Repetitive TLR3 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 receptor 3 (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 respiratory 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 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.

\section{1. Introduction}

Older adults have a diminished immune-response capacity and are more prone to suffer from viral lung infections such as respiratory syncytial virus (RSF) 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 (CDC1, n.d; CDC2, n.d; Murray and Chotirmall, 2015; Gavazzi and Krause, 2002). Viral-mediated inflammatory responses can also trigger exacerbations of age-related chronic lung diseases (Ritchie et al., 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 function. Viral-mediated inflammatory responses can also trigger exacerbations of age-related chronic lung diseases (Ritchie et al., 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 function.
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 et al., 2016).

We recently developed a mouse model of chronic viral-mediated pulmonary inflammation utilizing repetitive intranasal administration of polynucleosinic-polyribytidylic acid [poly(I:C)] (Tian et al., 2016, 2017). Structurally similar to dsRNA (double stranded RNA), poly(I:C) activates the Toll like receptor 3 (TLR3) pathway. TLR3 is a 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) (Jensen and Thomsen, 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-κB (Nuclear factor kappa-light-chain-enhancer of activated B cells) to initiate cytokine production (Jensen and Thomsen, 2012).

Acute treatment with poly(I:C) induces significant pulmonary inflammation and reduction of lung function (Stowell et al., 2009), edema with similarities to RSV in mice (Aeffner et al., 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 et al., 2013). Poly (I:C) administration also induces bronchial epithelial cell apoptosis (Koizumi et al., 2016) and airway remodeling in a rat asthma model (Takayama et al., 2011). Repetitive treatment causes additional airway remodeling, pulmonary fibrosis, lung structural changes, and loss of body mass in mice (Tian et al., 2017).

In previous work with the repetitive poly(I:C) administration model, we noted a decline in body mass in the treated mice (Tian et al., 2017). Cachexia develops in 20–40% of patients with COPD (Remels et al., 2013), and can have similar consequences to sarcopenia [impaired quality of life, difficulties with activities of daily living, increased morbidity and mortality (Sanders et al., 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 et al., 2012; Gan et al., 2004; Langen et al., 2013; Lemire et al., 2012; Van Remoortel et al., 2013; Wüst and Degens, 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.

2. Methods

2.1. Animals

All animal experiments were approved by 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. Fig. 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 50μl 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 50μl 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 et al., 2016, 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.

2.2. 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 immunochemistry. 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 (NaO7P4), 1 mM ethylenedinitrilotetraacetic 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 μg/ml soybean trypsin inhibitor; pH 7.4) at a 1:9 ratio (mass: 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).

2.3. 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 et al., 2013) and the grip test (Fry et al., 2015) have been previously published, however a brief description follows. All functional testing was performed by the same individual.

2.3.1. 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 et al., 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 min; the outcome measure was latency to falling. We report the best of three trials, each trial administered with a minimum 15 min rest period.

2.3.2. 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 et al., 2015). 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.

2.3.3. 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 min) before a test day in which the mice ran on the treadmill as it accelerated by 1 cm/s per 20 s 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 et al., 2011).

2.4. Contractile function
Both general details of the procedure and those specific to the SOL, EDL, and diaphragm have been previously published (Graber et al., 2015, 2013; Durham et al., 2004). In brief, we isolated the SOL, EDL and diaphragm from the mice, maintaining viability in Krebs-Ringer 2015, 2013; Durham et al., 2004). In brief, we isolated the SOL, EDL, and diaphragm have been previously published (Graber et al., 2011).

In brief, we isolated the SOL, EDL and diaphragm from the mice, maintaining viability in Krebs-Ringer buffer, 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 h 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), cSMA (#sc-130,616, 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 1 h incubation at 37 °C. 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.

2.7. 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.

2.8. 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-μm 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 et al., 2016, 2017).

2.9. 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-1α; 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.
2.10. Statistics

Data are reported as means ± standard error or as percent change ± 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 \( d = \frac{\text{difference in means}}{\text{pooled standard deviation}} \) with the G*Power 3.1.9.2 (Franz Faul, Universität Kiel, Germany) software package. We used the Kolmogorov-Smirnov test to determine if a difference existed between 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).

3. Results

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

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

3.1. Functional capacity (see Fig. 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, \( p > 0.10 \)).

3.2. Skeletal muscle contractile function (Fig. 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).

3.3. Muscle atrophy

3.3.1. Muscle wet mass and body mass (Fig. 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.

3.3.2. Muscle fiber cross-sectional area (Figs. 6 and 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 (Fig. A1) for SOL, plantaris, 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. Fig. A1 and Table A1 for further details, including skew and kurtosis of distributions.

3.4. Muscle oxidative capacity (Fig. 7 and Table B1)

3.4.1. 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).

3.4.2. Succinate dehydrogenase (Fig. 8)

Succinate dehydrogenase is a marker of mitochondrial activity as

Table 1

| Muscle contractile function: Only the soleus demonstrated significant changes in the measured parameters. 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, Pt = peak twitch force, Po = 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 at samples at specific values. |
|---|---|---|---|---|
| Group | SOL | EDL | DIA |
| | PBS | Poly(I:C) | %dif | PBS | Poly(I:C) | %dif | PBS | Poly(I:C) | %dif |
| n | 4 | 4 | −25.0 | 4 | 4 | −17.1 | 4 | 4 | N/A |
| Mass (mg) | 10.4 | 7.8 | 2.3 | 12.6 | 13.2 | 4.7 | 8.9 | 9.2 | N/A |
| L0 (mm) | 10.9 | 11.2 | −0.6 | 0.74 | 0.58 | −0.21 | 0.21 | 0.21 | N/A |
| Fiber length (mm) | 10.9 | 11.2 | 2.3 | 5.5 | 5.8 | 4.7 | 6.1 | 6.3 | N/A |
| Pt (mN) | 41.7 | 30.8 | −26.1 | 80.4 | 67.2 | −16.4 | 20.9 | 19.5 | N/A |
| Sp. Pt (mN/mL) | 46.3 | 46.7 | 0.8 | 108.9 | 119.1 | 9.3 | 103.7 | 92.0 | −11.3 |
| Po (mN) | 233.8 | 173.0 | −26.0 | 418.3 | 307.6 | −26.5 | 50.3 | 44.1 | N/A |
| Sp. Po (mN/mL) | 259.9 | 263.8 | 1.53 | 573.45 | 525.64 | −8.3 | 251.0 | 209.6 | −16.5 |
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 significant 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.

3.4.3. Capillary and arteriole content (Fig. 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 capillary content in the other muscles or in arteriole content.

3.4.4. PGC-1α, biomarker of mitochondrial biogenesis (Fig. 10)
Poly(I:C)-treated mice showed a subtle, non-statistically significant reduction in PGC-1α expression in Gastroc (−15% PGC-1α density, n = 4; p = 0.300) when compared to PBS.

Fig. 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).

Fig. 6. Muscle fiber cross-sectional area (CSA): A. Average muscle fiber CSA measured in μm². * 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 CSA.
4. 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 et al., 2009; Aeffner et al., 2011; Harris et al., 2013; Koizumi et al., 2016; Takayama et al., 2011). Chronic administration of poly(I:C) has demonstrated airway remodeling, fibrosis, unique gene expression signatures, and lung structural changes (Tian et al., 2017, 2016). In Fig. 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 et al., 2017). Building on 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 and Celli, 2009; Gan et al., 2004). The loss of fat-free mass alone is a risk factor for mortality independent of airflow capacity (Sanders et al., 2016). COPD-related cachexia develops in 20–40% of patients (Remels et al., 2013), and can have similar consequences to sarcopenia (Sanders et al., 2016). 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 et al., 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 et al., 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 et al., 2014). COPD is also the fourth largest cause of death globally, and the third largest in the United States (Adeloye et al., 2015; CDC3, 2008; NIH NHBLI Website, n.d; Rycroft et al., 2012). One hallmark of COPD, is the progressive loss of exercise capacity (Punekar et al., 2017; Spruit et al., 2016). 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 et al., 2009; Doucet et al., 2004; Levine et al., 2013).

Fig. 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)]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 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 = 100 μm. * Significant effect of poly(I:C) treatment (p < 0.05).
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 et al., 2009; Levine et al., 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.

4.1. 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 fiber type distribution toward a smaller mean, error bars = standard error. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
4.2. 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 et al., 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 et al., 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 et al., 2013).

4.3. 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 et al., 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 et al., 2004) and the quadriceps changing from type 1 to type 2 (Gosker et al., 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 et al., 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-1α. PGC-1α is considered the master regulator of mitochondrial biogenesis, and modulates mitochondrial remodeling (Austin and St-Pierre, 2012). PGC-1α 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 and St-Pierre, 2012). In the current study, the difference in PGC-1α expression in the Gastroc between PBS- and poly(I:C)-treated mice was indeterminate (~15.5%, non-significant; p = 0.30, see Fig. 9). However, after eliminating a single poly(I:C)-treated 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 et al., 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 et al., 2007; Maltai et al., 2014; Krüger et al., 2015; Hansen et al., 2013; Vlachos and Bozinovski, 2015). Future studies will extend the period of treatment to establish the time course of peripheral muscle adaptation.

5. 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.

6. 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 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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Appendix A. Muscle cell cross-sectional area distributions


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Conflicts of interest

The authors report no conflicts of interest, whether financial or otherwise.

Author contributions

TGG and CSF wrote manuscript and are responsible for all content. TGG: main author, designed experiments, conducted research, collected and analyzed data; BLR: collected data, conducted experiments, edited manuscript; BT: edited manuscript, conducted experiments, produced figures, scientific review; WJD: assisted with writing, edited manuscript, conducted experiments, scientific review; CRB: analyzed data, produced figures, reviewed manuscript; ARB: assisted with writing, edited manuscript, designed experiments, scientific review; CSF: wrote manuscript, designed experiments, conducted research, collected and analyzed data, study guarantor.

Fig. 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. 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.

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Group</th>
<th>n</th>
<th>K-S p-val</th>
<th>Mean</th>
<th>T-test p-val</th>
<th>Median</th>
<th>Mean Rank</th>
<th>M-W p-val</th>
<th>Min</th>
<th>Max</th>
<th>Skew</th>
<th>Kurtosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soleus</td>
<td>PBS</td>
<td>2226</td>
<td>&lt; 0.001</td>
<td>1211.9 ± 16.0</td>
<td>&lt; 0.001</td>
<td>1025.5</td>
<td>2404.9</td>
<td>&lt; 0.001</td>
<td>85.3</td>
<td>7720.2</td>
<td>2.35</td>
<td>± 0.05 10.34 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>poly(I:C)</td>
<td>2133</td>
<td></td>
<td>950.8 ± 10.1</td>
<td></td>
<td>839.1</td>
<td>1945.3</td>
<td></td>
<td>275.4</td>
<td>72.0</td>
<td>2.11 ± 0.05</td>
<td>15.75 ± 0.11</td>
</tr>
<tr>
<td>Plantaris</td>
<td>PBS</td>
<td>2280</td>
<td>&lt; 0.001</td>
<td>1412.6 ± 17.9</td>
<td>&lt; 0.001</td>
<td>1249.1</td>
<td>3208.6</td>
<td>&lt; 0.001</td>
<td>250.0</td>
<td>8328.4</td>
<td>1.62 ± 0.05</td>
<td>5.50 ± 0.10</td>
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<tr>
<td></td>
<td>poly(I:C)</td>
<td>3161</td>
<td></td>
<td>999.0 ± 9.7</td>
<td></td>
<td>860.8</td>
<td>2369.3</td>
<td></td>
<td>43.6</td>
<td>4892.9</td>
<td>1.71 ± 0.04</td>
<td>5.26 ± 0.09</td>
</tr>
<tr>
<td>Gastroc.</td>
<td>PBS</td>
<td>5241</td>
<td>&lt; 0.001</td>
<td>2204.3 ± 12.1</td>
<td>&lt; 0.001</td>
<td>2149.6</td>
<td>8063.8</td>
<td>&lt; 0.001</td>
<td>270.0</td>
<td>7267.9</td>
<td>0.95 ± 0.03</td>
<td>2.74 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>poly(I:C)</td>
<td>7722</td>
<td></td>
<td>1586.8 ± 8.5</td>
<td></td>
<td>1504.1</td>
<td>5408.4</td>
<td></td>
<td>257.0</td>
<td>6973.8</td>
<td>0.59 ± 0.03</td>
<td>0.50 ± 0.06</td>
</tr>
<tr>
<td>Diaphragm</td>
<td>PBS</td>
<td>2407</td>
<td>&lt; 0.001</td>
<td>978.0 ± 11.4</td>
<td>&lt; 0.001</td>
<td>847.0</td>
<td>2246.4</td>
<td>&lt; 0.001</td>
<td>250.9</td>
<td>7437.3</td>
<td>2.87 ± 0.05</td>
<td>16.90 ± 0.10</td>
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<tr>
<td></td>
<td>poly(I:C)</td>
<td>1871</td>
<td></td>
<td>835.4 ± 7.6</td>
<td></td>
<td>795.9</td>
<td>2002.0</td>
<td></td>
<td>250.0</td>
<td>3123.1</td>
<td>0.94 ± 0.06</td>
<td>2.20 ± 0.113</td>
</tr>
</tbody>
</table>

Appendix B. Table B1 peripheral fiber type distribution by muscle

Table B1
Fiber type distribution by muscle. 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.

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Gastrocnemius (n = 9)</th>
<th>Plantaris (n = 9)</th>
<th>Soles (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHC Type</td>
<td>1</td>
<td>2a</td>
<td>2b</td>
</tr>
<tr>
<td>PBS Mean</td>
<td>1.33 ± 0-</td>
<td>10.00 ± -</td>
<td>82.17 ± -</td>
</tr>
<tr>
<td>poly(I:C) Mean</td>
<td>1.86 ± 0-</td>
<td>13.17 ± -</td>
<td>79.56 ± -</td>
</tr>
<tr>
<td>% Difference</td>
<td>+40%</td>
<td>+32%</td>
<td>-3%</td>
</tr>
<tr>
<td>p-Val. (t-test)</td>
<td>0.269</td>
<td>0.177</td>
<td>0.370</td>
</tr>
</tbody>
</table>
