Detailed characterization of a long-term rodent model of critical illness and recovery

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Abstract

Objective: To characterize a long-term model of recovery from critical illness, with particular emphasis on cardiorespiratory, metabolic and muscle function

Design: Randomized controlled animal study

Setting: University research laboratory

Subjects: Male Wistar rats

Interventions: Intraperitoneal injection of the fungal cell wall constituent, zymosan or n-saline.

Measurements and Main Results: Following intervention, rats were followed for up to two weeks. Animals with zymosan peritonitis reached a clinical and biochemical nadir on day 2. Initial reductions were seen in body weight, total body protein and fat, and muscle mass. Leg muscle fiber diameter remained subnormal at 14 days with evidence of persisting myonecrosis, even though gene expression of regulators of muscle mass (e.g. MAFbx, MURF1, myostatin) had peaked on days 2-4 but had normalized by Day 7. Treadmill exercise capacity, forelimb grip strength and *in vivo* maximum tetanic force were also reduced. Food intake was minimal until Day 4 but increased thereafter. This did not relate to appetite hormone levels with early (6h) rises in plasma insulin and leptin followed by persisting subnormal levels; ghrelin levels did not change. Serum IL-6 level peaked at 6h but had normalized by Day 2 whereas IL-10 remained persistently elevated and HDL cholesterol persistently depressed. There was an early myocardial depression and rise in core temperature, yet reduced oxygen consumption and respiratory exchange ratio with a loss of diurnal rhythmicity that showed a gradual but incomplete recovery by Day 7.

Conclusions: This detailed physiological, metabolic, hormonal, functional and histological muscle characterization of a model of critical illness and recovery reproduces many of the findings reported in human critical illness. It can be used to assess putative therapies that may attenuate loss, or enhance recovery, of muscle mass and function.

Introduction

Insults such as infection, trauma and hemorrhage can trigger a dysregulated release of pro- and anti-inflammatory mediators that, through a final common pathway, may progress to multi-organ failure and death. In survivors, a combination of severe inflammation, critical illness neuromyopathy, catabolic loss of protein, metabolic modifications, and prolonged immobility frequently results in loss of muscle mass and weakness. Recovery of muscle function can take many months and may be incomplete (1-3). Interventions shown to clearly ameliorate and/or reverse this muscle dysfunction are lacking. This is, in part, due to the dearth of good pre-clinical models that can evaluate both pathophysiologic mechanisms and the use of putative therapies.

Animal, particularly rodent, models of critical illness abound. However, they generally focus on short-term outcomes ranging from hours to a few days (4). Models examining the prolonged recovery phase of critical illness are rare, despite inadequate recovery being increasingly recognized as having a major negative impact upon long-term quality of life (2,5). Muscle biopsies from affected patients confirm significant atrophy and wasting (6-10), though direct inferences from single fiber level pathology to functional tests of muscle strength and fatigue are limited. Experiments utilizing *in situ* muscle force generation by direct nerve stimulation have been reported (11,12), but also have limitations, being pre-terminal and/or requiring deep anesthesia.

Zymosan, a cell wall glucopolysaccharide of the fungus *Saccharomyces Cerevisae*, signals via Toll-like receptor-2 to induce a non-bacterial, non-endotoxic sterile local inflammatory stimulus with sustained release of lysosomal enzymes, reactive oxygen species and cytokines, which triggers a systemic inflammatory, sepsis-like response (13). When suspended in paraffin and injected intraperitoneally, zymosan produces a prolonged peritonitis (14-16). Clinical features initially resemble bacterial sepsis with piloerection, tachycardia, fever and reduced spontaneous movement. Catabolism, evidenced by reduced body mass and muscle atrophy, occurs after five days or so,

followed by clinical recovery with resumption of feeding and restoration of lost body weight and muscle mass (16).

The aim of our study was to develop, comprehensively characterize and validate a long-term model of rodent critical illness and recovery, based on zymosan peritonitis, which would simulate the metabolic and physiological responses seen in humans. We wished to demonstrate initial weight loss, muscle weakness and anorexia that would recover in line with clinical recovery. We describe accompanying changes in total body oxygen consumption and respiratory exchange ratio, overall body composition, hemodynamics, muscle mass, function and histology during the recovery phase from critical illness. Importantly, we also describe a new electromyographic technique for sequential assessment of muscle function.

Materials and Methods

Animal model

Male Wistar rats (Charles River, Margate, UK) of approximately 300g body weight, were singly housed for at least 72 hours pre-induction of peritonitis. They had free access to water and standard rat chow (Harlan Teklad, Madison, WI) containing 18% protein and 5% fat. Ambient temperature was controlled between 19-23°C and humidity at 55±10%. Lights were automatically turned on at 07.00 hours and off at 19.00 hours. Studies were performed under UK Home Office project licence and guidelines under the Animals (Scientific Procedures) Act 1986. Ethical approval was given by the University College London Ethics Committee.

Zymosan A (Sigma Aldrich, St Louis, MO), mixed with liquid paraffin (Merck, Darmstadt, Germany) to a concentration of 25 mg/ml (14), was homogenized with an Ultra-Turrax T25 electric homogeniser (Janke & Kunkel IKA, Staufen, Germany) at 24000 rpm for five mins, then sterilized in boiling water for 90 mins, aliquoted into 25 ml samples and stored at 4°C until required. Zymosan (30 mg/100 gm body mass) was administered intraperitoneally (i.p.) via a 19G needle under a brief

period of isoflurane anesthesia. ‘Sham-treated’ animals received an equivalent volume of saline injection only, whereas ‘naive', non-operated animals received no i.p. injection.

Animals were returned to their cages following injection. Fluid resuscitation was not given over the duration of study. Animals were checked at least four times daily and scored to assess insult severity (Table 1) (17). When clinical scores had returned to normal, they were scored on a daily basis. Any animal displaying signs of distress, or an inability to move, right itself or respond to external stimuli, was culled. Some animals did not display any clinical signs of illness (thus having a clinical severity score of ‘0’), or lose body mass, and had normal or near-normal food intake during the course of the study. Animals scoring ‘0’ at 24 hours after induction of sepsis (when illness severity was most pronounced in affected animals) were excluded from subsequent analysis.

Temporal analysis

Procedures and tests described below were not all performed on the same animals. Apart from body weight and food intake, which were measured daily, the tests described below were performed at up to eight timepoints: 6 hours, and 1, 2, 4, 7, 10, 12, and 14 days’ post-administration of zymosan or n-saline.

Food intake, body mass and tissue mass

Body weight and food intake were measured daily at 08.00 hours. Care was taken to collect and account for discarded food. After culling, gastrocnemius and soleus muscles were rapidly dissected and weighed before freezing in liquid nitrogen for subsequent biochemical analysis (see later).

Core temperature

Body temperature was measured via a rectal probe attached to a Homeothermic Monitor (Harvard Apparatus).

Exercise capacity and muscle function tests:

Videos demonstrating the functional tests are available within the Supplementary Materials

Forelimb grip strength

Muscle function tests were performed using a grip strength meter (Linton Instrumentation, Diss, Norfolk, UK) on Days 0, 2, 5, 8 and 12. Familiarization with the grip strength meter was ensured over a week prior to study commencement. The forelimbs were placed on a T-bar attached to a force transducer shaft connected to a peak amplifier, and allowed to flex before being pulled horizontally away gently by the base of the tail (18). The maximum grip force exerted by the rat until it released its grip was recorded. This was repeated five times for each animal (at 20 second intervals). The mean maximum peak force (in grams) was calculated from the top three values obtained (18).

Treadmill

Exercise capacity was determined using a rodent treadmill (Harvard Apparatus, Edenbridge, Kent, UK) at baseline (prior to sepsis induction) and then repeated on Days 2, 4, 7 and 14. Animals were pre-acclimatized to the treadmill in five sessions over two days, with increasing belt speed exposure over a 5 min interval. Formal assessment was then made by increments in belt speed rate (by 5 cm/s every 2 min) until animal fatigue occurred. This was determined by the inability to keep up with the belt speed despite a mild electric shock deterrent applied to the hind-legs when the animals touched the metal grid immediately behind the belt. If this occurred >3 times at one speed (19), the protocol was stopped. Performance time (in minutes) was used as a marker of exercise capacity.

Hind-limb myography

This was performed under isoflurane anesthesia with animals placed supine and temperature maintained at 37°C on a purpose-built rig (Harvard Apparatus) on Days 0, 2, 4, 7 and 14. Alternate hindlimbs, from which the fur was shaved, were tested at alternate timepoints. The hindlimb was fixed into position so that two needle electrodes could be inserted into the posterior muscle bulk. The electrodes provided an electrical stimulus via PowerLab 4/35 (ADInstruments, Oxford, UK) resulting in plantar flexion. A fixed length chain connecting the foot to a force transducer measured

the force generated by this movement. The mean of three measures of force (Newtons) was taken at increasing single stimuli between 1-15 V. This was followed by continuous submaximal electrical stimulation (electrical pulses at 12V for 0.05 seconds set at 40Hz) for 3.5 min to generate tetany. This enabled calculation of the submaximal tetanic force at the start of the fatigue protocol, and the fatigue index at 2 min (20). This index was derived from the proportion of tetanic force maintained at 2 min compared with the initial tetanic force during the fatigue test. Examples of traces obtained are shown in Supplementary Figure 1. Following myography, animals recovered fully from anesthesia and a full range of movement was observed in the tested hind limb.

Blood cytokine, gut hormone, and adipokine measurements

Blood samples were taken after either culling by decapitation (truncal blood: mixed arterial-venous) or by cardiac puncture, and divided equally into two chilled lithium-heparin test tubes, one of which contained 5 mg 4-(2-Amino-ethyl)benzenesulfonylfluoride hydrochloride (AEBSF) (Sigma Aldrich, St Louis, MO) and 100 µl aprotinin (10,000 kIU/ml, Sigma Aldrich) for analysis of gut hormone levels[21]. Both tubes were spun immediately at 4000 g for 15 mins and the plasma aliquoted into plastic Eppendorf tubes, one of which contained 25 µl 1M HCl, to which 500 µl plasma from the 'gut hormone' test tube was added for preservation of active ghrelin (21). The Eppendorf tubes were snap-frozen in liquid nitrogen before storage at -80°C. Plasma biochemistry was analysed by The Doctors Laboratory, London, UK and the Department of Clinical Biochemistry, Charing Cross Hospital, London UK using standard analysers. Gut hormones, leptin and cytokines were measured in duplicate using rat-specific multiplex bead-based assays (Millipore, Billerica, MA). Readings greater than two standard deviations from the mean for each gut hormone at each timepoint were not included in the final analysis (to account for extreme outliers).

In vivo metabolic behavior

The Comprehensive Laboratory Animal Monitoring System (CLAMS) and Oxymax for Windows software (Columbus Instruments, Columbus, OH) were used to measure oxygen consumption (VO_2) and carbon dioxide production (VCO_2) in naïve rats, or sham-operated and zymosan-treated rats

immediately after i.p. injection. The CLAMS is an open-circuit system where a continuous supply of room air (set at 400 ml/min) is delivered to, in our lab, four closed tightly-sealed cages. Gas sensors were calibrated before each study against known concentrations of oxygen (20.5%) and carbon dioxide (0.05%). O_2 and CO_2 concentrations were measured from air sampled from each cage for two minutes in turn, and of room air after every fourth sample. The rats remained in the metabolic carts for 7 days, apart from a 5-10 min period each morning when they were weighed and their cages cleaned.

Cardiac function

Under brief isoflurane anesthesia, spontaneously breathing rats with shaved chests underwent echocardiography (Vivid 7 Dimension™, GE Healthcare, Bedford, Beds, UK) with a 14 MHz probe at 0-2 cm depth, as previously described (22). Fractional shortening was calculated from internal left ventricular dimensions during at end-diastole and systole taken from parasternal short-axis views by M-mode (two-dimensional) echocardiography. Heart rate and respiratory rate were measured from these recordings. Pulsed wave Doppler measured aortic flow velocity in the aortic arch, with colour Doppler confirming the direction of flow. Stroke volume was calculated as the product of the velocity-time integral of each waveform and aortic cross-sectional area, assuming an aortic diameter of 0.28 cm (23). Cardiac output was computed as the product of stroke volume and heart rate. Studies were performed sequentially in the same animals pre-intraperitoneal injection, and on days 2, 4, 7 and 14. In separate studies, echocardiography was performed at 6h or 24 h post-injection just prior to culling for blood and tissue sampling.

Rodent body composition

In some studies, rats were culled on Day 12 by cervical dislocation under isoflurane anesthesia. The contents of the gastrointestinal tract were cleared manually, and any intra-abdominal fluid removed. The carcass was then frozen at -80°C, before being dissolved in 1 ml 3M KOH in 65% ethanol (VWR, Radnor, PA) per 1g carcass mass, sealed in a plastic pot and placed in an oven at 70°C for 5

days. The bones were removed by passing the liquid through a sieve and the total amount of liquid was then made up to 1000 ml by addition of 100% ethanol. From this a sample of fluid was removed and stored for analysis of protein and fat content.

Carcass protein and fat content were determined using a modified Lowry Protein Assay kit (Thermo Scientific, Rockford, IL) and a glycerol assay (Randox, Crumlin, Co. Antrim, N Ireland), respectively. The glycerol assay kit included all of the necessary reagents with the exception of the glycerol standard, for which 1M glycerol (VWR) was used.

RNA isolation, cDNA synthesis and real-time PCR

Total RNA was extracted from 20-30 mg gastrocnemius muscle using the RNeasy Fibrous tissue mini kit (Qiagen, Courtaboeuf, France) (24). cDNA was generated from 400 ng RNA using iScript cDNA synthesis kit (Bio-Rad, Marnes-la-Coquette, France). The selected forward and reverse primer sequences are listed in Supplementary Table 1. Real time PCR was performed in 20 µl final volume and optimized concentrations for each primer using the SsoFast EvaGreen Super mix (Bio-Rad) and a CFX96 Real Time PCR Detection System, C1000 Thermal Cycler (Bio-Rad). Beta-actin was used to normalize the expression levels of genes of interest. Primers were designer using Primer 3 software from gene sequences obtained from GenBank. Primer specificity was determined using a BLAST search.

Muscle histology

Animals culled on Days 2, 7 and 14 had gastrocnemius and soleus muscle dissected and rapidly frozen in 2-methylbutarate (Merck) cooled with liquid nitrogen before storage at -80°C until sectioning. These animals did not undergo exercise training nor the tests of muscle function described above. Serial cryostat sections were cut at 8 µm (OTF, Bright, Huntingdon, UK) and embedded in OCT (Sakura Finatek, Tatcham, UK).

Staining with hematoxylin and eosin (H&E) was performed on a ST5020 Multistainer (Leica Biosystems, Milton Keynes, UK). Muscle pathology was assessed by a neuropathologist blinded to

group and sampling time. H&E stained sections were assessed semi-quantitatively for necrosis, regeneration and atrophy in five randomly selected high power fields on a Leica bright field microscope: 0, 1-3 (mild), 4-9 (moderate), and ≥10 and/or focal clustered or circumscribed pathology (marked). Assessment of regenerating fibers was limited to recognition of early phase regeneration (intensely basophilic small fibers with large central nuclei resembling myotubes) and late phase regeneration (mature sized fibers with internalised nuclei). It was not possible to reliably distinguish regenerating fibers between these two phases from fibers undergoing atrophy as both can appear slightly basophilic and granular in H&E stained sections. Inflammation was assessed for its cellular type, location (perimysium, endomysium and/or blood vessels) and graded semi-quantitatively (0 none, 1 mild, 2 moderate, 3 severe). Intramuscular motor nerves were not assessed.

For cell morphometry, myofiber size was assessed by measuring fiber diameter (FD). Immunohistochemical labelling with alpha sarcoglycan (Leica NCL-L-a-SARC, Novocastra™), a muscle-specific membrane protein, (concentration: 1:200, incubation period: 1 hour at room temperature) was performed using an automated staining machine (IntelliPATH FLX™, A. Menarini Diagnostics, Wokingham, UK). Horseradish peroxidase-conjugated streptavidin complex and diaminobenzidine were used as chromogen. Images were digitized on a LEICA SCN400 scanner for subsequent digital image analysis using Definiens. Each section was screened prior to analysis to ensure inclusion of regions sectioned optimally in the transverse plane and to exclude regions of perimysial connective tissue, focal folds and other artefacts. The mean normal fiber size range in naïve animal gastrocnemius and soleus muscle was established by measuring lesser fiber diameter in approximately 100 optimally transversely sectioned fibres in random fields. In all samples, myofibers <15 micron diameter with angular or polygonal profiles were designated atrophic. Fibers adjacent to myotendinous insertions show increased fiber size variability and internal nuclei as a normal histological feature and were therefore excluded from the atrophy and regeneration count.

Statistical analysis

Animals that developed clinical signs of recurrence of illness (after previous recovery), or began to lose weight after having previously been gaining weight, were *a priori* excluded from analysis. Data were checked for normality using the Shapiro-Wilk test and are presented as mean and standard deviation. For comparisons between unpaired groups for change in body mass, food intake, and area-under-the curve from the metabolic carts, the Kruskal-Wallis test with Dunn's *post hoc* analysis was used. Unpaired Student's t tests were performed for comparison of the change in body composition between zymosan and naïve groups. Two-way ANOVA with post hoc Bonferroni analysis was performed for statistical analysis of biochemical, gut hormone, cytokine, blood gas, echocardiography, atrogene and muscle strength and fiber diameter measurements. Statistical analyses were performed with GraphPad Prism computer software (Version 5.00 for Windows, GraphPad Software, San Diego, CA). Statistical significance was set at the 5% level.

Results

For details of animal numbers tested in the separate studies, see Supplementary Table 2. No naïve or sham-treated animal died prematurely. In accord with previous reports (14), of the 150 zymosan-treated animals, 15 were excluded as they were not clinically affected, while one had a fluctuating course of weight loss and intermittent partial weight gain without ever surpassing its original body mass and so was also excluded *a priori*. The 24 hour clinical severity score was similar between groups of zymosan-treated animals culled at 24 hours, Day 2, 4, 7 or 12 ($p=0.40$). The naïve and sham groups remained healthy throughout (Score 0). Of note, despite receiving the same insult, the macroscopic appearances of the peritoneal cavity in zymosan-treated animals varied greatly on post-mortem examination, from normal to showing considerable fibrous tethering of the visceral organs, small abscesses and serous fluid. While not formally assessed, these changes appeared to correlate with both clinical severity and recovery of body mass.

Food intake, body mass and impact of clinical severity (Fig 1) The initial body mass pre-insult (range 274-333 g) was similar between groups, stratified by either intervention or by day culled. In

naïve and sham animals, body weight increased in a similar, near-linear fashion (Fig 1a), with both groups gaining 29% at 12 days. Animals receiving zymosan initially lost weight, reaching a nadir of 12% weight loss at Day 4. They regained baseline weight by Day 7 and, by Day 12, had increased body weight by 13.3%. Loss of weight at Day 4 was severity-dependent (Fig 1b). Cumulative food intake (Fig 1c) was significantly reduced in the zymosan group ($p<0.001$ compared to naïve and sham animals) with daily intake being significantly lower until Day 6. Animals with a 24-hour severity score ≥1 (range 1-4, mean 3) had a significantly lower food intake over 12 days compared to clinically unaffected (Score 0) zymosan-treated animals. (Fig 1d)

Muscle mass and body composition (Fig 2)

Over the first week, zymosan-treated animals (n= 8) had reduced muscle mass with significant reductions in wet gastrocnemius mass at Days 2, 4 and 7, and soleus mass at Day 4 and 7 (Fig 2a, b). Total body protein content (Fig 2c) and, in particular, fat content (Fig 2d) were significantly lower in the zymosan group.

Regulators of skeletal muscle mass (Fig 3)

The mRNA levels of several critical regulators of skeletal muscle mass ('atrogenes') were determined in naïve and zymosan-treated animals, including myostatin, a muscle-secreted protein that negatively regulates skeletal muscle mass (25, 26); MAFbx and MuRF1, two E3-ubiquitin ligases involved in the control of ubiquitin-proteasome-dependent proteolysis (27, 28); microtubule-associated protein 1 light chain 3 β (LC3b), an important regulator of the autophagy-lysosome-dependent pathway[29]; and cathepsins B and L, two lysosomal proteases (29, 30). Zymosan induced a marked increase in the mRNA level of these genes, the expression of which peaked at day 2 (MAFbx, MuRF1, LC3b, cathepsin B and cathepsin L) or day 4 (myostatin). Transcript levels returned to baseline values by day 7.

Metabolic monitoring (Fig 4)

In separate 7-day experiments, oxygen uptake and carbon dioxide production were measured continually in metabolic cages, from which the respiratory exchange ratio (RER) was calculated. Naïve and sham animals had virtually identical traces with clear diurnal variation. In contrast, zymosan induced a rapid and marked depression of VO_2 and VCO_2 with a significant fall in RER that did not recover until Days 5-6. Notably, there was complete loss of diurnal rhythmicity with zymosan that had not recovered by Day 7.

Cardiac function (Fig 5)

Cardiac function measured sequentially by echocardiography under brief anesthesia on Days 2, 4, 7 and 14 revealed initial myocardial depression in the zymosan-treated animals, with a significant decrease in stroke volume at Day 2 ($p=0.002$). Heart rate was initially unchanged but was significantly lower in the zymosan group from Day 4 onwards, and this persisted until Day 14 ($p=0.006$). In a separate group of animals (culled immediately after echocardiography), no difference was seen in any variables measured at 6 hours' post-zymosan. However, by 24 h, heart rate was significantly raised and stroke volume had fallen by approximately 50% in the zymosan animals. Cardiac output was correspondingly reduced (Supplementary Table 3). By 24 hours, core temperature was significantly elevated in the zymosan group (Supplementary Fig 2a).

Muscle function (Fig 6)

Forelimb grip strength was measured on Days 0, 2, 5, 8 and, 12 (Figs 6 a,b). Whereas grip strength in naïve rats progressively increased from Day 5 onwards with a 18±7% rise by Day 12, grip strength in the zymosan animals initially fell (-14±6% on Day 2) and had still not returned to baseline values by Day 12. The loss of grip strength seen in the zymosan animals reflected their reduced body mass (Figure 6b).

In separate studies, treadmill testing undertaken on Days 0, 2, 4, 7 and 14 showed a significant decrease in exercise capacity in the zymosan group that had not recovered by Day 14 (Fig 6c). *In vivo* myography performed in these animals at the same timepoints revealed a significant fall in

contractile strength at Day 2 (Fig 6d). Maximum tetanic force measured at the start of the fatigue studies (where a submaximal electrical stimulation was applied) also decreased significantly in the zymosan group at Day 2 (Supplementary Fig 3). The persistently lower mean fatigue index in zymosan-treated animals, did not reach statistical significance (p=0.12) (Fig 6e).

Plasma electrolyte, hepatic, lipid and hormone biochemistry, and cytokine levels (Fig 7)

Sham-treated and naïve animals showed similar electrolyte, lipid and hormone profiles. There was an initial deterioration in renal function, as denoted by significant elevations in plasma urea and creatinine (Figs 7 a,b). Alkaline phosphatase showed a later peak (Day 4) in the zymosan group but normalized by Day 7, whereas alanine transaminase fell significantly at 24h and remained low thereafter (Fig 7c and 7d). Bilirubin levels did not change (data not shown). Total cholesterol was significantly lower in zymosan-treated animals at 1-2 days' post-insult while HDL cholesterol remained suppressed from Day 1 through to Day 7 (Figs 7e,f).

Interleukin-6 (IL-6) was significantly elevated at 6 hours in zymosan-injected animals compared to naive, but had normalized by Day 2 (Fig 7g). On the other hand, interleukin-10 (IL-10) remained elevated throughout (Fig 7h).

Plasma leptin and insulin levels showed a similar pattern after zymosan, with an early elevation at 6 hours, but a significant fall from Day 2 which had not recovered by Day 7 (Figs 7i,j). There were no significant differences in either plasma glucose or ghrelin levels between groups at any timepoint (data not shown).

Hemoglobin levels in zymosan treated-animals were elevated compared to naïve rats at Day 2 (14.8 ± 0.24 g/dl vs. 12.9 ± 0.44 g/dl, $p<0.05$) (Suppl Fig 2b) but fell to similar levels by Day 7 and continued to fall thereafter (Days 12 and 14).

Muscle histology (Figs 8,9)

Muscle from naïve animals was histologically normal with no necrosis or inflammation and <1% of fibers showing atrophy or internalised nuclei. Across both sham and septic groups, gastrocnemius was histologically normal or showed rare isolated atrophic or necrotic fibers. However, soleus demonstrated mild-to-moderate scattered atrophic and necrotic fibers and perimysial inflammation in varying combinations in 9 of 10 sham animals. The septic group showed pathological changes from Day 2 through Day 14 with moderate-to-marked pathology in one or more domains (predominantly scattered myofiber atrophy and necrosis), most frequently seen in Day 7 samples. None of the septic animals showed complete resolution by Day 14, with moderate-to-severe changes persisting in half. Inflammation primarily centred on the perimysium with macrophages predominating, and with accompanying edema and focal extension into the fascicles. Regeneration was mostly observed in Day 7 and Day 14 septic samples, with scattered smaller and mature-sized fibers as well as focally accentuated or prominent circumscribed regeneration. Very early stage regenerating fibers resembling myotubes were virtually never observed. Examples of histological abnormalities seen in soleus muscle are shown in Fig 8.

The mean myofiber diameter of gastrocnemius ($p=0.02$) and soleus ($p<0.001$) was significantly reduced in the zymosan-treated group. Maximal change (-12%) was seen for gastrocnemius at Day 2, while for soleus a nadir of 15% was seen on both Days 7 and 14. In comparison to naïve samples, the spread of myofiber size was smallest in both muscle types on Day 2 (Fig 9).

Discussion

We aimed to develop a well-characterized long-term zymosan peritonitis model that would provide a useful and clinically relevant laboratory model of human critical illness and recovery by extensive characterization of cardiovascular, muscle, hormonal, and metabolic profiles. An early loss of body mass, muscle mass, total protein and fat, with reduced food intake, metabolic derangement and cardiovascular and functional muscle compromise correlated with the clinical severity of illness. Recovery in body weight and muscle functionality was slow with many aspects of muscle function

and strength, and metabolic and hormonal profiles not recovering, even after 1-2 weeks. Soleus muscle histology showed persisting inflammation, atrophy and necrosis, with some evidence of regeneration in the recovery phase. This model thus reflects changes reported in patients suffering from critical illness (10, 31, 32).

As with critically ill patients, there is considerable heterogeneity in the individual host response in these rats. Regardless of an identical insult being given to animals of similar age, gender and rearing, some responded with negligible clinical severity whereas others became critically ill and even succumbed. Acknowledging this variability, zymosan-treated animals were excluded *a priori* if their 24-hour clinical severity score was zero, or if a secondary deterioration occurred after several days of clinical improvement. Clinically unaffected animals maintained a near-normal food intake throughout, and with minimal consequences on body mass, plasma biochemistry and echocardiography. Of note, we previously demonstrated variability at clinical, biochemical and molecular levels in a 3-day rat model of fecal peritonitis, with distinct hemodynamic and gene transcriptomic profiling as early as 6 hours that enabled survival prognostication (17, 22).

The choice of species is important in extrapolating findings to the human response to critical illness. Seok *et al* demonstrated marked differences in the transcriptomic response between mice and humans suffering burn, trauma and endotoxemic insults (33). We recently reported that mice become rapidly hypothermic, hypoglycemic and profoundly hypometabolic within hours of a fecal peritonitis insult; by contrast, the phenotype in rats is more comparable to septic humans (34).

The focus of this present model was on the recovery phase following critical illness. We therefore specifically opted to minimize interventions such as vascular line placement, fluid administration and antibiotics, though *ad libitum* access to food and water was permitted. Timepoints were chosen to capture the clinical nadir and subsequent recovery period.

Our zymosan model suffered an initial 10-15% weight loss before recovering to baseline between days 5-8. Food intake was low for the first two days, but gradually increased to normal over

the next 3-4 days. These findings were less severe than those reported by Minnaard *et al* (16). In conjunction with the weight loss there was a marked reduction in total body protein and fat mass.

Each degree rise in temperature is associated with a 10-15% increase in oxygen consumption (35). Notwithstanding an early rise in core temperature that persisted for at least 24 hours in the zymosan group, there was an early marked fall in oxygen consumption (~25-30%) that persisted for several days, with an ongoing loss of diurnal rhythmicity. While decreased activity levels and food intake contribute to this decrease in metabolic rate, by Day 7 there was an obvious improvement in clinical severity and near-normalization of food intake. Other mechanisms need to be considered, including decreases in ATP-coupled respiration as a consequence of mitochondrial dysfunction and a greater degree of uncoupled respiration that would account for the pyrexia despite decreased muscular activity and food oxidation (36-38). In man, oxygen consumption rises in the early phase of uncomplicated sepsis, but then normalizes as organ dysfunction develops (39). In survivors, a rebound increase in oxygen consumption occurs during the recovery phase (39, 40). In human sepsis there are many interventions that either increase (e.g. inotropes, feeding, fluid resuscitation) or suppress (e.g. sedation, mechanical ventilation, antipyretics) metabolism, but which are avoided in a 'purer' laboratory model. This human pattern was not directly reflected in our zymosan model.

The fall in respiratory exchange quotient reflects the shift towards oxidation of endogenous fat and protein. This is due to both the decrease in food intake and to an inflammation-related shift in substrate utilization. This is consistent with the marked decrease in muscle mass and total body fat and protein stores. A very similar pattern was seen with plasma leptin and insulin levels in response to zymosan, with an early increase over naïve and sham groups at 6 hours, normalization at 24 hours, and marked and persisting subnormality from Day 2 onwards, despite progressive clinical recovery and increased food intake. The early rise in leptin may be related to an involvement in the acute inflammatory response, while the subsequent fall may reflect reduced body fat stores (41). Plasma insulin, like leptin, correlates with body fat when fasting and after ingestion of food or

carbohydrate (42, 43). Insulin resistance is a well-recognised feature of critical illness (44). Peripheral insulin has intrinsic anabolic actions that can prevent muscle protein breakdown (45, 46). The low levels seen in our model during recovery may partly explain the enduring reduction in muscle mass observed in the zymosan-treated animals. Unlike humans (47), no change in ghrelin was detected in the septic animals.

The initial rise in hemoglobin following zymosan likely reflects initial hemoconcentration due to volume depletion from decreased fluid intake and increased capillary leak, whereas the subsequent anemia seen after Day 7 is probably related to bone marrow suppression and erythropoietin resistance caused by sepsis.

To our knowledge, no temporal assessment has been made of muscle function during sepsis. Treadmill exercise capacity has been assessed in animal models of heart failure (48, 49), diabetes (50), aging (51, 52) and skeletal muscle pathophysiology (53-56). Motorised rodent treadmills have been used in sepsis models though as a means of determining the impact of endurance training on outcomes (57-60). The early and persisting fall in treadmill exercise capacity reflected the decrease in grip strength and the myography fatigue index shown with repetitive twitch. Our *in vivo* myography technique offers a novel means of monitoring sequential changes in muscle function as it allows full recovery of the animal following assessment while employing the same principles of electrical stimulation and fatigue as described elsewhere. As electrode placement is in the hind-limb muscle bulk, the set-up limits determination of individual muscle performance, but is rather more comparable to compound muscle forces that occur physiologically.

Previous sepsis models have used terminal experiments to assess muscle contractility at a single timepoint. Following cecal ligation and puncture, reduced contractile force and increased fatigue were shown in rat soleus at 7 days (7), and extensor digitorum longus at 10 days (8). In both these studies, the distal tendon was attached to a force transducer with complete denervation of the surrounding musculature. Minaard and colleagues assessed neuromuscular function in zymosan peritonitis by direct peroneal nerve stimulation, primarily affecting the tibialis anterior. Reduced

torque measurement was seen in zymosan treated rats compared to controls by day 6 and improvement was seen by day 11, albeit not to pre-insult levels. Tibialis anterior is a fast twitch muscle with predominantly type II fibers (61) that causes dorsiflexion. This may be affected differently from muscle groups causing plantar flexion, as assessed in our model. Here, multiple muscle fiber types are active, in particular soleus which is predominantly a slow-twitch, mitochondria-rich muscle with a large percentage of type I fibers.

Histopathological analysis revealed tissue damage in the form of macrophage-predominant fascial inflammation, edema and a necrotising myopathy consistent with observations in septic humans. Interestingly, gastrocnemius was virtually unaffected, whereas soleus bore the brunt of the pathological changes. Rat gastrocnemius is composed of multiple fiber types with type II subtypes predominating in the white, superficial portion and a higher type I content in the red, deep portion. Importantly, the histopathological changes do not represent an artefact of secondary damage induced by either electromyographic electrode insertion or exercise as samples were also taken from non-exercised, non-tested animals and showed similar changes. Despite improvements in functional, biochemical and metabolic parameters, at a cellular level there was ongoing inflammation, necrosis, atrophy and regeneration in the septic animals beyond day 7, with considerable variation in severity between individual animals.

The increased expression of MAFbx and MuRF1 strongly suggest involvement of the ubiquitin-proteasome pathway in zymosan-induced muscle mass loss, as previously reported (16). Increased expression of MAFbx and MuRF1 was demonstrated in C2C12 myotubes incubated for 24 hours with plasma pooled from septic patients at different timepoints in their ICU admission (62). Importantly, plasma incubation was associated with a concomitant fall in myosin content. We previously reported activation of the ubiquitin-proteasome pathway in skeletal muscle biopsies taken from septic patients (63). As observed for MAFbx and MuRF1, an increase in LC3b, cathepsin B and cathepsin L mRNA levels paralleled the decrease in muscle mass induced by zymosan injection, implying involvement of the autophagy-lysosome pathway in muscle catabolism. These data agree

with previous observations that cathepsin L mRNA and protein level are both increased in skeletal muscle of septic rats (64). Overall, these data support a role for both ubiquitin-proteasome and autophagy-lysosome dependent proteolysis in the zymosan septic model. Myostatin expression did not parallel expression of either ubiquitin-proteasome- or autophagy-lysosome-related genes, suggesting that myostatin signalling is probably not involved in the regulation of these genes.

In summary, we have performed a detailed metabolic, hormonal, muscle functional and histological characterization of a long-term rodent model of critical illness induced by intraperitoneal injection of zymosan, with a novel assessment of muscle functionality. This reflects many, though not all, of the features reported in human critical illness. This model appears useful for investigating changes occurring during critical illness, and for assessing putative therapies that may attenuate loss, or enhance recovery, of muscle mass and function.

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Figure legends:

Figure 1. Effect of zymosan on body mass and food intake.

Rats were studied at 1, 2, 4, and 7 days after i.p. injection of zymosan (30 mg/100 g body mass), n-saline (Sham), or no injection (Naive). Zymosan-injected animals with a clinical score of 0 at 24 hours were excluded from analysis. Change in (a) body mass and (c) cumulative food intake are shown (zymosan-injected animals with a clinical score of zero at 24 hours were excluded from analysis.) Data are expressed as mean (±SD). n=4 (Naive); n=4 (Sham) n=5-8 (Zymosan). Also shown are the effects of zymosan on 12-day body mass and food intake according to clinical score at 24 hours. (b) Change in body mass and (d) total food intake are shown for naïve animals (n=5) and zymosan-treated animals with a clinical severity score of either 0 (n=8) or 1-4 (n=22). Data are expressed as median ± IQR (box) and range (whiskers). ** p<0.01 vs. Naive; ***p<0.0001 vs. Naive; $^{+}$p<0.05 vs. Score 0; $^{++}$p<0.01 vs. Score 0

Figure 2. Effect of zymosan on wet muscle mass and body composition.

Mass of (a) gastrocnemius and (b) soleus muscles taken from the left leg at 2, 4, or 7 days after i.p. Zymosan (n=5-8), n-saline (Sham, n=4), or no injection (Naïve, n=4) are shown. Data are expressed as mean (±SD). Day 12 total body protein (c) and fat (d) mass are shown for animals receiving no intervention (Naïve, n=8) or Zymosan (n=12). Data are expressed as mean (±SD). **p<0.01 vs. Naive; ***p<0.0001 vs. Naive. $^{+}$p<0.05 vs. Sham

Figure 3. Effect of zymosan on atrogene RNA expression in skeletal muscle.

Relative expression of (a) MAFBx, (b) MuRF1, (c) LC3b, (d) Cathepsin B, (e) Cathepsin L, and (f) Myostatin as a percentage of control samples. Data are expressed as mean (±SD). *p<0.05 vs. Control; *p<0.05 vs. Control **p<0.01 vs. Control; $^{+}$p<0.05 vs. Day 2; $^{++}$p<0.01 vs. Day 2

Figure 4. The effect of a single intraperitoneal injection of zymosan on oxygen consumption and respiratory exchange ratio over 7 days. (a) Oxygen consumption, (b) carbon dioxide production and (c) respiratory exchange ratio (VCO_2/VO_2) were measured for 7 days after injection of i.p. zymosan (n=8), i.p. n-saline (Sham, n=4), or no injection (Naïve, n=4). Readings are shown from 12.00 pm on the first day. The insert graphs depict the area under the curve, analysed by ANOVA. Data are expressed as mean (±SD). *p<0.05 vs. Naïve and Sham

Figure 5. Effect of zymosan on physiologic and echocardiographic parameters.

Sequential measurement of zymosan injected (n=6) and naïve (n=6) animals at Baseline, 2, 4, 7, and 14 days of (a) heart rate, (b) stroke volume, and (c) cardiac output were undertaken. Zymosan-treated animals with a clinical score of 0 at 24 hours were excluded. Data are expressed as mean (±SD). *p<0.05 vs. Naïve

Figure 6: Effect of zymosan on forelimb grip strength, exercise capacity, sequential force generation with increasing electrical stimulation, and fatigue studies.

Grip strength was measured at baseline and on Days 2, 5, 8 and 12 after injection of i.p. zymosan (n=9), or no injection (Naïve, n=5) using a grip strength meter (a). Results corrected for body mass (b) are also shown. Treadmill performance time (c), *in vivo* myography (d), and change in the maximal tetanic force generated on submaximal electrical stimulation (e) was measured at baseline, 2, 4 and 7 days following zymosan (n=7-16) and naive (n=7-10), with additional animals undertaking treadmill and myography tests on Day 14 (zymosan n=5-8, naive n=3-4). Data are expressed as mean (±SD). *p<0.05 vs. Naive

Figure 7: Effect of zymosan on plasma electrolyte, hepatic & lipid biochemistry, cytokine, insulin, and leptin levels.

Plasma levels of (a) urea, (b) creatinine, (c) ALP, (d) ALT, (e) total cholesterol, (f) HDL cholesterol, (g) IL-6, (h) IL-10, (i) insulin, and (j) leptin were measured at 6 and 24 hours, and Days 2, 4, and 7 after injection of i.p. zymosan (n=5-10), i.p. n-saline (Sham, n=3-4), or no injection (Naïve, n=3-5). Data are expressed mean (±SD). *p<0.05 vs. Naïve; **p<0.01 vs. Naïve; ***p<0.001 vs. Naïve

Figure 8. Examples of histopathological changes.

Near-normal histology was frequently seen in the septic gastrocnemius (a). Sham soleus samples had mild-moderate change with scattered necrotic fibers (black arrows, b). Areas of clustered necrotic and atrophic fibers with fascial inflammation (black arrow, c) seen in septic soleus. More florid change with myofascitis and confluent necrosis occurred in some septic soleus samples (d).

Figure 9: Effect of zymosan on myofiber diameter of soleus and gastrocnemius.

Mean fiber diameters on Days 2 (n=4), 7 (n=6) and 14 (n=8) following i.p. zymosan are shown in frequency histograms for gastrocnemius (a) and soleus (b) with comparison against naïve specimens (n=4). Sham controls (n=4 per timepoint) are also shown (c and d).

Supplementary Figure 1: Schema of typical myography traces.

Following calibration with a known weight (1), three measures of plantar flexion force are made at sequentially increasing voltages from 1 to 15V (2). Fatigue is generated by applying repetitive electrical impulses with a height and width of 12V and 0.05s respectively set at 40Hz for 3.5 min

(3). Maximum tetanic force ($T^{max)}$ and tetanic force at 2 min ($T^{2\ min}$) can be recorded to calculate the fatigue index such that; Fatigue index = $T^{2\ min}/T^{max}$

Supplementary Figure 2. Effect of zymosan on core temperature and hemoglobin.

Core temperature (a) was measured at 6 and 24 hours after injection of i.p. zymosan (n=7-8), i.p. n-saline (Sham, n=4), or no injection (Naïve, n=4-5). Hemoglobin (b) was also measured at 6 and 24 hours, and Days 2, 4, 7, 12 and 14 after injection of i.p. zymosan (n=5-10) or no injection (Naïve, n=3-5). Zymosan-treated animals with a clinical score of 0 at 24 hours were excluded. Data are expressed mean (±SD). **p<0.01 vs. Naïve; $^{+}$p<0.05 vs. Sham**.**

Supplementary Figure 3: Changes in sequential force generation with increasing electrical stimulation

In vivo myography was performed at baseline (a), Day 2 (b), Day 4 (c) and Day 7 (d) following zymosan (n=16) and no injection (n=10). Further tests were done on Day 14 (e) in the septic (n=8) and naïve group (n=4). Data expressed as mean (±SD). 2-way ANOVA used for data analysis with post-test Bonferroni. * p<0.05, ** p<0.01, *** p<0.001

Supplementary Table 1: Oligonucleotide primers used for PCR analysis

Gene	**GeneBank accession no.**	**Primer sequences 5'–3'**
Actb	NM_031144.2	Fwd: GTC CAC CCG CGA GTA CAA CCT T

(Actin beta)		Rev: TTG CAC ATG CCG GAG CCG TT
Atg5 (Autophagy related gene 5)	NM_001014250.1	Fwd: TGT CTC TGC TGT CCT GTT GG Rev: GGA GCC AAA AAG GAA AAA GG
Bnip3 (BCL2/adenovirus E1B 19kDa interacting protein 3)	NM_053420.3	Fwd: AGA TTG GAT ATG GGA TTG GTC AAG Rev: CCC TTT CTT CAT AAC GCT TGT G
Ctsb (Cathepsin B)	NM_022597.2	Fwd: CCA TCG CAC AGA TCA GAG AC Rev: CCA CAT TGA CTC GGC CAT TG
Ctsl1 (Cathepsin L1)	NM_013156.2	Fwd: CAC AAT GGG GAG TAC AGC AAC Rev: TGT GCT TCT GGT GGC GAT AG
LC3b (microtubule-associated protein 1 light chain 3 beta)	NM_022867.2	Fwd: ACG GCT TCC TGT ACA TGG TC Rev: GTG GGT GCC TAC GTT CTG AT
MAFbx/atrogin-1 (F-box protein 32)	NM_133521.1	Fwd: TCC GTG CTG GTG GGC AAC AT Rev: AAG CAC ACA GGC AGG TCG GT
MuRF1 (Muscle RING finger 1)	NM_080903.1	Fwd: TGC AGC GGA TCA CTC AGG AGC A Rev: TGA GCG GCT TGG CAC TCA GA
Mstn (Myostatin)	NM_019151.1	Fwd: TGA CGG CTC TTT GGA AGA TGA CGA Rev: ATA TCC ACA GCT GGG CCT TTA CCA

Supplementary Table 2: Animal use

		Temperature, muscle mass, 6 & 24 hr echo, biochemistry, cytokines, gut hormones‡[$]	Grip	Body composition[$]	Twitch, treadmill, histology[#]	Sequential echo	Metabolic cart ††
Zymosan	**Used**	37	10** †	12**	24	6	9
	Died/culled	21	5	0	0	4	4
	Excluded*	6	5	3	0	0	1
Naïve		21	5	8	16	6	5
Sham		20	5	–	–	–	5

* On the basis of clinical score 0 at 24 hours

‡ Not all animals were used for all measurements

† One of these animals was unwilling to perform grip strength measurements so was excluded from the subsequent analysis

[$] Hemoglobin concentrations were obtained from animals already counted in the Body composition and Temperature groups, and from 5 additional animals (3 zymosan, 2 naive) from another experiment not listed here

** These groups were combined to obtain the food intake and body mass data (Fig 1)

†† Incomplete data was obtained from one animal in each group and these three animals were not included in the subsequent analysis

[#] Histological analysis included 4 additional naïve animals

Supplementary Table 3: Echocardiographic parameters

	Heart rate (beats/min)		Stroke volume (ml)		Cardiac output (l/min)	
	6 hours	**24 hours**	**6 hours**	**24 hours**	**6 hours**	**24 hours**
Zymosan	433 (29)	479 (29)$^{+++}$	0.29 (0.07)** $^{+++}$	0.22 (0.05)	125 (25)	106 (27) ** $^{++}$
Sham	427 (35)	391 (30)	0.35 (0.02)	0.39 (0.06)	146 (10)	151 (16)
Naive	435 (28)	436 (48)	0.33 (0.04)	0.35 (0.06)	143 (10)	152 (22)

At 6 and 24 hours after i.p. zymosan (n=7-8), i.p saline (sham, n=4) or no intervention (naïve, n=4-5). Data are expressed as mean (±SD). **p<0.01 vs. Naive; $^{++}$p<0.01 vs. Sham: $^{+++}$p<0.001 vs. Sham