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Repetitive TLR-3 Activation in the Lung Induces Skeletal Muscle Adaptations and Cachexia

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Abstract

Due to immunosenescence, older adults are particularly susceptible to lung-based viral infections, with increased severity of symptoms in those with underlying chronic lung disease. Repeated respiratory viral infections produce lung maladaptations, accelerating pulmonary dysfunction. Toll like 3 receptor (TLR3) is a membrane protein that senses exogenous double-stranded RNA to activate the innate immune response to a viral infection. Polyinosinic-polycytidylic acid [poly(I:C)] mimics double stranded RNA and has been shown to activate TLR3. Utilizing an established mouse viral exacerbation model produced by repetitive intranasal poly(I:C) administration, we sought to determine whether repetitive poly(I:C) treatment induced negative muscle adaptations (i.e. atrophy, weakness, and loss of function). We determined skeletal muscle morphological properties (e.g. fiber-type, fiber cross-sectional area, muscle wet mass, etc.) from a

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treated group ((poly(I:C), n=9) and a sham-treated control group (PBS, n=9); age approximately 5 months. In a subset (n=4 for both groups), we determined *in vivo* physical function (using grip test for strength, rotarod for overall motor function, and treadmill for endurance) and muscle contractile properties with *in vitro* physiology (in the EDL, soleus and diaphragm). Our findings demonstrate that poly(I:C)-treated mice exhibit both muscle morphological and functional deficits. Changes of note when comparing poly(I:C)-treated mice to PBS-treated controls include reductions in fiber cross-sectional area (–27% gastrocnemius, –25% soleus, –16% diaphragm), contractile dysfunction (soleus peak tetanic force, –26%), muscle mass (gastrocnemius –19%, soleus –23%), physical function (grip test –34%), body mass (–20%); and altered oxidative capacity (140% increase in succinate dehydrogenase activity in the diaphragm, but 66% lower in the gastrocnemius). Our data is supportive of a new model of cachexia/sarcopenia that has potential for future research into the mechanisms underlying muscle wasting.

Keywords

Aging; Mouse Models; TLR3; Skeletal Muscle; Physical Function; COPD Cachexia

Introduction

Older adults have a diminished immune-response capacity and are more prone to suffer from viral lung infections such as respiratory syncytial virus (RSV) or influenza. These viral infections can progress to pneumonia and/or more severe symptoms especially in older adults with pre-existing conditions such as congestive heart failure (cdc.gov; Murray, 2015; Gavazzi, 2002). Viral-mediated inflammatory responses can also trigger exacerbations of age-related chronic lung diseases (Ritchie, 2015). For example, viral respiratory tract infections are responsible for two thirds of acute exacerbations in COPD (chronic obstructive pulmonary disease), leading to enhanced inflammation, respiratory decompensations, emergency room visits, and accelerating decline in pulmonary capacity (Wedzicha, 2004). There is mounting clinical evidence that frequent exacerbations of disease, caused by viral infections, trigger inflammation-induced remodeling and a decline in pulmonary function (Dransfield, 2016).

We recently developed a mouse model of chronic viral-mediated pulmonary inflammation utilizing repetitive intranasal administration of polyinosinic-polycytidylic acid [poly(I:C)] (Tian, 2016; Tian, 2017). Structurally similar to dsRNA (double stranded RNA), poly(I:C) activates the Toll like receptor 3 (TLR3) pathway. TLR3 is a family 1 membrane protein that helps initiate the innate immune response to viral infection by detecting exogenous dsRNA, or, as in the case of the current study, its mimetic poly(I:C) (Jenson, 2012). Upon dsRNA or poly(I:C) binding to TLR3, it dimerizes and recruits TRIF (TIR domain-containing adaptor protein inducing interferon- β), which in turn recruits TRAF6 (TNF receptor-associated factor 6). Downstream signaling cascades lead to type 1 interferon transcription, and activate NF- κ B (Nuclear factor kappa-light-chain-enhancer of activated B cells) to initiate cytokine production (Jenson, 2012).

Acute treatment with poly(I:C) induces significant pulmonary inflammation and reduction of lung function (Stowell, 2009), edema with similarities to RSV in mice (Aeffner, 2011), increased production of chemokines/cytokines, infiltration of the lungs by neutrophils and lymphocytes (primarily natural killer cells), and gene expression signatures relevant to COPD (Harris, 2013). Poly (I:C) administration also induces the bronchial epithelial cell apoptosis (Koizumi, 2016), and airway remodeling in a rat asthma model (Takayama, 2011). Repetitive treatment causes additional airway remodeling, pulmonary fibrosis, lung structural changes, and loss of body mass in mice (Tian, 2017).

In previous work with the repetitive poly(I:C) administration model, we noted a decline in body mass in the treated mice (Tian, 2017). Cachexia develops in 20–40% of patients with COPD (Remels, 2013), and can have similar consequences to sarcopenia [impaired quality of life, difficulties with activities of daily living, increased morbidity and mortality (Sanders, 2016)]. We hypothesized that chronic induction of acute viral-like TLR3 activation using poly(I:C) treatment would create a model for the mechanistic study of lung damage-associated cachexia. Investigation into the etiology of pulmonary disorder-associated cachexia credits many potential causes [e.g. reduced activity, hypermetabolism, high levels of systemic inflammation, increased catabolism, anabolic resistance, oxidative stress, and inefficient post-prandial splanchnic extraction of amino acids] (Fermoselle, 2012; Gan, 2004; Langen, 2013; Lemire, 2012; Van Remoortel, 2013; Wüst, 2007). With limited current treatment strategies for cachexia, research on disease etiology, progression and treatments are of paramount importance. In the current study, we sought to characterize skeletal muscle adaptations in a preclinical model of repetitive pulmonary viral-like TLR3 activation.

Methods

Animals

All animal experiments by were approved the University of Texas Medical Branch Institutional Animal Care and Use Committee, and experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Experimental Animals. Figure 1 illustrates the study design.

The mice used for this study were male C57BL/6, aged approximately 5 months. Experimental mice were treated with 500 µg of poly(I:C) (in 50 µl PBS, phosphate buffered saline) via intranasal administration on alternating days (total of 15 times) for a period of 30 days. Control mice received intranasal administration of 50 µl PBS (phosphate buffered saline) on alternating days (total of 15 times) for a period of 30 days. For immunohistochemical and wet muscle mass analysis we had n=9 for both groups. For the functional and contractile measurements, we used a subset from each of the two groups, n=4 per group. Further details on the model and the treatment regimen have been previously published (Tian, 2016, Tian 2017). Animals underwent functional testing one week after their last poly (I:C) treatment and then were sacrificed at approximately 2 weeks after the last treatment.

Tissue Collection

At sacrifice, the soleus (SOL), extensor digitorum longus (EDL), and diaphragm were collected and kept viable for contractile physiology. Other skeletal muscle [gastrocnemius (Gastroc), plantaris] and additional diaphragm portions were immediately mounted on cork in optimal temperature cutting compound (Tissue-Tek #4583), pinned at near optimal length and then frozen in liquid nitrogen-cooled 2-methylbutane (isopentane) or flash frozen in liquid nitrogen. The muscles were stored at -80°C until used for analysis. After establishing the viability of one of the soleus muscles for *in vitro* contractile physiology, the other soleus was frozen in isopentane as noted above for immunohistochemistry. The Gastroc that was frozen in liquid nitrogen was homogenized in buffer (50 mM Tris, 250 mM mannitol, 50 mM NaF, 5 mM sodium pyrophosphate (NaO_7P_4), 1 mM ethylenedinitrilo tetraacetic disodium salt (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 1% V/V Triton X-100, 1 mM dithiothreitol (DTT), 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 5 $\mu\text{g}/\text{ml}$ soybean trypsin inhibitor; pH 7.4) at a 1:9 ratio (mass, mg: volume, μl) of muscle to buffer; and the protein concentration was determined utilizing the Bradford assay (Biorad #500-0006) on a 96-well plate reader (Biorad iMARK).

Functional Capacity

To determine the functional ability of the mice we incorporated three commonly used assessment tools: rotarod, grip and treadmill tests. Procedures for the rotarod (Graber, 2013) and the grip test (Fry, 2015) have been previously published, however a brief description follows. All functional testing was performed by the same individual.

Rotarod Test (overall motor function)—We used a Panlab LE 8205 rotarod to measure the motor function (balance, coordination, stamina, power production) of the mice. The mice were acclimated to the device over 2 practice sessions consisting of 1 session per day with 3 trials of varied protocols per session (see Graber, 2013 for details). This was followed by the testing day where the mice ran on the device as it accelerated from 4 to 40 rpm over 5 minutes; the outcome measure was latency to falling. We report the best of three trials, each trial administered with a minimum 15 minutes rest period.

Grip Test (upper limb strength)—We tested the mice for strength with a Bioseb GT3 model grip strength tester using the trapeze style grip. Our standard operating procedure is based upon the published and oft used protocol from the European Mouse Phenotyping Resource of Standardised Screens (EMPreSS, 2007), and was published previously (Fry, 2105). For one trial, the mouse is removed from its cage, gently held by the tail and positioned so that its paws grasp the bar/grid in a pronated grip. Then the mouse is smoothly pulled backwards until it releases the bar. The highest of 5 trials was recorded as max grip strength and reported herein.

Treadmill Test (endurance/volitional fatigue)—A PanLab LE 8710 treadmill was used to determine the endurance capacity of the mice. We acclimated the mice to the device with two daily sessions (one trial of walking for a 5-minute maximum at 5 cm/s on day one; two trials on day two, trial one walking for 5.5-minute maximum at 5 cm/s, and trial two the same as the test day protocol but with a maximum time of 5.5 minutes) before a test day in

which the mice ran on the treadmill as it accelerated by 1 cm/s per 20 seconds starting at 5 cm/s. The trial was terminated after the mice had touched the shock pad (set at 0.6 mA) 3 times. This protocol was adapted from previously published work (Marcaletti, 2011).

Contractile Function

Both general details of the procedure and those specific to the SOL, EDL, and diaphragm have been previously published (Graber, 2015; Graber 2013; Durham, 2004). In brief, we isolated the SOL, EDL and diaphragm from the mice, maintaining viability in Krebs-Ringer buffer perfused with 95% O₂/5% CO₂ at 25°C. The SOL and EDL were tied with #4 suture line tied at the myotendinous junctions, with the distal tendon attached via the line to a static clamp and the proximal origin tendon attached to the force transducer. For diaphragm studies, the diaphragm was carefully extracted from the mouse and fiber bundles were cut parallel to the longitudinal axis of the fibers to form a span from a small section of rib to the central tendon. The rib was tied to a metal bracket with #4 suture line. Likewise, silk suture was carefully tied to the central tendon, for later attachment to the force transducer. We then mounted the diaphragm by securing the metal bracket to the static clamp and tying the silk suture line to the force transducer arm. Upon completion of physiology testing, diaphragm fiber bundles were removed from the rib, weighed, and flash frozen.

Using an Aurora Physiology system (Model 6650LR Force Transducer, Dual Mode Lever System, Hi-power Bi-Phase Stimulator, Signal Interface, and software: Dynamic Muscle Control v5.500 and Dynamic Muscle Analysis version 5.300) we then measured peak twitch force (P_t) and peak isometric force (P_0). The length of the muscle from the origin myotendinous junction to the insertion myotendinous junction at P_t was L_0 (optimal muscle length). The muscle was kept at L_0 throughout the protocol. P_0 was determined by generating a force-frequency curve. Exact protocols for the EDL and SOL were described previously, and we used the same procedures for DIA as for the SOL (Graber, 2015; Graber 2013).

Muscle Size

To measure wet mass, the muscles were blotted dry and massed prior to being frozen.

Immunohistochemistry

These techniques have previously been published (Fry, 2015). In brief: The muscles were cut into 7-micron sections on a cryostat (ThermoScientific HM525-NX) and mounted on slides. For fiber typing, unfixed slides were incubated overnight at room temperature with antibodies against myosin heavy chain (MyHC) isoforms type 1, type 2a and type 2b (#BA.D5, SC.71 and BF.F3 from Developmental Studies Hybridoma Bank) and laminin (#L9393 Sigma Aldrich). The next day slides were incubated with species- and isotype-specific fluorescent secondary antibodies (#A21242, A21121, A21426 and A21068, ThermoFisher) followed by a post-staining fixation in methanol. Fiber type was manually assessed by determining the predominantly expressed MyHC isoform per fiber, and fibers were denoted as Type 1 (pink), 2a (green), 2b (red) or 2x (unstained), with one blinded researcher doing all assessments. Type 2x primary antibody was not applied to the sections,

and Type 2x expression was assumed from fibers that did not stain positive for either Types 1, 2a or 2b.

For muscle cross-sectional area staining, slides were fixed in 4% paraformaldehyde (PFA) for 10 min at RT. Slides were then incubated with AF488-conjugated wheat germ agglutinin for 2 hr at RT (# W834, ThermoFisher). Mean myofiber and binned distribution cross-sectional area (CSA) were determined through semi-automated analysis using AxioVision (Zeiss v 4.9.1).

For laminin, capillary and arteriole staining, slides were fixed in ice-cold acetone and blocked in normal horse serum. Slides were incubated overnight in primary antibodies against CD31 (#550274, BD Biosciences), α SMA (#sc-130616, Santa Cruz Biotechnology) and laminin at 4°C. The following day, slides were incubated in secondary antibodies (#A10522, A21131, A21244, ThermoFisher) and then mounted. Capillary and arteriole density were determined through semi-automated analysis of capillary and arteriole content and normalized to number of fibers or per 100 fibers, respectively.

For succinate dehydrogenase (SDH) staining, slides were unfixed and incubated for 1hr incubation at 37°C in the following solution: nitro blue tetrazolium (Sigma) and succinate acid disodium (Sigma), dissolved in 0.2 M of phosphate-buffered saline (PBS). The reaction was performed in a light protected coplin jar, and sections were then sequentially rinsed with 30 and 60% acetone before being mounted with aqueous mounting media. Images were manually assessed for SDH staining intensity, and fibers were categorized as strongly positive, weakly positive, or negative (++ , + , -), by a single blinded researcher.

Image Acquisition and Analysis

Images were captured at RT at 100X, 200X or 400X total magnification with a Zeiss upright M1 Imager microscope and analysis conducted using the AxioVision Rel software (v 4.9.1). Image analysis was performed blinded to experimental group.

Lung Histology Examination

Mice were sacrificed two weeks later after the last poly(I:C) administration to allow resolution of the acute inflammation. For lung histological examination, animals were anesthetized and lungs were inflated under 25 cm H₂O pressure with 10% (v/v) neutral buffered formalin through the tracheal cannula and immersed in 10 % buffered formalin for at least 24 h. After being processed into paraffin blocks, the lungs were cut into 5- μ m sections and stained with Masson Trichrome to assess lung histological changes. The lung histological images were taken at magnifications of 100X, 200X, and 400X respectively on a NIKON Eclipse Ti System (Tian, 2016; Tian, 2017).

Western Immunoblotting

We used standard Western Blotting technique to probe for the protein expression levels of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α ; ABCAM #54481). The primary antibody was IgG of rabbit origin and diluted at 1:1000 in blocking buffer (tris buffered saline with 3% bovine serum albumin). We used a 1:2000 dilution of

donkey anti-rabbit horse radish peroxidase conjugated IgG secondary antibody (GE Healthcare ECL # NA934V) with Super Signal Dura West (ThermoScientific, #80196) as the substrate developing agent.

Statistics

Data are reported as means $\pm$ standard error or as percent change $\pm$ standard error, as appropriate. Significance was set at $p < 0.05$, and trends are reported at $0.05 < p < 0.10$. Independent samples 2-tailed t-tests determined differences between the 2 groups (poly(I:C) and PBS). Effect size is reported for some measures with $d > 0.08$ considered a strong effect and calculated using $d = (\text{difference in means})/(\text{pooled standard deviation})$ with the G*Power 3.1.9.2 (Franz Faul, Universitat Kiel, Germany) software package. We used the Kolmogorov-Smirnov test to determine if the pooled fiber size distributions, and the Mann-Whitney U-test to determine if the mean ranks, were different between the two groups ($\alpha = 0.05$). Statistical analysis was performed using SPSS v23 (IBM).

Results

Repetitive intranasal poly(I:C) treatment of wild-type C57BL/6 mice induces airway epithelial injury, interstitial collagen deposition, and mesenchymal transition (Tian, 2017; and Figure 2). Chronic poly(I:C) administration also results in gene expression signatures common in viral-exacerbations (Tian, 2016). In addition to the severe pulmonary fibrosis and airway remodeling (the right panel, Figure 2), poly(I:C) administration also induces structural airway degradation, including alveolar destruction, thickening and hypercellularity of the alveolar septa (two middle panels, Figure 2).

We observed in previous work that poly(I:C)-treated mice lost a significant fraction of body weight (Tian, 2017), and focused the current study on skeletal muscle and functional adaptations after chronic poly(I:C) administration.

Functional Capacity (See Figure 3)

We determined that there was an overall reduction in functional capacity in the *Grip Test* (upper limb strength, $p < 0.05$) and a trend for reduced capacity in the *Treadmill Test* (endurance/volitional fatigue, $0.05 < p < 0.10$), but there was no change in *Rotarod Test* (overall motor function, $p > 0.10$).

Skeletal Muscle Contractile Function (Figure 4 and Table 1)

In SOL (a mixed fiber-type muscle, $n=4$, used for *in vitro* contractile physiology), mass, physiological cross-sectional area and peak isometric force (effect size $d=3.19$) were reduced ($p < 0.05$); but there was no reduction in specific force. We discovered a trend for lower twitch force in poly(I:C) SOL, but specific twitch force was not different. There were no significant reductions in the poly(I:C)-treated mice in the fast twitch fiber type EDL (See Table 1) ($p=0.145$, with effect size $d=1.18$). Specific force in the poly(I:C) diaphragm also did not change significantly ($p=0.209$, effect size 1.01) (See Table 1).

Muscle Atrophy

Muscle Wet Mass and Body Mass (Figure 5)—There was atrophy exhibited in the Gastroc, TA, SOL, and plantaris (n=9 per group, all $p<0.05$, effect size $d=1.78, 1.64, 2.39$, and 3.35 , respectively), but not in the EDL (n=4, $p>0.05$). Body mass (n=9, $p<0.05$, effect size $=2.02$) was reduced in poly(I:C) compared to PBS. Relative peripheral muscle mass (muscle mass in mg/body mass in grams, n=4 per group) was significantly lower with poly(I:C) treatment in the SOL (PBS 0.350 ± 0.005 and poly(I:C) 0.331 ± 0.007 ; -5.6% , $p=0.020$) and plantaris (PBS 0.350 ± 0.005 and Poly(I:C) 0.331 ± 0.007 ; -17% , $p=0.020$), but not in the TA, Gastroc, or EDL.

Muscle Fiber Cross-Sectional Area (Figure 6, Figure A1, Table A1)—In comparison to PBS, Gastroc, plantaris and diaphragm from poly(I:C)-treated mice had lower mean muscle fiber cross-sectional areas of -27 , -28 and -16% (all $p<0.05$, effect size $1.95, 1.18$, and 1.92 , respectively), and lower median fiber areas of -18.2 , -30.0 and -6% respectively (all $p<0.05$). SOL cell area means tended to be lower by 25% in the poly(I:C)-treated group ($0.05<p<0.10$, effect size $d=1.39$) and the median -26.2 lower ($p<0.05$). The distributions (**Figure A1**) for SOL, plantaris, and Gastroc and diaphragm were all significantly different ($p<0.001$), between the two groups indicating a leftward shift and a higher percentage of smaller fibers in the poly(I:C)-treated mice. The mean ranks were significantly different for all the tested muscles as well (all $p<0.001$). See Appendix A. Figure A1 and Table A1 for further details, including skew and kurtosis of distributions.

Muscle Oxidative Capacity (Figure 7 and Table B1)

Fiber Type Shift—The Gastroc, plantaris, SOL (n=9 muscles per group examined for each) and diaphragm (n=4 per group) were tested for fiber type (1, 2a, 2x and 2b) percentage. There was no significant difference in hind limb muscle fiber type distribution, see data in **Table B1** in Appendix B for specific details. Significant differences were found in the diaphragm, with 97% fewer type 2b fibers and a concomitant 23% greater frequency of 2x fibers in poly(I:C).

Succinate Dehydrogenase (Figure 8)—Succinate dehydrogenase is a marker of mitochondrial activity as this enzyme functions in the citric acid cycle and as Complex 2 in the electron transport chain. Thus, lower activity would tend to suggest reduced mitochondrial density and/or activity. In poly(I:C)-treated animals, we found a significantly lower frequency of highly SDH positive fibers in the hind limb Gastroc and a 140% greater frequency of highly SDH positive fibers in the respiratory diaphragm muscle. SOL and plantaris muscles showed no effect of poly(I:C) treatment.

Capillary and Arteriole Content (Figure 9)—We examined the capillary density and arteriole content of the diaphragm, Gastroc, SOL and plantaris (n=4 per group per muscle). The diaphragm of poly(I:C)-treated mice showed 88.4% more ($p=0.005$) capillaries, whereas the Gastroc muscles had 16.7% fewer capillaries ($p=0.036$) than the PBS samples. There was no difference in in capillary content in the other muscles or in arteriole content.

PGC-1 α , Biomarker of Mitochondrial Biogenesis (Figure 10)—Poly(I:C)-treated mice showed a subtle, non-statistically significant reduction in PGC-1 α expression in Gastroc (–15% PGC-1 α density, n=4; p=0.300) when compared to PBS.

Discussion

Acute poly(I:C)-mediated viral-like TLR3 activation has been shown to negatively impact lung function and morphology, and to activate the innate immune system (Stowell, 2009; Aeffner, 2011; Harris, 2013; Koizumi, 2016; Takayama, 2011). Chronic administration of poly(I:C) has demonstrated that airway remodeling, fibrosis, unique gene expression signatures, and lung structural changes (Tian, 2017, Tian, 2016). In Figure 2 of the current study, we show that repeated TLR3 stimulation results in lung damage similar to that observed in chronic lung diseases. We have previously shown that repetitive poly(I:C) treatment resulted in a loss of body mass (Tian, 2017). Building off of our previous findings, we sought to determine if repeated TLR3 stimulation via poly(I:C) treatment would result in a skeletal muscle cachexic phenotype that might present similarly to cachexia observed in COPD patients.

COPD patients have an increased risk for osteoporosis, cardiovascular disease and loss of peripheral muscle mass (Barnes, 2009; Gan, 2004). The loss of fat-free mass alone is a risk factor for mortality independent of airflow capacity (Sanders, 2016). COPD-related cachexia develops in 20–40% of patients (Remels, 2013), and can have similar consequences to sarcopenia (Sanders, 2015). There is mounting clinical evidence that frequent exacerbations of disease, caused by viral infections, trigger inflammation-induced pulmonary remodeling and a decline in function (Dransfield, 2016). COPD represents a spectrum of heterogeneous degenerative conditions of the lung associated with structural remodeling of the airway, primarily chronic bronchitis and emphysema (Celli, 2004). COPD is an age-related disease with clinical development typically starting at middle-age or later, with increased severity and a higher prevalence noted with advancing age (Abramson, 2014). COPD is also the fourth largest cause of death globally, and the third largest in the United States (Adeloye, 2015; CDCD Website; NIH NHBLI website; Rycroft, 2012). One hallmark of COPD, is the progressive loss of exercise capacity (Punekar, 2017; Spruit, 2017). Beyond changes in structure and function of lung tissues, there are skeletal muscle maladaptations that contribute to the loss of functional capacity. Notably, peripheral and respiratory muscles share some phenotypic changes, but can also manifest divergent adaptations (Caron, 2009; Doucet, 2004; Levine, 2013).

In the current study, we show poly(I:C)-treated mice exhibited significant muscle and myofiber atrophy that was accompanied by weakness, as assessed by both *in vitro* and applied functional measures. Additionally, poly(I:C) treatment elicited alterations in skeletal muscle fiber type distribution, mitochondrial functional capacity, and capillarization. The differential adaptations in hind limb and respiratory muscles of the poly(I:C) treated mice model human COPD cachexia in some respects (Caron, 2009; Levine, 2013). Below we discuss the parallels and differences in skeletal muscle adaptation in our repeated poly(I:C) lung damage model and human COPD-associated cachexia.

Functional Capacity and Muscle Contractile Function

We employed a battery of applied functional measures to evaluate the impact of repetitive poly(I:C) treatment and relate it to human disease presentation. Poly(I:C)-treated mice demonstrated a trend for reduced endurance (–42% time to failure during treadmill exercise, non-significant at $p=0.096$, but with a large effect value of $d=1.39$ indicating a large practical significance for this difference), reduced forelimb strength (significant –34% force production during grip testing, $p=0.049$ with a large effect value of $d=1.72$), and a non-significant –14% loss in overall motor function (rotarod). Overall, these results provide evidence that chronic poly(I:C) administration impairs mouse functional capacity. More research is needed to determine underlying mechanisms driving our observed functional deficits.

Complementary assessments of *in vitro* contractile performance, morphological characteristics, and fiber type distribution (see below) in both respiratory (diaphragm) and limb muscles provide further insight on the etiology of the impaired functional performance in poly(I:C)-treated mice. These treatment-induced adaptations are broadly consistent with findings in human COPD patients and other animal models of chronic airway obstruction. Muscle fiber atrophy in poly(I:C)-treated mice was evidenced by a shift in fiber size distribution toward a smaller cross-sectional area profile in both respiratory and limb muscles. Consistent with this shift, limb muscle mass and mean fiber size were also lower in most of the muscles examined. For example, the SOL, a chronically active postural muscle, had significantly lower mass which also manifested as a corresponding significant loss of maximal force production. Specific force did not change in the SOL, or the other muscles we studied, suggesting that changes in muscle force production were largely due to changes in muscle mass (i.e. atrophy) rather than changes in muscle (contractile) quality.

Muscle Atrophy

Previous studies have demonstrated that the quadriceps muscle fiber CSA is reduced 25–30%, while the diaphragm muscle fibers have been reported to atrophy 40–60% in COPD patients with severe emphysema (Caron, 2009). A 30% loss in MHC content has also been reported in patients presenting with a more moderate to mild manifestation of the disease (Caron, 2009). In the present study, poly(I:C) treatment resulted in peripheral limb muscle and diaphragm atrophy, as measured by both reduced muscle mass and fiber cross-sectional area, that resembles clinical manifestation of COPD-related cachexia (Remels, 2013).

Muscle Oxidative Capacity and Fiber type

Muscle is classified into different fiber types by MyHC. The typical designations in mice, from least oxidative capacity to most, include the highly glycolytic 2b, the glycolytic 2x, oxidative/glycolytic 2a and the oxidative type 1. COPD-associated skeletal muscle adaptations involve an increase in the oxidative capacity for the diaphragm and a reduced oxidative capacity in the limb muscles (Gea, 2015). Fiber type shifting has been observed in both peripheral and respiratory muscles of individuals with COPD, with the diaphragm exhibiting a relative shift from type 2 to type 1 (Doucet, 2004) and the quadriceps changing from type 1 to type 2 (Gosker, 2002). Gosker and colleagues also found that the proportion of Type 1 fibers in the quadriceps is markedly lower in COPD patients compared to controls.

Our poly(I:C)-treated mice demonstrated shifting fiber type shift in the diaphragm, resulting in relatively fewer type 2b fibers and more 2x fibers. Repeated poly(I:C) treatment did not induce a shift in limb muscle fiber type distribution significantly within the time course of our treatment.

Furthermore, poly(I:C) mice had alterations in oxidative capacity, namely a differential shift in succinate dehydrogenase activity in limb (66% lower in the Gastroc) and respiratory (140% higher in the diaphragm) muscles as compared to controls. Succinate dehydrogenase, or electron transport chain complex 2, is an enzyme in the mitochondria that acts in the citric acid cycle to oxidize succinate to fumarate and in the electron transport chain to oxidize ubiquinone (coenzyme Q) to ubiquinol (Rutter, 2010). Thus, succinate dehydrogenase activity can be used as a rough indicator of mitochondrial-based metabolism, with greater activity indicative of enhanced oxidative capacity. Another indirect marker of mitochondrial capacity is the expression of transcriptional coactivator PGC-1 α . PGC-1 α is considered the master regulator of mitochondrial biogenesis, and modulates mitochondrial remodeling (Austin, 2012). PGC-1 α also plays a role in the biogenesis of peroxisomes, organelles that break down complex fatty acids into smaller chains that are then exported to the mitochondria where these smaller lipids chains are broken down and ultimately metabolized to create energy (Austin, 2012). In the current study, the difference in PGC-1 α expression in the Gastroc between PBS- and poly(I:C)-treated mice was indeterminate (-15.5%, nonsignificant; $p=0.30$, see Figure 9). However, after eliminating a single poly(I:C) outlier, we observe a trend for a 26% difference in the poly(I:C)-treated mice, $p=0.078$.

Another hallmark of respiratory capacity is capillary and arteriole density. Greater capillary density allows for greater delivery of oxygen to skeletal muscles. In COPD cachexia, peripheral limbs have reduced capillary density, while the diaphragm shows increased capillarization (Gea, 2015). The diaphragmatic adaptations likely stem from an increased workload to overcome the less compliant and fibrotic lung. Conversely, as the peripheral limb muscles become more glycolytic, the reduction in capillary density would reflect an opposing adaptation indicative of reduced oxidative capacity. Our poly(I:C)-treated mice demonstrated a similar adaptation in the gastrocnemius and diaphragm, although the hind limb plantaris and soleus showed no change. We detected no change in arteriole density, which is unsurprising given their relatively low abundance.

The limb muscles demonstrated a less robust fiber type/metabolic response to poly(I:C) treatment. There are two factors that may have contributed to this: 1) variability in the response to poly(I:C) treatment, and 2) the relatively short duration of the treatment. While a shorter induction time is a strength of the model, alterations in fiber type distribution take time, and may not be evident to the same degree as atrophy with a relatively short experimental period. COPD-associated muscle maladaptations in humans occur incrementally over years/decades and even longer-term mouse models, such as the cigarette-smoke model, require up to six-eight months to manifest cachectic phenotypes (Hopkinson, 2007; Maltais, 2014; Kruger, 2015; Hansen, 2013; Vlahos, 2015; Marcolino 2017). Future studies will extend the period of treatment to establish the time course of peripheral muscle adaptation.

Caveats

In humans, COPD pathology develops insidiously over many years, typically being diagnosed in middle or later life. In the current proof-of-concept study of chronic poly(I:C)-treated mice as a model of COPD-associated cachexia, the mice were treated for a relatively short period of time. Thus, although our recent studies suggest that this model reproduces important aspects of human disease, additional research will be necessary to document the full extent to which this model portrays human pathology. Nevertheless, our findings of functional decline, contractile deficit, muscle atrophy, and shifts in oxidative capacity in the setting of an overall reduction in body mass correspond with changes observed in human presentation of COPD-associated cachexia and other chronic lung diseases. While most animal models do not perfectly model human disease, we suggest that the evidence in this exploratory study validates repetitive poly(I:C) treatment as a viable model of COPD-associated cachexia. Future studies on the time course of disease progression and mechanisms will be informative. As the treatment was only for one month, there may not have been sufficient time for discernable adaptations to occur in peripheral muscles (e.g. MyHC has a very long half-life), therefore another future goal will be to extend the treatment period to determine the degree of peripheral muscle deficits. In addition, although our primary focus is on the effect of repeated intranasal poly(I:C) administration on skeletal muscle, not on the lungs, more work is also needed to more completely determine and document the effect of repetitive poly(I:C) administration on both the lungs and overall physiology of the mice.

Another caveat in this study is that with the relatively small 'n', we did not separate cachexic from non-cachexic poly(I:C)-treated mice (only poly(I:C)-treated versus PBS sham-treated). For example, within our functional subset of 4 poly(I:C)-treated mice, one mouse appeared to exhibit severe frailty (kyphosis, tremors, very low body mass, and poor outcome measurements across all the tests), two of the mice appeared to be more mildly affected, while one mouse appeared to be relatively unaffected (non-cachexic). This suggests substantial biological variability. We will be expanding this study utilizing more animals in future work to understand this biological variability, potentially enabling mechanistic understanding of the causes of cachexia, and allowing us to control for these variables to produce more consistent changes. One source of this variability could include general malaise induced by the treatment, thus monitoring habitual activity and food intake during treatment will be needed to differentiate what part of the cachexic effect arises from these two variables.

Conclusion

We observed adaptations in function, muscle contraction, atrophy, fiber type, and oxidative capacity that model human presentation of COPD-associated cachexia in many respects. Future studies will to determine the effects of poly(I:C) treatment in older mice to determine if sarcopenia and frailty onset are enhanced and severity exacerbated. We suspect that COPD cachexia comorbidity contributes to frailty and sarcopenia in the human population, so this may become a valuable model to examine the interaction between those syndromes and COPD cachexia. Our future work will also include dose-response experiments to extend the

period of illness out to 3–4 months so that we can evaluate various intervention treatment strategies designed to mitigate cachexic progression and to determine the mechanisms underlying this form of muscle wasting. We conclude that the evidence presented in the current study suggest that chronic poly(I:C) treatment may induce a phenotype in mice that could prove to be a valuable model to mimic COPD cachexia.

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Abbreviations

COPD	Chronic obstructive pulmonary disease
EDL	extensor digitorum longus
fl	fiber lengths
Gastroc	gastrocnemius
IFN- β	interferon-beta
P_{max}	maximum power output
mTORC1	mechanistic target of rapamycin complex 1
mN	milliNewton
MuRF-1	Muscle ring finger-1
MyHC	Myosin Heavy Chain
PBS	phosphate buffered saline
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
P₀	peak isometric force
P_t	peak twitch force
%P₀	percentage of maximum force
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
poly(I	C), polyinosinic-polycytidylic acid

L₀	optimal length of muscle
RSV	Respiratory syncytial virus
SOL	soleus
TRIF	TIR domain-containing adaptor protein inducing IFN- β
TRAF6	TNF receptor-associated factor 6
TLR3	Toll like receptor 3

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Appendix A: Muscle Cell Cross-Sectional Area Distributions

Figure A1. Pooled Distributions by Muscle

Error bars equal standard error of the percent of total from each measured muscle (n=9 for each group, except for diaphragm with n=4 per group). * = statistically significant differences in pooled distributions with p<0.05 (see **Table A1** for exact statistics).

Table A1

Distribution Statistics.

Pooled Distribution Statistics by Muscle and Group												
Muscle	Group	n	K-S p-val	Mean	T-test p-val	Median	Mean Rank	M-W p-val	Min	Max	Skew	Kurtosis
Soleus	PBS	2226	<0.001	1211.9±16.0	<0.001	1025.5	2404.9	<0.001	85.3	7720.2	2.35±0.05	10.34±0.10
	poly(I:C)	2133		950.8±10.1		839.1	1945.3		275.4	72.0	2.11±0.05	15.75±0.11
Plantaris	PBS	2280	<0.001	1412.6±17.9	<0.001	1249.1	3208.6	<0.001	250.0	8328.4	1.62±0.05	5.50±0.10
	poly(I:C)	3161		999.0±9.7		860.8	2369.3		43.6	4892.9	1.71±0.04	5.26±0.09
Gastroc.	PBS	5241	<0.001	2204.3±12.1	<0.001	2149.6	8063.8	<0.001	270.0	7287.9	0.95±0.03	2.74±0.07
	poly(I:C)	7722		1586.8±8.5		1504.1	5408.4		257.0	6973.8	0.59±0.03	0.50±0.06
Diaphragm	PBS	2407	<0.001	978.0±11.4	<0.001	847.0	2246.4	<0.001	250.9	7437.3	2.87±0.05	16.90±0.10
	poly(I:C)	1871		835.4±7.6		795.9	2002.0		250.0	3123.1	0.94±0.06	2.20±0.113

Data is ± standard error. K-S = Kolmogorov-Smirnov; M-W = Mann-Whitney U-Test. Bold indicates statistical difference between PBS and poly(I:C) groups.

Appendix B Table B1 Peripheral Fiber Type Distribution by Muscle

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Table B1

Fiber Type Distribution by Muscle

Muscle	Percentage of Total Fiber Count by Fiber Type											
	Gastrocnemius (n=9)				Plantaris (n=9)				Soleus (n=9)			
MHC Type	1	2a	2b	2x	1	2a	2b	2x	1	2a	2b	2x
PBS Mean	1.33±0.35	10.00±1.54	82.17±1.90	6.50±1.10	1.68±1.07	38.04±2.94	44.74±3.61	15.54±1.11	32.71±0.91	55.99±2.26	2.15±0.83	9.15±2.38
poly(I:C) Mean	1.86±0.31	13.17±1.64	79.56±2.11	5.41±0.42	0.83±0.23	43.71±2.89	41.52±3.43	13.93±1.52	35.70±1.63	55.26±2.32	1.28±0.53	7.77±1.47
% Difference	+40%	+32%	-3%	-17%	-51%	+15%	-7%	-10%	+9%	-1%	-40%	-15%
p-val. (t-test)	0.269	0.177	0.370	0.366	0.512	0.104	0.801	0.962	0.128	0.822	0.389	0.628

Data is means ± standard error. Percent change is due to treatment in poly(I:C) compared to PBS. P-value is taken from an independent samples t-test. **Bold underlined text** indicates p<0.05.

Highlights

1. Repetitive intranasal poly(I:C) treatment induces a loss of body mass in addition to significant muscle atrophy.
2. Poly(I:C)-treated mice exhibit muscle atrophy and loss of body mass, as well as some contractile and functional deficits.
3. Phenotypic changes in poly(I:C)-treated mice have similarities to human cachexia pathology associated with chronic lung disease, with differential responses in limb and respiratory muscles.

Figure 1. Study Design

C57BL/6 mice at approximately 5 months of age were subjected to n=15 challenges with intranasal poly(I:C) (polyinosinic-polycytidylic acid), or PBS (phosphate buffered saline), every other day for a total of 30 days. One week after the last poly(I:C) treatment, the mice were tested for function with the rotarod (overall motor function), treadmill (endurance), and grip test (forelimb strength). Two weeks after last poly(I:C) treatment, tissue was harvested for contractile physiology and downstream experimentation.

Figure 2. Repetitive Poly(I:C) Treatment Induces Airway Remodeling

Lung morphological changes: alveolar destruction, thickening and hypercellularity of the alveolar septa. Masson Trichrome staining of lung sections from PBS treated (left panel) or poly(I:C)-treated mice (middle and right panels). The lung histological images were shown at magnifications of 100X, 200x, and 400X respectively.

Figure 3. Functional Measurements

A. No Change in Rotarod (overall motor function). **B.** Treadmill (endurance) tended to be reduced by 42% in poly(I:C). **C.** Grip (forelimb strength) was 34% lower in poly(I:C). Each symbol [circle (PBS) or diamond (poly(I:C))] = one mouse. Rectangles = means. Error bars on means = standard error. * = $p < 0.05$.

Figure 4. Muscle Contractile Function in Soleus

A. Specific Force Unchanged **B.** -26% Peak tetanic isometric force (P_0) in poly(I:C). Each symbol [circle (PBS) or diamond (poly(I:C))] = one muscle from one mouse. Rectangles = means. Error bars on means = standard error. mN=milliNewton, * = p<0.05.

Figure 5. Muscle Wet Mass

In 4 of the 5 muscles examined there was significant atrophy with poly(I:C) treatment. Body mass was also decreased. EDL had n=4 per group, but all others had n=9 per group. EDL= extensor digitorum longus, TA = Tibialis Anterior. Each symbol [circle (PBS) or diamond (poly(I:C))] = one mouse, rectangles = means. Error bars on means = standard error. * Significant effect of poly(I:C) treatment (p<0.05).

Figure 6. Muscle Fiber Cross-Sectional Area

A. Average muscle fiber CSA measured in μm^2 . * Significant effect of poly(I:C) treatment ($p<0.05$). * Significant effect of poly(I:C) treatment ($p<0.05$). Each symbol [circle (PBS) or diamond (poly(I:C))] indicates count from an individual muscle sample, rectangle = mean value, error bars = standard error. **B.** Representative images (top poly(I:C), bottom PBS) demonstrating glycosaminoglycan used to assess gastrocnemius muscle fiber cross-sectional area (CSA).

Figure 7. Muscle Fiber Type

A. Relative distribution of fiber type in the diaphragm. * Significant effect of poly(I:C) treatment ($p < 0.05$). Each symbol [circle (PBS) or diamond (poly(I:C))], indicates count from an individual muscle sample, rectangle = mean value, error bars = standard error. **B.** Representative image demonstrating fiber type in mouse gastrocnemius muscle [myosin isoforms: Type 1 (pink), Type 2a (green), Type 2b (red), Type 2x (black)].

Figure 8. Succinate Dehydrogenase

A. Percentage of total cell count reported. Positivity equals increased activity thus more relative oxidative phosphorylation **B.** Succinate dehydrogenase (SDH) mitochondrial enzyme (TCA cycle) enzymatic stain, representative image demonstrating SDH staining to assess oxidative capacity. Fibers were scored as strongly positive (++), weakly positive (+) or negative (-). Scale bar = 100μm. * Significant effect of poly(I:C) treatment (p<0.05).

Figure 9. Capillary and Arteriole Density

Representative images of PBS-treated (A.) and poly(I:C)-treated (B.) mouse diaphragm denoting skeletal muscle capillaries (red), arterioles (green) and laminin (white). C. Average capillary density in various muscles as capillaries per fiber. D. Average arteriole density in various muscles as arterioles per 100 fibers. * = significant effect of poly(I:C) treatment ($p < 0.05$). Each symbol [circle PBS, diamond poly(I:C)] = data calculated from one cross section of an individual muscle, rectangle = mean, error bars = standard error.

Figure 10. PGC-1α not affected by poly(I:C) treatment in Gastroc

A. PGC-1α is a marker of mitochondrial biogenesis, less relative amount is indicative of a more glycolytic muscle. n=4 per group. **B.** Representative Image. Electrophoresis for PGC-1α and β-tubulin run on 4–15% Acrylamide Gel, 50 μg of protein from gastrocnemius whole muscle homogenate per lane. Blot made on PVDF membrane, 1:1000 1° Ab, 1:2000 2° Ab. Blot was stripped and reprobbed for β-tubulin load control. Each symbol, circle or diamond, indicates PGC-1α relative density for an individual muscle sample, kDa= kilodalton.

Table 1

Muscle Contractile Function

Only the soleus demonstrated significant changes in the measured parameters.

<i>in vitro</i> Muscle Physiological Properties										
Muscle		SOL			EDL			DIA		
Group		PBS	Poly(I:C)	%dif	PBS	Poly(I:C)	%dif	PBS	Poly(I:C)	%dif
n	#	4	4		4	4		4	4	
Mass	mg	10.4	<u>7.8</u>	<u>-25.0</u>	9.8	8.2	-17.1	1.9	2.0	N/A
L₀	mm	10.9	11.2	2.3	12.6	13.2	4.7	8.9	9.2	N/A
PCSA	mm ²	0.91	<u>0.66</u>	<u>-26.8</u>	0.74	0.58	-21.7	0.21	0.21	N/A
Fiberlength	mm	10.9	11.2	2.3	5.5	5.8	4.7	6.1	6.3	2.9
P_t	mN	41.7	30.8	-26.1	80.4	67.2	-16.4	20.9	19.5	N/A
Sp. P_t	mN/mm ²	46.3	46.7	0.8	108.9	119.1	9.3	103.7	92.0	-11.3
P₀	mN	233.8	<u>173.0</u>	<u>-26.0</u>	418.3	307.6	-26.5	50.3	44.1	N/A
Sp. P₀	mN/mm ²	259.9	263.8	1.53	573.45	525.64	-8.3	251.0	209.6	-16.5

Values given are the mean of n=4 for each group and muscle ± standard error. SOL= soleus, EDL=extensor digitorum longus, DIA=diaphragm, L₀ = optimal length of muscle, PCSA= physiological cross-sectional area, Sp. = specific, P_t = peak twitch force, P₀ = peak isometric force, %dif = percent difference between poly(I:C)-treated and PBS groups, n=4 muscles per group. **bold underlined text** indicates p<0.05, N/A=not applicable as diaphragm can only be measured accurately between samples at specific values.