistical analysis

All data are reported as means ± standard errors of the mean (SEM). The data were compared with a one-way ANOVA using JMP software (JMP 7, SAS Institute Inc., Cary, NC, USA) and Tukey’s a posteriori test. P values <0.05 were considered significant.
Results

*M. ulcerans* causes a significant loss of muscle force

To evaluate the functional impact of *M. ulcerans* on the biceps muscles, twitch tension (Pt, mN) and maximal isometric force (absolute P₀, mN) values were recorded in CTR, Sham, and PIB muscles on days 7, 21, and 42 post-infection. The Pt values for the PIB muscles were 12.8% and 25% lower than the Sham muscles on days 21 and 42, respectively (Fig. 1A). The absolute P₀ values were 18% and 31% lower for the PIB muscles than for the Sham muscles on days 21 and 42, respectively (Fig. 1B). The decline in muscle force was time dependent in PIB muscles and significant differences were observed between PIB muscles at 7, 21 and 42 days. These decrements were nearly constant since the force-frequency curves for the PIB muscles were significantly lower than the Sham and CTR muscles (Fig. 1C). However, *M. ulcerans* had no significant influence on speed-related contractile parameters, since the time to peak tension (TPT; ms) and half relaxation time (RT½; ms) values were not significantly different from those of the Sham muscles at all time points (data not shown). Although *M. ulcerans* causes myofiber atrophy, edema, and the accumulation of connective tissue, which can all change the size of skeletal muscles, we also found a significant decrease in muscle specific force (sP₀, N/cm²) at 21 and 42 days post-injection relative to sham muscles (data not shown).

*M. ulcerans* and mycolactone cause muscle edema, damage, atrophy, and fibrosis with impairment in satellite cell proliferation

The *M. ulcerans* infections resulted in significant edema in the biceps muscles as indicated by the wet weight and dry/wet weight ratios of PIB muscles compared to the Sham muscles. The edema progressed steadily, with the water content of the PIB muscles increasing from 2.6% to 12% between days 7 and 21 relative to the Sham muscles (table 1). Interestingly, the edema decreased thereafter, with no significant differences between the CTR, Sham, and PIB muscles on day 42. To assess muscle fibrosis, CTR and PIB muscle sections were stained with Masson’s trichrome. As expected, there was more collagen in the PIB muscles than in the CTR muscles (Figs 2A-B). The area of fibrotic tissue increased over time and reached 4.5% and 5.3% of the muscle CSA on days 21 and 42, respectively (Fig. 2C). The histological observations with hematoxylin and eosin suggested that the edema was mainly concentrated between muscle fascicles (Figs. 3A-B). Furthermore, the *M. ulcerans* infections caused a significant reduction in
myofiber CSA, with the mean values for the PIB muscles dropping by 17% and 29% on days 21 and 42, respectively, relative to the CTR muscles (Fig. 3C). The number of central nucleated myofibers was also determined to estimate the number of regenerating fibers. The CTR muscles did not display any fiber damage or central nucleated myofibers while the number of regenerating fibers remained stable at approximately 6-7 myofibers/mm² from days 7 to 42 in infected skeletal muscles (Fig 4A). Consistent with this observation, the levels of expression of myogenic regulatory factors MyoD and myogenin remained relatively low and stable at all experimental time points (Figs 4B-C).

Histological observations also showed that muscle injected with 70% ethanol (Sham) had minor inflammatory reaction (Fig 5A) whereas skeletal muscles injected with PBS (Sham) presented no sign of histological damage (Fig 5B). The injection of mycolactone (25μg) or bupivacaine (50μg) induced extensive damage with significant cell infiltration at 7 days post-muscle injury (Figs 5C-D). The extent of damage was particularly important following mycolactone injection (Fig 5C). More importantly, the level of expression of transcription factor Pax-7, expressed on quiescent and activated satellite cells, increased by more than 12-fold in bupivacaine-injected muscles but remained similar to Sham in mycolactone-injected muscles at 7 days post-injury (Fig.5E). These results clearly indicated for the first time that satellite cell proliferation is abrogated in presence of mycolactone (Fig. 5E). As expected, the PIB muscles displayed significant areas of necrosis on days 21 and 42 compared to day 7 (Fig. 6A). The area of necrotic tissue increased and reached 7% of the muscle CSA on day 42. This catabolic state was associated with an upregulation of the muscle-specific ubiquitin ligases (E3) atrogin-1/MAFbx (muscle atrophy F-box protein) and MuRF-1 (muscle-specific RING-finger 1). These two muscle specific genes for atrophy rose respectively 2.4 and 1.5-fold at day 42 when compared to CTR muscles (Figs. 6B-C).
Discussion

The loss of contractile proteins and normal sarcomeric organization associated with muscle wasting is the hallmark of diverse catabolic conditions such as muscle disuse, burn injury, cancer, renal failure, AIDS, chronic obstructive pulmonary disease, space flight, muscular dystrophy, and aging (14, 22, 30, 31, 34). Loss of muscle mass is also commonly seen in animal models and patients with sepsis (1, 17, 27). This catabolic state of skeletal muscles is mainly driven by polyubiquitination, which involves a cascade of proteolytic enzymes such as ubiquitin-activating enzyme, ubiquitin-conjugated enzyme, and ubiquitin-protein ligase. Ultimately, the loss of muscle mass results in weakness and fatigue that limit ambulation and prolong rehabilitation.

The present findings showed that the *M. ulcerans* infections caused a 31% and 29% decrease in the force and CSA of myofibers, respectively, which was associated with an upregulation of atrogin-1 and MuRF-1, two muscle-specific ubiquitin-ligases involved in protein degradation. The ubiquitin-proteasome system is the major pathway responsible for the turnover of muscle protein in general and, more specifically, in sepsis (35). For example, atrogin-1 and MuRF-1 play an essential role in sepsis-induced muscle wasting. More recent data have indicated that MuRF-1 acts by targeting myofiber proteins such as titin, troponin, myosin light-chain-2, and nebulin (33), leading to degradation and muscle loss of mass. Another major mechanism of sepsis-induced muscle wasting is the activation of the calpain system. Calpain and caspase-3 appear to play a pivotal role in ubiquitin-proteasome-mediated muscle wasting by promoting myofibrillar proteolysis (5, 29). Both the ubiquitin-proteasome and calpain proteolytic pathways have been shown to be partly controlled by proinflammatory cytokines (9, 12, 21) such as TNF-α and IL1β which increase during *M. ulcerans* infections (17). While the assessment of protein synthesis and degradation were not within the scope of the work reported here, other research on muscle atrophy and sepsis suggests that the loss of muscle mass and the reduction in the CSA of myofibers in mice with *M. ulcerans* infections are likely the result of an upregulation of protein degradation combined with a reduction in protein synthesis.
Muscle growth and regeneration are attributed to satellite cells. Our results showed that *M. ulcerans* and mycolactone have negative impacts on the regeneration process since the number of centronucleated myofibers and the level of myogenic regulatory factors MyoD and myogenin and Pax-7 did not increase over time in the PIB muscles. MyoD is one of the earliest markers of myogenic commitment, which is expressed in activated satellite cells (23), while myogenin is essential for the development of functional skeletal muscles (24, 27). The lack of a significant increase in MyoD and myogenin content suggested that the presence of *M. ulcerans* prevented muscle regeneration. More importantly, the level of Pax-7 expression increased by 12-fold in bupivacaine-injected muscles while the level of Pax-7 expression in mycolactone-injected muscle remains stable indicating that satellite cell proliferation is inhibited at 7 days post-injury. These results are in agreement with previous findings demonstrating that injecting skeletal muscles with mycolactone totally abrogates muscle regeneration (16). The fact that the number of regenerating fibers did not significantly increase in these skeletal muscles may thus be due to an increase in satellite cell apoptosis, satellite cell inactivation, and/or the decreased unavailability of cytokines and growth factors that govern the muscle regeneration process. Furthermore, mycolactone can diffuse far from the site of infection (11, 15), penetrate membranes of other cell types, induce cytoskeleton rearrangement, and ultimately cause cell death by apoptosis (10, 28).

Alternate interpretations for muscle dysfunction are also possible. Firstly, an in vivo study of mice footpad infections demonstrated that *M. ulcerans* can invade and produce nerve necrosis (8). It is thus possible that biceps nerves and/or neuromuscular junctions may be partly dysfunctional, leading to progressive muscle atrophy. Secondly, the accumulation of leukocytes and fibrotic tissue over time indicates that infected biceps muscles are subject to an inappropriate chronic inflammatory reaction leading to scar formation instead of muscle regeneration (17). The inadequate chronic inflammatory response may lead to fibrosis caused by persistent irritant or an upregulation of fibrogenic growth factors and cytokines, which together stimulate the synthesis and deposition of extracellular matrix (36). Previous observations have shown that muscle regeneration does not occur in the presence of mycolactone and that the collagen content of skeletal muscle increases by 134%, indicating that mycolactone stimulates fibroblasts, which are a major cell type in skeletal muscles (16). These results are consistent with the increase in the
fibrotic areas of the PIB muscles observed on days 21 and 42. Further in vitro and in vivo studies are needed to clarify the molecular mechanisms by which *M. ulcerans*, and/or mycolactone, and/or recruited leukocytes that secrete proinflammatory cytokines are involved in muscle atrophy and muscle regeneration impairment.

**Perspectives and significance**

BU is a neglected disease. Little research has been conducted on the impact of *M. ulcerans* on skeletal muscles. The present study demonstrated that *M. ulcerans* infections lead to a progressive and significant decrease in tetanic force production and twitch tension of biceps muscles close to the site of infection. Histological, morphological and biochemical analyses also indicated that *M. ulcerans* infections and mycolactone induce myofiber atrophy, fibrosis and inhibit satellite cell proliferation. These results confirmed the involvement of skeletal muscles at the functional level in our model of subcutaneous infection with *M. ulcerans*. The subcutaneous injection of the *M. ulcerans* was chosen because it mimics the mode of contamination of the disease from bug bites to human. Furthermore, mice injected with *M. ulcerans* present a discrete nodule at 7 days post-infection. Several mice exhibit sequentially a local edema and skin ulceration at 21 and 42 days post-infection, respectively (17). The same chronological manifestation of the disease is found in human making this animal model useful and relevant. We trust that the present work will set the stage for an extensive research program on skeletal muscle rehabilitation directed toward a better understanding of muscle contracture and weakness that often lead to a loss of function, autonomy, and dignity in BU patients (see the following address for details on this disease: http://www.who.int/buruli/en).
Acknowledgments

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**Table I:** The morphological characteristics were obtained from control (CTR), Sham, and proximate-infected biceps (PIB) muscles on days 7, 21, and 42 post-injection.

<table>
<thead>
<tr>
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<th>Day 7</th>
<th>Day 21</th>
<th>Day 42</th>
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<tr>
<td></td>
<td>CTR (n = 6)</td>
<td>Sham (n = 6)</td>
<td>PIB (n = 6)</td>
</tr>
<tr>
<td></td>
<td>(n = 9)</td>
<td>(n = 9)</td>
<td>(n = 9)</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>25.13 ± 0.55</td>
<td>25.28 ± 0.57</td>
<td>25.04 ± 0.39</td>
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<tr>
<td>Biceps dry weight (mg)</td>
<td>3.89 ± 0.12</td>
<td>3.90 ± 0.11</td>
<td>3.88 ± 0.20</td>
</tr>
<tr>
<td>Biceps dry weight / wet weight</td>
<td>0.23 ± 0.01</td>
<td>0.23 ± 0.01</td>
<td>0.23 ± 0.01</td>
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<tr>
<td>Biceps water content (mg)</td>
<td>12.76 ± 0.20</td>
<td>12.90 ± 0.14</td>
<td>13.24 ± 0.12</td>
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All values are means ± S.E.M.; n = 6 to 9. \(^{a}\) Significantly different from the matched Sham muscle on day 7; \(^{b}\) significantly different from the matched PIB muscle on day 7; \(^{c}\) significantly different from the matched Sham muscle on day 21; \(^{d}\) significantly different from the matched PIB muscle on day 21; (p<0.05).
Figure legends

**Fig. 1**: Isometric contractile properties of biceps muscles. Maximum twitch tension $P_t$ (A), maximum tetanic force $P_0$ (B), and force-frequency curves (C) are shown for control (CTR), sham (Sham), and proximate-infected biceps (PIB) muscles on days 7, 21, and 42 post-injection. Isometric force decreased progressively during the infection. All values are means ± SEM; n = 6 to 9. No significant differences were observed between CTR and Sham muscles * Significantly different from the matched CTR and Sham biceps muscles; # significantly different from the matched PIB muscle on day 7; ‡ significantly different from the matched PIB muscle on day 21, (p<0.05).

**Fig. 2**: Histological staining of cross-sectional biceps muscles. Control (CTR) and proximate-infected biceps (PIB) muscles on day 42 were stained with Masson’s trichrome (A-B). Masson’s trichrome-stained fibrotic tissues in blue which are indicated in brackets (B). The fibrotic muscle area (C) was quantified for control (CTR) and proximate-infected biceps (PIB) muscles on days 7, 21, and 42 post-injection. All values are means ± SEM; n = 6 to 9. * Significantly different from the matched CTR muscle; # significantly different from the matched PIB muscle on day 7; (p<0.05). Bar = 100 µm.

**Fig. 3**: Histological and morphological analyses of biceps muscles. Control (CTR) and proximate-infected biceps (PIB) muscles on day 42 were stained with hematoxylin/eosin (A-B). The cross-sectional area of muscle fibers was quantified for control (CTR) and proximate-infected biceps (PIB) muscles on days 7, 21, and 42 post-injection. The arrow indicates a central nucleated fiber. All values are means ± SEM; n = 6 to 9. * Significantly different from the matched CTR muscle; # significantly different from the matched PIB muscle on day 7; (p<0.05). Bar = 100 µm.
**Fig. 4:** Central nucleated myofibers and expression of myogenic regulatory factors. The number of central nucleated myofibers (A), was quantified for control (CTR) and proximate-infected biceps (PIB) muscles on days 7, 21, and 42 post-injection. Levels of expression of MyoD (B) and myogenin (C), two myogenic regulatory factors. MyoD and myogenin are usually expressed during muscle growth, development, and regeneration. MyoD and myogenin levels were similar over time in the control (CTR) and proximate-infected biceps (PIB) muscles, suggesting that muscle regeneration was not initiated in the presence of *M. ulcerans*. Alpha-tubulin was used as a loading control. All values are means ± SEM; n = 6 to 9. * Significantly different from the matched CTR muscle; (p<0.05).

**Fig. 5:** Histological damage and Pax-7 expression in injured skeletal muscles. Skeletal muscles injected with 70% ethanol (Sham) had minor inflammatory reaction (A) whereas skeletal muscles injected with PBS (Sham) presented no sign of histological damage (B). The injection of mycolactone (25 μg) or bupivacaine (50 μg) induced extensive damage with significant cell infiltration at 7 days post-muscle injury (C-D). The extent of damage was particularly important following mycolactone injection (C). However, Pax-7 expression increased drastically in bupivacaine-injected muscles but remained low in mycolactone-injected muscles (E). The levels of Pax-7 were expressed relative to control muscles. All values are means ± SEM; n = 4. * Significantly different from mycolactone and Sham groups. Bar = 50 μm.

**Fig. 6:** Necrotic area and expression of two ubiquitin ligases. The necrotic area (A) was quantified for control (CTR) and proximate-infected biceps (PIB) muscles on days 7, 21, and 42 post-injection. Expression levels of atrogin-1 and MuRF-1, two ubiquitin ligases associated with muscle atrophy. The levels of atrogin-1 (B) and MuRF-1 (C) increased progressively during the infection. Alpha-tubulin was used as a loading control. All values are means ± SEM; n = 6 to 9. * Significantly different from the matched CTR muscle; # significantly different from the matched PIB muscle on day 7; ‡ significantly different from the matched PIB muscle on day 21; (p<0.05).
References


Fig. 1

A

Maximum twitch tension (mN)

Days post-infection

B

Maximum tetanic force $P_{\text{max}}$ (mN)

Days post-infection

C

Isometric force (mN)

Frequency (Hz)
Fig. 2

A

B

C

Muscle fibrotic area (% of muscle CSA)

CTR    7    21    42

Days post-infection
Fig. 3

A

B

C

Cross sectional area (µm²)

<table>
<thead>
<tr>
<th>Days post-infection</th>
<th>CTR</th>
<th>PIB</th>
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<tbody>
<tr>
<td>7</td>
<td></td>
<td></td>
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<tr>
<td>21</td>
<td></td>
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<tr>
<td>42</td>
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Legend:
- CTR
- PIB

Note: * and # indicate significant differences.
Fig. 4

A

![Bar graph showing central nucleated myofibers per mm² over days post-infection.](image)

B

![Western blot images of MyoD and α-tubulin with densitometry graphs.](image)

C

![Western blot images of Myogenin and α-tubulin with densitometry graphs.](image)
Fig. 6

A

B

C

Atrogin-1

α-tubulin

CTR  7D  21D  42D

MuRF-1

α-tubulin

CTR  7D  21D  42D

Atrogin-1

MuRF-1

Densitometry (arbitrary units)

CTR  7  21  42

Densitometry (arbitrary units)

CTR  7  21  42

Days post-infection

Days post-infection