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Influenza A Virus Infection Induces Muscle Wasting via IL-6 Regulation of the E3 Ubiquitin Ligase Atrogin-1

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Muscle dysfunction is common in patients with adult respiratory distress syndrome and is associated with morbidity that can persist for years after discharge. In a mouse model of severe influenza A pneumonia, we found the proinflammatory cytokine IL-6 was necessary for the development of muscle dysfunction. Treatment with a Food and Drug Administration–approved Ab antagonist to the IL-6R (tocilizumab) attenuated the severity of influenza A-induced muscle dysfunction. In cultured myotubes, IL-6 promoted muscle degradation via JAK/STAT, FOXO3a, and atrogin-1 upregulation. Consistent with these findings, atrogin-1−/− mice had attenuated muscle dysfunction following influenza infection. Our data suggest that inflammatory endocrine signals originating from the injured lung activate signaling pathways in the muscle that induce dysfunction. Inhibiting these pathways may limit morbidity in patients with influenza A pneumonia and adult respiratory distress syndrome. The Journal of Immunology, 2019, 202: 484–493.

Influenza A pneumonia is the most common cause of morbidity from an infectious agent, responsible for at least 20,000–50,000 deaths in the United States yearly (1, 2). The lung epithelium is the primary target of the influenza A virus (IAV) infection, and the resulting diffuse damage to the epithelium can impair gas exchange, resulting in severe pneumonia and development of acute respiratory distress syndrome (ARDS). About 30–40% of patients with ARDS die, and most survivors often suffer multiple organ dysfunctions related to their prolonged critical illness (3, 4). Muscle weakness is evident in up to 50% of ARDS survivors, in whom it is associated with prolonged lengths of stay in the intensive care unit and the development of the multiple-organ dysfunction syndrome (5–7). In patients who survive ARDS, muscle weakness results in substantial morbidity and long-term reductions in quality of life (8–11).

As survival from ARDS continues to improve (12, 13), it is increasingly important to prevent muscle dysfunction and other long-term sequelae of the syndrome (14). Our understanding of the pathophysiology of muscle weakness associated with critical illness is incomplete. Some investigators have argued that skeletal muscle dysfunction in ARDS is largely attributable to the prolonged immobility associated with critical illness (14, 15). Others have shown that the ubiquitin proteasome system is activated in skeletal muscle within hours or days of the onset of critical illness, suggesting a role for active signaling processes that promote muscle dysfunction (7, 16).

Maintenance of muscle mass requires an equilibrium between the synthesis and breakdown of myofiber proteins. Muscle atrophy occurs when there is a net loss of muscle mass, causing shrinkage of the myofibers. There are several different types of skeletal muscle atrophy, including sarcopenia, disuse atrophy, and cachexia (9, 10, 17). The forkhead box O (FoxO) class of transcription factors has been implicated in the control of muscle degradation (18, 19). Specifically, FoxO1 and FoxO3 upregulate the muscle-specific ubiquitin ligases: muscle atrophy F-box protein (MAFbx/atrogin-1) and muscle RING finger 1 (MuRF1), which target muscle proteins for degradation by the ubiquitin proteasome system (20, 21).

The proinflammatory cytokine IL-6 has been reported to regulate atrogin-1 and thus promote muscle atrophy (22–24). Cellular signaling induced by IL-6 has been shown to activate JAK/STAT3 (25). Phosphorylation of STAT3 leads to the translocation of FoxO3 to the nucleus, leading to upregulation of atrogin-1 (19, 26).

In animal models and healthy volunteers infected with IAV, IL-6 and TNF-α are rapidly detected in bronchoalveolar lavage (BAL) fluid and nasal washings (27, 28). IL-6 is also increased in the serum of patients with lung injury (29, 30) and in mouse models of influenza infection (28). Increased levels of IL-6 and other inflammatory cytokines are associated with prolonged mechanical...
ventilation (29, 30) and increased mortality (29–31). In this study, we sought to determine whether IL-6-mediated activation of STAT3/atrogin-1 was necessary for the development of muscle dysfunction in a murine model of influenza A infection.

Materials and Methods

Reagents

All cell culture reagents were from Corning Life Sciences (Tewksbury, MA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Mice

Adult male (12–16 wk) C57BL/6 wild-type mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Age-matched male atrogin-1/− and atrogin-1+/+ mice as well as wild-type littermates (atrogin-1+/+) on a 129S/C57BL/6 background have been described previously and were obtained from Regeneron Pharmaceuticals and S. Lecker at Beth Israel Deaconess Medical Center and Harvard Medical School (20). Mice were provided with food and water ad libitum, maintained on a 14-h light/10-h dark cycle, and handled according to National Institutes of Health and Northwestern University Institutional Animal Care and Use Committee guidelines. Northwestern University’s Institutional Animal Care and Use Committee approved all animal experimental protocols.

IAV and infection

IAV (A/WSN/33 [H1N1]), a mouse-adapted virus, was provided by R. Lamb, Ph.D., Sc.D., Northwestern University, Evanston, IL. Mice were anesthetized with isoflurane, their lungs were intubated with a 20-gauge angiocath (32, 33), and two 25-μl sterile aliquots of PBS (control) or IAV (A/WSN/33 [H1N1]) (1500 or 150 PFU/mouse) were instilled through the catheter as we have previously described (28). We continuously observed mice infected with IAV for signs of distress (slowed respiration, failure to respond to cage tapping, lack of grooming, huddling, and fur ruffling). Mice that developed these symptoms were sacrificed and the death classified as an infectious-induced mortality. Weight was measured at infection time and prior to harvest 7 d postinfection (dpi) and recorded as percentage of weight loss from baseline.

Administration of tocilizumab

Tocilizumab is a humanized mAb against the IL-6R; for our studies, we used a mouse-adapted form of tocilizumab (Genentech, San Francisco, CA). Briefly, adult male C57BL6 mice were anesthetized with isoflurane and were administered tocilizumab (8 mg/kg) or PBS alone by a retroorbital injection in a total volume of 150 μl. After 24 h, the mice were infected with IAV or PBS as described above.

Measurement of muscle dysfunction

Immediately prior to muscle harvest, forelimb skeletal muscle strength was assessed using a digital grip strength meter (Columbus Instruments, Columbus, OH) as described previously (34, 35). Grip strength was measured in each animal six successive times, and the average of the highest four values for each mouse was used. The same operator performed all tests. The mice were then terminally anesthetized with Euthasol (pentobarbital sodium/phenytoin sodium). The soleus and extensor digitorum longus (EDL) muscles were excised, and the tendons were trimmed under a microscope to ensure optimal accuracy for weight measurement. The muscles were then blotted dry and weighed. Muscles were either frozen in liquid nitrogen–cooled isopentane for cryosectioning or snap frozen in liquid nitrogen for protein extraction. Immunohistochemistry and fiber size assessment

Soleus and EDL serial transverse cryosections (8 μm) were obtained from the Northwestern University Mouse Histology and Phenotyping Laboratory and mounted on glass slides. Sections were fixed in 4% formaldehyde, permeabilized, and blocked. Immunostaining was performed with laminin primary Ab (catalog: L9393; 1:50; Sigma-Aldrich) followed by Alexa Fluor 568–conjugated secondary Ab (catalog: A-11011; 1:200; Life Technologies, Carlsbad, CA). Images were acquired with a Zeiss LSM 510 confocal microscope using a 40× objective (Northwestern University Center for Advanced Microscopy) and analyzed using Fiji (National Institutes of Health) (36). Fiber size and cross-sectional area (CSA) were determined as previously described (34). All analysis was performed by one blinded operator.

Cell culture, small interfering RNA transfection, drug treatment, myotoxin analysis, and viral plaque assay

C2C12 mouse myoblasts (CRL-1772; American Type Culture Collection, Manassas, VA) were cultured and differentiated as we have previously described (34). For experimental procedures, myoblasts were grown to 90–95% confluence, and then media was switched to 2% horse serum DMEM (differentiation media) and renewed daily for optimal myotube formation. For small interfering RNA (siRNA) transfection experiments, 90–95% confluent C2C12 myoblasts were switched to differentiation media (day 0). On day 1 of differentiation, the media was replaced with antibiotic-free differentiation media, and the transfection protocol was performed 12 h later. On day 2 of differentiation, cells were transfected with 60 pmol of either mouse STAT3, FKHRLI, FKHR, or control siRNA (scrambled [scramb]) pools (Santa Cruz Biotechnology, Dallas, TX) using Lipofectamine RNAiMax transfection reagent (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer’s instructions. On day 4 of differentiation, cells were treated with 10 ng/ml of recombinant mouse IL-6 (BD Biosciences, San Jose, CA) for 16 h. For the dose-response treatment of IL-6, cells were treated on day 4 of differentiation with various concentrations of IL-6 (2.5, 5, and 10 ng/ml) for 16 h. For experiments with tocilizumab, cells were treated with 10 ng/ml of Ro3 and 1 mg/l of phosphatase (sodium fluoride 30 mM, β-glycerophosphate 250 mM, sodium orthovanadate 1 mM) inhibitors (38). Tissue was then homogenized with a Tissue-Tearor (BioSpec Products, Bartlesville, OK) for 1 min. Samples were centrifuged at 20,000 × g and the supernatant was collected. Soleus and EDL muscles were lysed with ice-cold lysis buffer (Nonidet P-40 0.1%, glycerol 10%, NaCl 137 mM, Tris-HCl [pH 7.5] 20 mM) containing phosphatase (1 cOmplete Mini, EDTA-free tablet, Roche) and protease (sodium fluoride 30 mM, β-glycerophosphate 250 mM, sodium orthovanadate 1 mM) inhibitors (38). The proteins were quantified by one blinded operator measuring ∼375–679 tube diameters (5–10 measurements per fiber) from five random fields from a minimum of three independent experiments using MetaMorph Software (Molecular Devices, Sunnyvale, CA; version 6.3) as previously described (34). Madin–Darby canine kidney cells (CCL-34; American Type Culture Collection) were maintained as described previously and used to perform a viral plaque assay with homogenized soleus muscle or lungs from mice infected with influenza (37). Western blot analysis

C2C12 myotubes were washed with ice-cold PBS and then lysed with 2× Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA). Lysates were centrifuged at 20,000 × g and the supernatant was collected. Soleus and EDL muscles were lysed with ice-cold lysis buffer (Nonidet P-40 0.1%, glycerol 10%, NaCl 137 mM, Tris-HCl [pH 7.5] 20 mM) containing phosphatase (1 cOmplete Mini, EDTA-free tablet, Roche) and protease (sodium fluoride 30 mM, β-glycerophosphate 250 mM, sodium orthovanadate 1 mM) inhibitors (38). Tissue was then homogenized with a Tissue-Tearor (BioSpec Products, Bartlesville, OK) for 1 min. Samples were centrifuged at 15,000 rpm for 10 min at 4°C, and the supernatant was collected. Protein concentrations were determined with Protein Assay Dye (Bio-Rad, Hercules, CA) using the Bradford method (39). Equal amounts of protein were loaded on an SDS-PAGE apparatus, and Western blot analysis was performed as described previously (40). Incubation with primary Abs was performed overnight at 4°C. Immunobots were quantified by densitometry using Image J 1.48r (National Institutes of Health) or Image Studio Software (LI-COR Biosciences, Lincoln, NE) (41). The following Abs were used: rabbit monoclonal to Fbx32 (catalog: ab168372; 1:1000; Abcam, Cambridge, U.K.), rabbit polyclonal to MuRF1 (catalog: MP3401; 1:1000; ECM Biosciences, Versailles, KY), rabbit monoclonal to GAPDH (D16H11) (catalog: 5174s; 1:1000; Cell Signaling Technology), rabbit monoclonal to STAT3 (7D9P) (catalog: 4904s; 1:1000; Cell Signaling Technology), rabbit monoclonal to FoxO3a (75D8) (catalog: 2497s; 1:1000; Cell Signaling Technology), and rabbit polyclonal to FoxO1 (L27) (catalog: 9454s; 1:1000; Cell Signaling Technology). Primary Abs were detected with HRP-conjugated secondary Abs, including anti-rabbit IgG, HRP linked (catalog: 7074s; 1:2000 dilution; Cell Signaling Technology) and goat anti-rabbit IgG, HRP conjugate (catalog: 170-6515; 1:16,000 dilution; Bio-Rad).

BAL fluid and blood collection for measurement of cell count, protein, and IL-6

Animals were euthanized with Euthasol (pentobarbital sodium/phenytoin sodium) prior to BAL and blood collection. A midline dissection through the abdominal cavity up to the thoracic cavity was performed, followed by a small incision at the edge of the diaphragm, which resulted in a fatal pneumothorax. Then, a right ventricular puncture for the collection of blood was performed. Blood was collected in a microvette microtube with EDTA (Kent Scientific), kept on ice, and then spun at 2000 rpm. Serum was
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collected and used for IL-6 determination by ELISA (KMC0062; Thermo Fisher Scientific) (37). BAL fluid was obtained through a 20-gauge angiocath ligated into the trachea through a tracheostomy. A total of 1 ml of PBS instilled into the lungs was aspirated three times to collect BAL fluid for cell counts (Cellometer K2; Nexcelom Bioscience), protein quantification (Bradford assay), and IL-6 determination by ELISA.

Statistics

Results are shown as mean ± SEM. Data were analyzed and statistical differences were compared by a Student t test between two groups and by a one-way ANOVA with Dunnett or Tukey post hoc corrections for three or more groups using GraphPad Prism version 7.0 for Windows (GraphPad Software, La Jolla, CA). All in vitro experiments were repeated a minimum of three times. We considered specific data points outliers if they deviated by more than two times the SD from the mean, and they were excluded automatically from the statistical analysis. Results were considered significant when the p value was <0.05.

Results

C57BL/6 wild-type mice lose muscle weight and strength after influenza infection

We infected C57BL/6 wild-type mice with either a low dose (150 PFU/mouse) or high dose (1500 PFU/mouse) of IAV (A/WSN/33 [H1N1]) and assessed total body weight, forelimb grip strength, soleus and EDL muscle wet weight, and fiber CSA. Compared with control (PBS-treated) mice, IAV-infected mice experienced significant weight loss after both low-dose and high-dose IAV infection (Fig. 1A). Forelimb grip strength was significantly decreased in the IAV-infected mice (Fig. 1B). In addition, mice infected with either low- or high-dose influenza had significantly decreased soleus and EDL wet muscle weight (Fig. 1C, 1D). Fig. 1E and 1H show representative images of soleus and EDL laminin-stained cryosections that were used to determine the mean CSA. Mice infected with high-dose IAV had significantly decreased mean soleus and EDL CSA (Fig. 1F, II), with the histograms showing a left shift of frequency distribution indicating a predominance of thinner fibers in IAV-infected mice (Fig. 1G, 1J).

To determine that IAV infection was restricted to the lung, we measured by plaque assay the viral load in lungs and soleus muscle of mice infected with IAV for 7 d. As expected, the viral load was almost undetectable in soleus muscle homogenates compared with lung homogenates of wild-type IAV-infected mice (soleus: 38.33 PFU/ml ± 6.009 SEM; lung: 1.25 × 10^7 PFU/ml ± 8.3660 × 10^5 SEM). A Student t test between two groups was used to show statistical differences, with p < 0.0001 (n = 3–5 mice per group).

Influenza-induced muscle dysfunction is attenuated in mice with genetic loss of atrogin-1

The ubiquitin proteasome system regulates muscle degradation via the E3 ubiquitin ligases MAFbx/atrogin-1 and MuRF1 (20, 21). We set out to determine if one or both of these muscle-specific E3 ubiquitin ligases were upregulated in our murine model of IAV infection. C57BL/6 wild-type mice were infected with IAV (1500 PFU/mouse), and 7 dpi, the mice were sacrificed and the soleus muscle weight for soleus and EDL after IA V infection, wet muscle weight loss was slightly less severe (Fig. 3A). Additionally, IL-6 levels were measured in serum and BAL fluid. Total protein and cell count were also determined in the BAL fluid. IL-6 levels were markedly increased in the IAV-infected atrogin-1+/− mice in both serum and BAL fluid as compared with IAV-infected atrogin-1+/− and atrogin-1−/− mice (Fig. 3B, 3C). There was no difference in total protein and cell count of the BAL fluid among the different atrogin-1 genotypes infected with IAV (Fig. 3D, 3E).

The IL-6R Ab tocilizumab attenuates influenza-induced muscle dysfunction

To investigate the role of IL-6 in mediating influenza A–induced muscle atrophy in vivo, we treated C57BL/6 mice with tocilizumab, which is a mAb with specificity for the IL-6R that prevents IL-6 from binding to both the soluble and membrane-bound IL-6R through competitive blockade (42). C57BL/6 mice were administered tocilizumab (8 mg/kg) or PBS (control) by retro-orbital injection 24 h prior to infection with IAV (1500 PFU/mouse) or PBS, and then 7 dpi, weight loss and grip strength were assessed. There was no difference in total-body IAV-induced weight loss between the PBS- and tocilizumab-treated mice (Fig. 4A). The reduction of grip strength observed in IAV-infected mice treated with vehicle was attenuated in the IAV-infected and tocilizumab-treated mice (Fig. 4B). Consistent with these findings, soleus and EDL wet muscle weights were significantly reduced in influenza-infected compared with uninfected wild-type mice, whereas there was no significant decrease in either the soleus or EDL wet muscle weight in uninfected compared with tocilizumab-treated mice (Fig. 4C, 4D). The induction of atrogin-1 protein by influenza A was attenuated in the soleus muscle of tocilizumab-treated mice (Fig. 4D). Similarly, the influenza A–induced reductions in soleus and EDL CSA were attenuated in mice that had been pretreated with tocilizumab compared with vehicle, as was the left shift of muscle fiber size frequency distribution for both the soleus and EDL (Fig. 4F–M).

IL-6 acts via STAT3, FoxO3a, and atrogin-1 to induce skeletal muscle atrophy

To obtain insight into the mechanisms by which IL-6 induces muscle atrophy, we treated differentiated C2C12 myotubes with various doses of rIL-6 (2.5, 5, and 10 ng/ml) or with 1 MOI of IAV or media alone, and blinded measurements of myotube diameters were performed 16 h later. Treatment of C2C12 cells with IL-6 resulted in a dose-dependent reduction in myotube diameter, whereas treatment with IAV had no effect on fiber diameter (Fig. 5A). To determine if STAT3 was involved in the IL-6–induced reduction in myotube diameter, we transfected well-differentiated C2C12 cells with Scramb siRNA or siRNA specific for STAT3 followed by treatment with rIL-6, and then we measured myotube diameters. Silencing of STAT3 prevented the decrease in diameter seen with IL-6 treatment
To determine whether STAT3 acts through FoxO1 or FoxO3a to reduce myotube diameter, we compared the effect of IL-6 on myotube diameter in control and FoxO1- and FoxO3a-silenced cells. Silencing of FoxO3a attenuated the decline in myotube diameter induced by IL-6 (Fig. 5C), but silencing of FoxO1 did not prevent the decrease in myotube diameter (Fig. 5D). Consistent with our in vivo findings, pretreatment of myotubes with tocilizumab prevented the IL-6–induced decrease in myotube diameter as compared with the myotubes that received the mock treatment and IL-6 (Fig. 5E). Moreover, treatment of myotubes with IL-6 increased the expression of atrogin-1 in vehicle-treated cells, and this effect was attenuated in cells pretreated with tocilizumab (Fig. 5F). Accordingly, we provide evidence that IL-6 released from the lungs as a result of influenza infection leads to the upregulation of the E3 ubiquitin ligase atrogin-1, which results in muscle degradation (Fig. 6).

**Discussion**

Severe infection with IAV is an important cause of lung injury and ARDS (1, 2). A significant proportion of patients with ARDS develop skeletal muscle weakness that is associated with prolonged duration of mechanical ventilation and with increased short-term and long-term mortality (43, 44). In patients who survive ARDS, muscle dysfunction may persist for years after hospital discharge, impairing quality of life and affecting survival (1, 2, 9, 10, 17).

Using a murine model of influenza A infection, we show that IL-6 released from the influenza A–infected lung upregulates atrogin-1 to promote the active degradation of muscle proteins via the ubiquitin proteasome system. Muscle mass and function could be preserved during influenza A infection through genetic deletion of atrogin-1 or through pharmacologic blockade of the IL-6R. The fact that mice infected with IAV had negligible viral load detected in the soleus muscle homogenates and that differentiated C2C12 mouse myotubes have reduced muscle fiber size when treated with IL-6 and not when incubated with IAV suggest an endocrine mechanism for the muscle loss observed in influenza A–infected mice. The effects of IL-6 on fiber size were attenuated by knockdown of STAT3 or FoxO3a and by pharmacologic inhibition.
FIGURE 2. Influenza-induced muscle dysfunction is attenuated in mice with genetic loss of atrogin-1. (A and B) Adult male C57BL/6 mice were infected with 1500 PFU/mouse of IAV or PBS (control), and 7 dpi, soleus muscles were excised and atrogin-1 (n = 6 mice per group) and MuRF1 (n = 3 mice per group) expression was determined by Western blot. β-Actin was used as loading control. A Student t test between two groups was used to show statistical differences. (C) Soleus muscle from atrogin-1 (+/+, +/-, and −/−) mice was excised, and atrogin-1 expression was determined by Western blot. GAPDH was used as loading control. (n = 3–4 mice per group). A one-way ANOVA with Dunnett post hoc corrections for comparison with more than three groups was used to show statistical differences. (D–P) Atrogin-1 (+/+, +/-, and −/−) mice were infected with 1500 PFU/mouse of IAV or PBS (control), and 7 dpi. (D) Forelimb grip strength was determined. (n = 4–10 mice per group). A one-way ANOVA with Tukey post hoc corrections for comparison with more than three groups was used to show statistical differences. (E) Soleus (n = 4–9 mice per group) and (F) EDL (n = 3–9 mice per group) wet muscle weights were determined after muscles were excised, frozen, and cryosectioned. A one-way ANOVA with Tukey post hoc (Figure legend continues)
of the IL-6R but not by knockdown of FoxO1. These data suggest that muscle loss during influenza A infection is an active process initiated by endocrine signals from the injured lung.

Our data in mice are potentially relevant for humans. IL-6 and TNF-α are the first detectable cytokines after influenza infection (27, 37) and are increased in the serum of patients with lung injury (29–31). In humans with influenza A infection, IL-6 levels peak within 2 d of infection and remain elevated for up to 7 d, with levels that correlate with symptom severity (27). In patients with ARDS, the activation of proteolytic pathways follows a similar time course, with increases in ubiquitination of muscle proteins detected within hours of onset of critical illness that remain active for up to 1 wk following discharge from the intensive care unit (7, 45). Our in vivo measurements were performed at 1 wk following infection with IAV (A/WSN/33 [H1N1]), administered intratracheally, at which point proteolytic pathways are active and muscle weakness is evident. In atrogin-1 mice, we observed an increase in IL-6 in both serum and BAL fluid after IAV infection in all the genotypes, but even though it was profoundly increased in the atrogin-1−/− mice, the lack of atrogin-1 in the muscle is enough to protect against muscle atrophy.

Although our studies demonstrate a deleterious role for IL-6 during IAV-induced lung injury, IL-6 can also be produced by muscle fibers during exercise, where it acts as a myokine (46). For example, with extreme exercise, IL-6 may be sufficiently produced by working muscle to increase circulating levels up to 100-fold (47) without signs of muscle tissue damage (48) or a change in IL-6–expressing immune cells (49). During exercise, IL-6 is released from muscle in response to low glycogen stores (50) or activation of AMPK, and it acts through autocrine and paracrine mechanisms to increase fat oxidation and enhance insulin-stimulated glucose uptake through GLUT4 translocation (51). As a myokine, IL-6 also provides an endocrine signal to enhance hepatic glucose production during exercise (52). Importantly, IL-6 is produced as the predominant myokine in response to exercise, and yet, as we observed, it can also induce muscle dysfunction as part of the cytokine storm associated with IAV infection. We speculate that differences in the kinetics of IL-6 release and its

![Graphs and images](https://via.placeholder.com/150)
association with other proinflammatory cytokines may underlie the differential response of muscle to IL-6. After exercise, IL-6 levels peak rapidly and then fall (53, 54), whereas sustained elevations are present for days after influenza infection (27). Further, IL-6 generated during exercise is not accompanied by an increase in other cytokines elevated during influenza A infection (55). Our data are consistent with those of others who have shown that prolonged exposure to rIL-6 can induce muscle atrophy (22, 56) and with observations from others that high levels of IL-6 are associated with the age-related decline in muscle function due to sarcopenia (57).

We found that pharmacologic inhibition of the IL-6R using the Food and Drug Administration–approved drug tocilizumab attenuated muscle loss during influenza A infection. We did not see adverse effects of tocilizumab in the muscle as there was no difference in muscle mass of the soleus and EDL. Furthermore, we saw no change in total body weight in mice treated with tocilizumab. Nevertheless, in health, IL-6 has an important role in maintenance of muscle bulk, modulating muscle carbohydrate and lipid metabolism (47, 58–60). The differing roles for IL-6 signaling in the muscle during health and disease are poorly understood and suggest that the effects of IL-6 in the muscle might be modulated by other factors present in the muscle microenvironment during disease. Furthermore, influenza A–induced muscle loss was only partially inhibited by tocilizumab, suggesting that other cytokines released in response to influenza A infection (e.g., IL-1β, IL-18, and others), changes in inflammatory cell populations (e.g., regulatory T cells), or nutrient deprivation might independently contribute to muscle dysfunction (61–66). Influenza A infection in mice provides an excellent model system to further explore these possibilities.
We observed increased levels of atrogin-1 in muscle following influenza A infection, and heterozygous and homozygous loss of atrogin-1 resulted in a dose-dependent inhibition of influenza A–induced muscle degradation. In cultured myotubes, the induction of atrogin-1 by IL-6 required the activation of STAT3 and FoxO3a but not FoxO1. These data suggest inhibiting atrogin-1 directly or the pathways required for its induction may preserve muscle mass during influenza A infection. These results should be interpreted with some caution, however, as others have observed increased expression of mRNA for both atrogin-1 and MuRF1 in the skeletal muscle following influenza infection (67). Further, it is not clear whether the IL-6– and atrogin-1–mediated degradation of muscle proteins is an adaptive process during influenza A infection. For example, the degradation of muscle proteins might provide metabolic substrates to fuel inflammatory cell responses to pathogen challenge. Alternatively, the systemic release of IL-6 might be a maladaptive consequence of the excessive inflammation thought to underlie the development of multiple-organ dysfunction syndrome. Although our study does not answer this question, we saw no worsening in weight loss or mortality in influenza A–infected atrogin-1 compared with wild-type controls, nor did we observe any impact of tocilizumab on these parameters. However, tocilizumab has been associated with transient neutropenia, increased body weight, and elevated cholesterol in clinical trials (68, 69).

There are a number of limitations to this study. First, because the expression of atrogin-1 is largely limited to the muscle, the effects of atrogin-1 loss in other cells is less of a concern. However, it is possible that partial or complete developmental loss of atrogin-1 might activate pathways in atrogin-1+/2 and atrogin-1−/− mice, respectively, which protect against influenza A–induced muscle loss. Further studies using inducible knockouts or pharmacologic inhibitors could address these concerns. Additionally, our model system is limited to infection with a single mouse-adapted IAV. Studies using other strains of IAV or...
administering tocilizumab to pigs, which are susceptible to circulating IAVs, could address this limitation. Finally, although IL-6 levels are often elevated in patients with ARDS secondary to other causes, the importance of IL-6 in muscle degradation in these conditions is unknown.

In summary, we provide evidence that influenza infection results in the release of IL-6 from the injured lung, which activates STAT3 and FoxO3a in the muscle to induce the expression of atrogin-1. Atrogin-1 is an E3 ligase that targets key muscle proteins for active degradation via the ubiquitin proteasome system. Thus, as depicted in Fig. 6, IL-6 acts as an endocrine signal to promote active degradation of the skeletal muscle during influenza A pneumonia. Inhibition of the IL-6R and genetic deletion of atrogin-1 attenuates the loss of muscle mass and function associated with influenza A infection, suggesting these pathways might be therapeutically targeted to preserve muscle mass in patients with influenza A–induced ARDS.

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Disclosures
The authors have no financial conflicts of interest.

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