Influenza-induced Muscle Degradation: Mechanisms of Flu-induced Disability with Aging

Spencer Ryan Keilich

University of Connecticut - School of Medicine, keilich@uchc.edu

Follow this and additional works at: https://opencommons.uconn.edu/dissertations

Recommended Citation
https://opencommons.uconn.edu/dissertations/2450
The goal of this dissertation is to expand our understanding of how influenza (flu) infection negatively impacts skeletal muscle and leads to future disability with aging. We hypothesize that flu infection triggers prolonged inflammation and increased immune cell presence in skeletal muscle leading to skeletal muscle atrophy and impaired muscle function accompanied by diminished regenerative capacity in aged mice. We first determined that despite lack of direct flu infection, flu led to skeletal muscle atrophic gene expression and impaired muscle function which was prolonged and heightened in aged mice (Chapter 2). Next, we determined that muscle atrophy primarily occurs in MyHC type IIB muscle fibers (Chapter 3). This occurred concomitantly with disrupted architecture, fibrosis, and increased nuclei in aged muscle IHC staining. Importantly, protective immunity via vaccination mitigated these effects. These studies culminated into a hypothesis generating transcriptomic project, which has painted a fully unbiased look into aged muscle processes during flu infection (Chapter 4). Interestingly, these experiments point to an immune-mediated, primarily T cell, driven myopathy during flu infection in aged muscle. The studies in this thesis are the first to examine in depth how flu impacts skeletal muscle with aging and leads to muscle dysfunction and disability. While many questions remain, this research has laid the ground work for others to test pathways/genes as mechanism(s) of flu-induced muscle atrophy with aging as the first step of developing prevention and treatment options. Indeed, development of prophylactic and therapeutic treatments for flu-induced myopathies could save lives of countless older adults, prevent catastrophic disability, and increase overall resilience and healthspan.
Influenza-induced Muscle Degradation: Mechanisms of Flu-induced Disability with Aging

Spencer Ryan Keilich

B.S. Worcester Polytechnic Institute, 2015

A Dissertation
Submitted in Partial Fulfillment of the
Requirements for the Degree of
Doctor of Philosophy
at the
University of Connecticut School of Medicine
2020
Influenza-induced Muscle Degradation: Mechanisms of Flu-induced Disability with Aging

Presented by

Spencer Ryan Keilich, B.S.

Major Advisor _________________________________________________________________
Laura Haynes

Associate Advisor _____________________________________________________________
Lynn Puddington

Associate Advisor _____________________________________________________________
George A. Kuchel

Associate Advisor _____________________________________________________________
Robert B. Clark

Associate Advisor _____________________________________________________________
Jenna M. Bartley

University of Connecticut School of Medicine
2020
ACKNOWLEDGMENTS

I would first like to acknowledge my mentor, Laura Haynes, Ph.D., who was there for me in the beginning, during my first interview weekend dinner and all the way through to my defenses. I will forever be grateful for her mentorship, support, and the strength that she helped instill in me.

I would like to thank my committee members Jenna Bartley, Ph.D., Robert Clark, M.D., George Kuchel, M.D., and Lynn Puddington, Ph.D. for their guidance, support, and constructive criticism. Their perspectives were crucial to shaping my project into the story it became. Special thanks to Jenna and Laura who came up with this muscle flu project; Jenna who trained me on nearly every technique I utilized, helped improve my scientific writing, and gave her constant support.

My past and present lab mates deserve special acknowledgements. April Masters, Ph.D., Erica Lorenzo, Ph.D., Jake Hopkins, Sandra Jastrzebski, Judy Kalinowski, Jenna Bartley, Ph.D., Blake Torrance, and all of the other members of the Center on Aging have been a wonderful team to work with. Countless times they dropped everything they were doing to help me with an experiment or gave me the month’s batch of aged mice. Thank you for putting up with all my questions and listening to me present at our lab meetings.

My friends have truly made graduate school an unforgettable experience. They were the ones who had everyone’s backs and knew how to help each other break from the grind of Ph.D. life.

I acknowledge my brother, Stefan, for never letting me forget that life is fun, helping me return to myself amidst stressful times, and always ready for a laugh or adventure. I owe much to my parents who raised me to be strong minded, determined, and patient. They encouraged me to strive to be better, be respectful of others, and follow my dreams. They have always supported
me and been there with unconditional love. I love you all very much and could not have done this without all of you.

I would like to give special thanks to Darcy Ahern and Linrica Golly Miss Molly. If not for Darcy I would not have made it through this program. She was the one who was there for me day and night, knew how to pick me up when I was down, and put up with me in times good and bad. She has made me a better man in many areas of my personal, academic, and professional life. Words cannot describe my gratitude and love for her. About half way through my Ph.D., Darcy and I found love in the form of a fluffy puppy/gremlin, Molly. Molly the Cavalier greets us when we come home with the same level of full excitement and cannot wait to be in our laps. She has supported us with her cuddles, gotten us to go outside more, and blissfully distracted us from the stress of day-to-day life. It is their love that has given me the strength and courage to carry on.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE PAGE</td>
<td>i</td>
</tr>
<tr>
<td>COPYRIGHT</td>
<td>ii</td>
</tr>
<tr>
<td>APPROVAL PAGE</td>
<td>iii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF FIGURES BY CHAPTER</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xi</td>
</tr>
<tr>
<td>CHAPTER ONE</td>
<td>1</td>
</tr>
<tr>
<td><strong>INTRODUCTION</strong></td>
<td>1</td>
</tr>
<tr>
<td>- Influenza and Older Adults</td>
<td>1</td>
</tr>
<tr>
<td>- Age-related immune Deficits</td>
<td>3</td>
</tr>
<tr>
<td>- Impact of aging on immune responses to influenza</td>
<td>4</td>
</tr>
<tr>
<td>- Common complications of influenza</td>
<td>8</td>
</tr>
<tr>
<td>- Secondary bacterial infections</td>
<td>9</td>
</tr>
<tr>
<td>- Acute Respiratory Distress Syndrome</td>
<td>10</td>
</tr>
<tr>
<td>- Extrapulmonary and other uncommon complications in older adults</td>
<td>11</td>
</tr>
<tr>
<td>- Cardiac Complications</td>
<td>11</td>
</tr>
<tr>
<td>- Musculoskeletal</td>
<td>13</td>
</tr>
<tr>
<td>- Central and peripheral nervous system complications</td>
<td>15</td>
</tr>
<tr>
<td>- Gastrointestinal and gut microbiota complications</td>
<td>17</td>
</tr>
<tr>
<td>- Hepatic Complications</td>
<td>18</td>
</tr>
<tr>
<td>- Renal Complications</td>
<td>19</td>
</tr>
<tr>
<td>- Diminished immune responses with aging predispose older adults to</td>
<td>20</td>
</tr>
<tr>
<td>common and uncommon influenza complications</td>
<td></td>
</tr>
<tr>
<td>- Skeletal muscle homeostasis</td>
<td>21</td>
</tr>
<tr>
<td>- Influenza vaccination</td>
<td>22</td>
</tr>
</tbody>
</table>
LIST OF FIGURES BY CHAPTER

CHAPTER ONE

Figure 1. Summary of age-related changes to the immune systems of humans and mice.....4
Figure 2. Influenza infection of the upper respiratory tract leads to complications in other tissues and organs................................................................................................................8

CHAPTER TWO

Figure 1. Prolonged weight loss and elevated flu titers in aged mice during flu infection .....53
Figure 2. Influenza infections induces functional decrements in both voluntary locomotor activity and gait kinematics that is more pronounced in the hind limbs and in the aged mice ..........................................................................................56
Figure 3. Influenza infection induces muscle-localized inflammatory gene expression in the gastrocnemius that is prolonged and elevated in aged mice.................................................................58
Figure 4. Influenza infection induced gastrocnemius expression of components of the ubiquitin proteasome pathway that is more dramatic in aged mice.........................................................60
Figure 5. Influenza infection reduced gastrocnemius expression of positive regulators of muscle mass and myogenic regulatory factors to a greater degree in aged mice..............61
Figure 6. Influenza induced weight loss is correlated with gastrocnemius gene expression of ubiquitin proteasome pathway components..................................................................................63
Figure 7. The pathogenesis of influenza-induced myopathies is likely not direct infection of skeletal muscle as viral copies are not seen in the gastrocnemius muscle .........................64

CHAPTER THREE

Figure 1. The NP/Alum vaccination hastens viral clearance while reducing body mass loss84
Figure 2. Vaccination against influenza reduced flu-induced functional decrements in voluntary locomotor activity, grip strength, and gait kinematics .................................................................87
Figure 3. Vaccination reduces flu-induced cytokines and chemokines in the BAL and Serum ..................................................................................................................................................89
Figure 4. Vaccination reduces flu-induced skeletal muscle gene expression and protein expression in gastrocnemius..............................................................................................................92
Figure 5. Vaccination reduces muscle morphological changes and fast twitch fiber atrophy94
Supplemental Figure 0. The Graphical Abstract made with biorender.com.........................105
Supplemental Figure 1. Experimental plan for the experiments described .......................106
Supplemental Figure 2. Vaccination to influenza reduced flu-induced functional decrements in voluntary locomotor activity, grip strength, and gait kinematics................................. 107

Supplemental Figure 3. Vaccination preserves architecture and nuclear infiltration of young and aged skeletal muscle H&E’s ........................................................................................................ 108

Supplemental Figure 4. Musculoskeletal protection is not NP-antibody mediated .............. 109

CHAPTER FOUR

Figure 1. Aged mice have increased weight loss and increased flu PA copies in the lung during infection, simultaneously with prolonged functional deficit ....................................... 121

Figure 2. Inflammation is elevated and prolonged with aging in BAL and serum ............ 123

Figure 3. Determination of age and DPI specific differences in global gene expression .... 125

Figure 4. Young and aged mice undergo different kinetics for inflammation and gene expression changes ........................................................................................................ 127

Figure 5. Pathway analysis of aged skeletal muscle RNA-Sequencing reveals strong leukocyte activity during pulmonary influenza infection .............................................. 130

Figure 6. Increased leukocyte and T cell infiltration in the skeletal muscle .................. 132
LIST OF TABLES

Table 1. Antibodies used for phenotyping infiltrating cells in skeletal muscle by immunofluorescence................................................................. 119

Table 2. Target genes for future hypothesis testing................................................................. 150

Supplemental Table 1. SRK_all_upregulated_pathways_table ........................ external document

Supplemental Table 2. SRK_all_downregulated_pathways_table ........................ external document
CHAPTER 1

INTRODUCTION

Influenza and Older Adults

Influenza viruses belong to the Orthomyxoviridae family and are enveloped, negative-sense, single-stranded RNA viruses with segmented genomes [1]. Influenza A is most often associated with human illness with H1N1 subtypes more often resulting in milder illness when compared to H3N2 subtypes, which are responsible for 80% of flu-related deaths [2]. Rapid mutations leave us with an ineradicable disease that has frequent epidemics. Although most influenza virus infections result in an acute, self-limiting illness, severe and fatal infections are associated with hemorrhagic bronchitis, bronchiolitis, and alveolitis with pulmonary edema and hemorrhage [3]. There are also several comorbidities that increase vulnerability to influenza infection, including underlying chronic respiratory and cardiovascular conditions, diabetes mellitus and immunosuppression [4, 5]. Immunocompromised individuals also have increased risk of enhanced disease after flu infection with prolonged viral shedding that can last for months [6]. There are severe age-related immune declines that lead to increased susceptibility and severity of flu infection in older adults. Additionally, older individuals often experience fewer clinical signs and symptoms of classical influenza infection, while also experiencing more severe complications of the disease [4-6].

It is well established that immune function declines with aging. Age-related declines of both the innate and adaptive immune systems result in increased susceptibility to infection, as well as increased severity of infection in older adults [7, 8]. Influenza (flu) is problematic in older adults with increased risk for serious complications and hospitalization. In addition, approximately 90%
of flu-related deaths occur in this population [9], with influenza and pneumonia being the eighth leading cause of death among persons over 65 years of age in the United States [10]. Even when death is avoided, older adults have an increased risk for secondary complications and morbidities from flu infection. The average annual infection rate of seasonal flu is 10-20 percent of the world population with 3-5 million hospitalizations and estimated United States economic burden of $87.1 billion USD [11, 12].

Flu infections in older adults often cause hospitalization due to prolonged illness and secondary infections, such as bacterial pneumonia. Hospitalizations attributable to flu vary depending on the flu season length and circulating strains [2]. Multiple models demonstrate a common trend for increased flu-related mortality over the past 30 years [13, 14], seemingly parallel to the increased older adult population and the growing aged population is at greatest risk. The mortality rate for adults over 85 is 16x greater than individuals 65-69 years of age [2]. Additionally, the average length of a hospital stay for flu-related complications is 2 days longer in patients over 75 compared to adults 50-64 years of age [2].

In addition to common flu symptoms, flu infection leaves older adults more susceptible to secondary infection and causes dysfunction in many other tissues. These complications are often both caused by and lead to other co-morbidities, which increases mortalities. For example, secondary bacterial infection following flu is responsible for five million cases of severe illness, which results in 250,000-500,000 deaths worldwide. Recently, these have been categorized together by the CDC as influenza/pneumonia deaths [14, 15]. Due to both immunosenescence and overall senescence that occurs with aging, the body is more prone to complications resulting from delayed and weakened responses to maintain homeostatic function. Extrapulmonary and other complications in older adults can include cardiovascular dysfunction, musculoskeletal atrophy, neurological degeneration, and worsening of preexisting
diseases. Importantly, many of these effects may be delayed so the association with flu infection may be initially masked.

Age-related declines in immune responses have been well-documented in both murine and human systems. Here, we will briefly review age-related immune changes and how they relate to the impaired response to flu infection, focusing more on the clinical significance of common and uncommon flu-associated complications in older adults. Identifying mechanisms of common and uncommon flu-associated complications in older adults can provide initial insight for future therapeutic investigations to enhance age-related immune declines and determine effective ways to prevent flu-related morbidity and mortalities.

**Age-related immune deficits**

The overall aging process leads to a decreased ability to respond to stressors and maintain homeostasis. Commonly, murine models of aging are utilized to investigate age-related changes in the immune system and the C57BL/6 mouse is considered to be one of the most studied animal models. Indeed, this mouse shares many important processes with the human immune system and in aging biology (Figure 1 [1-16]). Briefly, during the biological process of aging many immune system components are impacted. Age-related DNA damage in the bone marrow is associated with increased release of immature cells into the blood and a bias for differentiation toward myeloid lineage cells over lymphoid lineage cells from hematopoietic stem cells [22, 39, 1640, 41]. The innate immune system (specifically neutrophils, macrophages, and dendritic cells [DCs]) exhibits decreased migration and chemotaxis [29, 31, 37, 42], as well as decreased phagocytosis [33, 36, 39, 42, 46] and changes in population frequencies [28, 39, 40, 59] in both aging humans and mice. Similarly, the adaptive immune system exhibits decreased function with aging. In both mice and humans, thymic involution and dysfunction lead to
decreased naïve T cell output with aging [58, 60]. In addition, T cell function and memory T cell generation are negatively impacted by aging [18, 19, 27, 30, 64, 65, 69] and antibody quality is also reduced [77, 78]. In summary, the aged immune systems of both humans and mice exhibit delayed and reduced responsiveness with regards to both innate and adaptive aspects resulting in an overall poor response to an infectious challenge.

Figure 1. Summary of age-related changes to the immune systems of humans and mice [11-76]. Increases or decreases in cell numbers or particular functions are indicated by arrows pointing upward or downward. Figure made with biorender.com.

Impact of aging on immune responses to influenza

Along with immunosenescence, or the deleterious age-associated changes in immunity described above [81], “inflammaging” leads to cytokine imbalance, tipping towards constant
inflammation both within tissues and systemically. Indeed, immunosenescence and inflamming play a large role in exacerbating flu infection in older adults. Immunosenescence is associated with decreased phagocyte function, altered cellular migration, changes in cell population numbers, and reduced antibody production [45]. Further, we would expect the same trends during flu infection, which would impair adaptive immunity and hinder the immune responsiveness to infection.

Innate immune cells, specifically DCs, produce less interferon I/III in older adults, while showing decreasing phagocytic capacity [82]. Further, phagocytes and DCs have altered receptor signal transduction and systemic over population of neutrophils (neutrophilia) in older adults can promote increased serum levels of IL-6 and c-reactive protein that increases death risk [82]. Larger issues arise during a secondary infection (bacterial or viral) since the increased inflammatory/infection damage leaves the system both frail and exhausted [82]. DCs play a large role in generating innate and adaptive immune responses, but aged mouse studies have observed that the number of DCs recovered from spleens of aged mice is 50-70% of that found in young mice during flu infection [83]. Further studies indicated that during flu infection, DCs in aged mice had significant differences in maturation, migration, and recruitment [83-86], as well as low expression of the co-stimulatory molecules CD40 and CD86 [87].

Age-related declines in immune function contribute significantly and lead to multiple manifestations with regards to the CD4 T cell response to flu antigens [88]. Importantly, the proper functioning of CD4 T cells is essential for a robust CD8 T cell response to flu infection. Changes in T cell responses during flu infection with aging include impaired T cell receptor signaling via reduced immunological synapse formation [88-90] and deficits in CD4 T cell activation, differentiation, and proliferation [19]. With aging there is also an increase in FoxP3+ regulatory CD4 T cells (Tregs), which produce anti-inflammatory molecules, resulting in reduced expression of co-stimulatory molecules CD40 and CD86 [87].
CD8 T cells are indispensable for clearance of flu infected cells. Influenza virus-specific CD8 T cells from older adults exhibit decreased functionality with corresponding increases in CD57 and KLRG1 expression, indicating a senescent phenotype [91]. These same CD8 T cells also have reduced expression of inhibitory receptors such as PD-1, resulting in higher frequencies of flu-specific CD8 T cells that exhibit reduced lytic capacity and decreased migration and chemotaxis ability during flu infection. The overall result of these age-related changes is slower viral clearance [83, 92]. Increasing age also has a significant impact on the memory CD8 T cell response to flu infection due to the loss of protective effector memory cells from peripheral tissues over time [93, 94, 95].

Similarly, aging impacts B cell and antibody responses to flu infection. Declines in B cell precursors in old age are associated with preferential loss of lymphoid-biased hematopoietic stem cells [58]. With aging, there is decreased germinal center formation during flu infection and B cells produce lower quality antibody [77,78] in both humans and mice [77]. In addition, the increase in senescent B cells with aging is negatively associated with a protective response following flu vaccination. Importantly, many of these age-related declines in humoral responses following influenza infection and vaccination are partially due to declines in CD4 T cell function with aging [20, 71].

Related to these immune cell specific alterations, lymph node stromal cells and particularly fibroblastic reticular cells, are impacted by aging. Lymph node fibroblastic reticular cells are not significantly different between young and aged mice at steady state but have delayed proliferation and expansion in aged mediastinal lymph nodes during flu infection [96]. Further, in aged lymph nodes, mouse fibroblastic reticular cells exhibit notably reduced homeostatic chemokine production, which is associated with reduced T cell homing to these lymph nodes during flu infection. Ultimately, this is associated with delayed and impaired immune responses in aged mice [96].
Another factor to be considered regarding the immune response to flu infection is the gut microbiota, which is also important in regulating immune responses both locally and systemically [97]. Pulmonary immunity is directly related to gut microbiota, which mediates flu infection-related responses, including dendritic cell migration, T cell priming, cytokine secretion, and overall viral clearance [98]. Dysbiosis of the gut microbiota induced by antibiotic administration during flu infection influences helper T cell responses and can negatively impact flu outcomes and recovery [99]. This is of particular interest with aging, as older adults are more likely to have concurrent infections and may be on antibiotics at the time of flu infection itself or due to a secondary infection. Adequate probiotic intervention after antibiotic treatment may improve the intestinal ecosystem and prevent Th2-shifted immunity [100]. Indeed, without probiotic treatment there is further dysregulation of cytokines and cell mediated regeneration related to flu infection [100].
Figure 2. Influenza infection of the upper respiratory tract leads to complications in other tissues and organs. These complications are made worse by the diminished immune response in older adults. Figure made with biorender.com.

Common complications in older adults

Influenza infection of the upper respiratory tract leads to complications in other tissues and organs (see Figure 2). These complications are made worse by the diminished immune response in older adults. In this section, we will describe the common complications seen in older adults with influenza (flu) infection.
Secondary bacterial infections

The most frequent concurrent bacterial infections with flu are pneumopathogens: *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Haemophilus influenzae*, and occasionally other gram-negative bacilli [101, 102]. Opportunistic bacterial infections, which are common in older adults, occur during and post influenza through many mechanisms. For example, necrosis of airway epithelial cells can result in loss of mucociliary clearance, which then leads to increased colonization of the upper respiratory tract due to loss of physical barriers [103]. In fact, exposure of bacterial attachment sites can result from damaged surfaces of airways with associated fibrin deposition, tissue repair, regenerative process, and sialidase activity of the viral neuraminidase [104, 105]. Similar to humans, mice infected with flu have suppressed Th17 responses and macrophage function leading to inhibited NADPH-oxidase-dependent phagocytic bacterial clearance [106,107].

Secondary bacterial infection can cause physical damage resulting in blockage or small airway functional loss from cellular debris. Additionally, proteinaceous edema fluid and development of exudative transmural pleuritis can severely compromise the respiratory system [108, 109]. In older adults, there is a loss of respiratory epithelial progenitor basal cells resulting in epithelial cell damage, bacterial invasion and decreased lung repair [80]. During enhanced disease states the generation of a robust inflammatory response can lead to subsequent increase in anti-inflammatory responses, which restricts adaptive immune responses [110, 111]. Human studies examining the effects of the immunosuppressive cytokine interleukin (IL)-10 demonstrate that the ratio of interferon gamma to IL-10 (IFN-γ:IL-10) produced by ex vivo stimulation with flu virus and IL-7 of peripheral blood T cells is reduced in old age due to increases in IL-10 production [112] and this significantly correlates with patients suffering from laboratory diagnosed flu infection [113]. These studies also associated high IL-10 in the blood with increased longevity, but also greater susceptibility to flu infection in older adults. Thus, IL-10 seems to be important
in longevity to control overall age-related inflammation, but it can also have a negative impact on the adaptive immune response to infection, leaving older individuals more susceptible to severe infection.

Conversely, lack of control of proinflammatory cytokines can also aggravate disease in older adults and has been described in cases of flu and *Streptococcus pneumoniae* [114]. In some cases, there has been strong evidence of excessive neutrophil infiltration and early mortality [114]. In aged mice, susceptibility to *S. pneumoniae* infection was correlated with increased systemic proinflammatory cytokines, including TNF-α, prior to *S. pneumoniae* infection [114]. Indeed, we and others have shown that old mice have a prolonged and elevated inflammatory response following flu infection [24, 96, 115, 116, 117].

In summary, bacterial infection following flu leads to weakened lung efficiency, blood oxygenation, and multiple organ system dysfunction. In fact, hospitalizations and mortality resulting from flu infections are most commonly due to these complications, rather than the flu infection itself.

**Acute Respiratory Distress Syndrome**

In addition to secondary bacterial infections, acute respiratory distress syndrome (ARDS) is another common complication of flu infection, especially in the context of human aging [118, 119, 120]. ARDS is severe respiratory failure characterized by diffuse inflammation of alveolar and vascular lung structures which produces progressive hypoxemia [121]. ARDS is associated with the rapid onset of cyanosis, delirium, incontinence, and lungs filled with frothy blood-tinged sputum, all of which can be attributed to primary viral flu pneumonia [122, 123]. The rapid progression from viral pneumonia to ARDS in previously healthy adults suggests that the body’s reaction to infection leads to advanced disease states rather than flu-associated damage [124].
When combined with flu infection, ARDS can be devastating and can often result in death. In an ARDS study independent of flu, patients had a 67% survival rate at 5 years post hospital discharge and suffered decreased physical quality of life, increased costs and use of health care services, and irrecoverable severe lung injury [125, 126]. The H1N1 pandemic of 2009 caused severe hypoxemic respiratory failure defined as ARDS, independent of bacterial infection, but still with multi-organ involvement [127, 128]. Heart failure independent of myocarditis can also occur during flu infection and has higher prevalence in ARDS patients when compared to the general population [129]. In theory, older adults would not have the stem cell potential to repair the hypoxemic damage from this advanced disease nor the efficiency to clear damage quickly. Indeed, ARDS following flu leads to weakened lung efficiency, blood oxygenation, and disrupts multiple organ systems.

**Extrapulmonary and other uncommon complications in older adults**

In this section, we will examine the extrapulmonary and uncommon complications of flu infection in older adults (see Figure 2). These complications are often overlooked due to the distal nature from the primary site of infection and often times delayed onset.

**Cardiac Complications**

Cardiovascular complications during influenza infection are common in older adults. Myocarditis, defined as inflammation of the heart muscle affecting its ability to pump blood, is diagnosed with documented flu infection in 0.4-13% of cases, varying on the flu season [130, 131]. Cellular infiltration and myocyte necrosis have been found in 30-50% of flu and flu-related deaths at autopsy, despite no previous cardiac complications or involvement [132, 133, 134].
Further, myocarditis was not dependent on secondary pneumonia or other infections and was evident in cases of flu infection alone [135]. Age-related concerns arose when 69% of fatal cases, with both bacterial pneumonia and cardiac complications present post-flu, were in those over 18 years of age [132]. During the influenza A H1N1 pandemic of 2009, 70% (31/44) of studied patients with myocarditis were flu-related and, interestingly, only 32% were in people over 40 years of age [130, 131, 132, 133, 134, 135].

Ischemic heart disease (IHD), defined as the narrowing of the heart arteries, is also associated with flu infection and believed to stem from inflammation that plays a critical role in acute coronary syndrome [136]. This stems from systemic pro-inflammatory responses triggered by flu infection that are coupled with pro-coagulant effects [137]. No human study or autopsy results currently suggest the theory of direct virus invasion of the vascular bed. In rare cases, viremia in pandemic influenza strains has occurred [138, 139], but not typically in most patients [140]. In a mouse model of atherosclerosis, mice over 24 months of age with this condition and flu infection develop more prominent inflammatory and thrombotic effects on atherosclerotic plaques, with subendothelial and smooth muscle immune infiltration [141]. This suggests that older adults with atherosclerosis are at greater risk for increased severity of flu infection and potential cardiac complications.

Similar to ischemic heart disease are ischemic cerebral vascular accidents (CVAs), which also significantly increase in incidence after respiratory tract infection [142]. A study of adults (>18 years) found transient increases in adverse vascular events following an acute flu infection, wherein the median age at myocardial infarction was 72.3 years of age and the median age at first stroke following acute flu infection was 78.3 years of age [142]. Thus, suggesting an age-related at-risk population even though the direct link between flu infection and stroke events is unclear. A common theory is that increased damage and clotting factors in the blood during infection leads to greater risks of clotting in the arteries and the brain. Notably, reduced levels of
these clotting factors and reduced stroke incidence are present in vaccinated patients with most significant vaccine effects in men >65 years [143]. Similarly, in patients treated with oseltamivir, a common flu anti-viral, there is a 34% stroke risk reduction after 6 months in patients <65 years [144]. Interestingly, those who get an annual flu vaccine have a lesser likelihood of cardiac complications [145-147] and the largest treatment effect was seen among the highest risk patients (>65 years), with more active coronary disease. Indeed, vaccinated older adults were at a 2.9% risk for composite cardiovascular events versus 4.7% in those who were unvaccinated in a recent meta-analysis [145].

**Musculoskeletal**

Primary flu infection is limited to pulmonary epithelial cells, yet myalgias are a common symptom and older adults have increased risk for physical disability following flu infection. Skeletal muscle wasting in patients with lung diseases, including COPD, acute respiratory distress syndrome, and cystic fibrosis, contributes to worse outcomes and is associated with increased morbidity following flu infection [148]. Diaphragm dysfunction has also been reported in patients during flu infection and other related lung complications [149-151]. Importantly, skeletal muscle dysfunction plays a role in the pathophysiology of ARDS and is associated with prolonged mechanical ventilation, weaning failure (atrophy), and markedly elevated risk of returning to ICU post discharge. The median age of patients with ARDS who survived to be discharged from the ICU was 45 years (n=117) and the median age of those who died in ICU was 50 years (n = 78) [125]. This results in a vicious cycle of increased hospitalization and impaired quality of life associated with greater morbidity and mortality [126, 152]. These studies found age was significantly associated with physical performance post ARDS hospital discharge, in those >52 years [126]. In fact, critically ill patients with ARDS from flu exhibit
weakness, fatigue, reduced exercise capacity, and lose muscle mass even after ICU discharge and after lung recovery [125].

Interestingly, skeletal muscle more distal to the site of infection also is affected by flu infection. Despite the fact that cases of viremia are rare and direct skeletal muscle infection does not occur in influenza [153-155], the clinical association of flu infection and disability with aging has been repeatedly shown especially in those >65 years of age [156-158]. Indeed, following flu infection older adults (>65 years) are at increased risk to lose activities of daily living and develop disabilities [158, 159]. Our laboratory was the first to demonstrate a molecular link for this interaction by characterizing the impact of flu infection on muscle health in mice. Overall, flu leads to mobility impairments with induction of inflammatory and muscle degradation genes and downregulation of positive regulators of muscle growth. These effects are prolonged with aging (mice >21 months), suggesting a direct link between flu infection and increased risk of disability in older adults [115]. Others demonstrated in adult (12-16 week) mice that muscle wasting during/post flu infections is through IL-6 regulation of the E3 ubiquitin ligase atrogin-1 pathway [160], further supporting increased risk with aging as IL-6 is already implicated in aging.

In more serious situations, though much less common, extreme muscle wasting during flu infection has resulted in rhabdomyolysis, where extreme muscle breakdown causes leaking of muscle components into the blood stream that can lead to renal failure [155, 161-173]. Myositis and rhabdomyolysis have been reported during influenza A & B infection [168]. Additionally, a higher incidence of myositis has been observed in older adults (observed cases in 70-86 year patients) [169]. About 50% of adult patients hospitalized with influenza A had elevated serum creatine phosphokinase (CK), an indicator of muscle damage that can potentially lead to renal failure [174]. Between a quarter and half of people older than 85 years are estimated to be frail [175]. Frailty has also been associated with both an impaired antibody response to influenza and increased sarcopenia following flu infection [175]. Loss of muscle integrity and function is
clinically important, since flu infection has been associated with a greater incidence of falls in men and women over 70 years [176, 177]. A clinical study of 650 adults with confirmed flu cases and a mean age of 63 reported that two thirds of the subjects were at risk for flu-related morbidity and self-reported disability related to muscle pain/fatigue around 20.4% [178]. Thus, despite not commonly considered a complication of flu infection, skeletal muscle wasting during flu infection is more prevalent with aging [177] and leads to long term disability and loss of resilience.

Central and peripheral nervous system complications

There is a link between influenza and CNS manifestations. Neurologic complications from the flu include Reye's syndrome, encephalopathy, encephalomyelitis, transverse myelitis, aseptic meningitis, focal neurologic disorders, and Guillain-Barre syndrome [179]. While these complications are rare, they occur more frequently in children and older adults [179, 180]. Additionally, it seems that immunogenetics play a role in neurological associated influenza complications.

Influenza-associated encephalitis (IAE)/encephalopathy can be extremely severe and can affect all age groups, but the majority of patients were under 5 years of age and have 30% fatality rate [181, 182]. In a 21-person study of acute encephalopathy associated with flu infection, the ages spanned from 4-78 years with 5 patients >60 years, 8 patients 40-59 years, 2 patients 18-25 years, 6 patients <12 years [183]. Impaired consciousness with IAE can put patients in a dreamlike state and leave them confused or faint. Acute necrotizing encephalopathy (ANE) has severe and sudden onset and is characterized by multiple brain lesions, frequently involving the thalami. In Japan there are about 10 cases of ANE per year, 28% are fatal and age-associated prevalence occurs in children under 5 years of age [181]. Other forms of encephalitis associated
with influenza include, acute encephalopathy with biphasic seizures (AESD), febrile seizures, and subcortical white matter lesions. Flu can trigger genetic predispositions for AESD in children <2 years old and has a low fatality rate, but high probability of recurring neurological issues including intellectual, motor deficits and epilepsy [181]. In less severe cases there is only mild encephalitis/encephalopathy with a reversible lesion (MERS) of the splenium of the corpus callosum, which is often associated with good clinical outcome. MERS usually resolves in about 10 days and is most common type of encephalopathy among children aged 3-8 years [181]. Similar to MERS there is Posterior Reversible Encephalopathy Syndrome (PRES), where areas of edema via MRI are detected days to weeks after initial viral symptoms. In adult patient cases of PRES, the brain edema is often malignant, with elevated cytokines IL-8 and IL-10, but no detectable virus in the cerebral spinal fluid [184]. More severe conditions also arise from flu-associated CNS disease such as acute hemorrhagic leukoencephalopathy (AHLE), which affects adults and children. Gonzalez et al observes a 46-year old mother and her 11-year old daughter both with AHLE [182]. AHLE is characterized by rapid and fulminant demyelination and inflammation of the white matter with possible links to flu-associated multiple sclerosis flair ups [181, 182].

Flu-associated and multifaceted disease complications (observed in patients 4-78 years) do not occur in singlet, in fact, patients with IAE more frequently experience concurrent hepatic and renal function dysfunction, which could suggest a component of metabolic encephalopathy coexisting severe influenza illness rather than as a direct effect of virus itself [183]. Dysregulated immune responses have also been posited to drive neurologic complications in influenza. Serum levels of IL-6, TNF-α, and IL-10 were found to be significantly elevated in pediatric patients with IAE as compared to influenza-infected patients without neurologic involvement [185, 186]. Interestingly, elevated IL-6, TNF-α, and IL-10 has also been observed in the serum of older adults [187], suggesting that may also be at higher risk for these CNS
events. It is possible that CNS dysfunction in older adults may be overlooked due to pre-existing cognitive impairment, such as Alzheimer’s Disease or dementia, or may not associated with the flu infection itself due to confounding aging factors [187].

A correlative study on H3N2 flu virus-associated encephalopathy examined cytokine levels and NF-kB activation in peripheral blood mononuclear cells from 30 children with influenza virus-associated encephalopathy at the acute stage of flu infection [185]. This study linked serum levels of IL-6, soluble tumor necrosis factor receptor-1 (sTNFR1), and IL-10 levels with flu virus-associated encephalopathy. Higher NF-kB activation in peripheral blood mononuclear cells (PBMCs) in flu-associated encephalopathic patients’ groups was higher than patients with uncomplicated H3N2 influenza infection [185]. In young mice, A/Puerto Rico/8/1934 H1N1 flu virus induced neuroinflammatory responses paralleled loss of neurotrophic and glial regulatory factors in the hippocampus, and deficits in cognitive function. This suggests that flu-induced neuropathy that is observed clinically is likely due to neuroinflammation and can lead to cognitive declines [188]. Indeed, neuropathology with influenza is a real threat and it affects both mind and body resulting in observed loss of motor skills, reflexes, cognition, and more. Older adults are at greater risk for these complications due to many factors including elevated basal inflammation, pre-existing cognitive conditions, and other co-morbidities.

**Gastrointestinal and gut microbiota complications**

Flu infection can also be accompanied by gastrointestinal symptoms and other complications. Murine studies have supported these clinical associations as well. Flu infection itself induces gut microbiota dysbiosis through type I interferons (IFN-I) favoring proteobacteria overgrowth [189]. Such shifts can also impact T cell trafficking, differentiation, and even inflammatory responses.
In this instance there is a greater chance of secondary salmonella infection localized to the gut during or post flu infection [189].

Common gastroenteritis-like symptoms in many flu infected patients include abdominal pain, nausea vomiting, and diarrhea [190-192]. In 6-10 week old male mice, lymphocytes derived from the respiratory mucosa migrated into the intestinal mucosa during flu lung infection via CCL25-CCR9 chemokine signaling, which destroyed the intestinal microbiota homeostasis in the small intestine and led to intestinal injury [193]. Respiratory influenza virus infection can also induce intestinal immune injury via microbiota-mediated Th17 cell-dependent inflammation [193]. Age-related changes, whether direct or indirect due to antibiotic treatment, may impair flu responses in older adults. In 8-16 week old CBA/J female mouse models, respiratory influenza virus infection induces intestinal immune injury via microbiota-mediated Th17 cell-dependent inflammation [117, 194].

Flu RNA has been found in stools of patients with confirmed flu infection and this ranged from 3 to 71% in studies with children and 7.2% to 47% in studies on adults [192,195-202]. Altogether, the prevalence of flu virus in stool was 20.9% for patients in the above-mentioned studies [203]. Gastrointestinal symptoms, most commonly vomiting, were reported in 30.9% of the pandemic 2009 H1N1 flu infections, 2.8% prevalent in seasonal H1N1 infection, 25.3% of influenza B virus infections and 21.9% for influenza A(H3N2) [203-205]. In theory, the already weakened immune system of older adults would be less prepared for these types of infections.

**Hepatic Complications**

Liver injury, from secondary systemic inflammation, mediated by viral infection is also an uncommon complication during flu infection, however this is observed in some older patients [120, 206]. Papic et al. examined pandemic 2009 flu patients (mean age of 46.79) compared to
seasonal flu patients (mean age of 47.06) and determined both illicit significant immune responses to infection leading to hepatocellular injury [206]. Additionally, Polakos et al. infected humans aged 18-45 years with $10^7$ TCID$_{50}$ influenza A/Kawasaki/86 (H1N1) virus to observe the flu-induced changes to the human liver [207]. Indeed, severe cases of flu have reported liver damage evident through elevated blood transaminases [alanine transaminase (AST) and aspartate transaminase (ALT)], bilirubin, and Gamma-glutamyl transferase (GGT) [206, 207]. Additionally, these elevations were associated with duration of hospitalization, hypoxia, and serum C-reactive protein (CRP) levels [206, 207]. Others have even noted centrizonal hepatic hemorrhagic necrosis in deceased pandemic flu patients ages 24-65 years upon autopsy [208, 209], while Avian A(H5N1) and A(H7N9) were specifically connected with transaminase elevations [209, 210]. Beyond these threats, the liver is also susceptible to clots, hemorrhage, and less severely metabolic dysfunction [208, 209]. It is likely since there is increased systemic inflammation in older adults during flu infection, and that they may have pre-existing hepatic deficiencies, older adults may be more susceptible to hepatic complications during flu infection. In the context of future directions, more research should focus on the metabolic impact of flu infection on organs such as the liver, particularly in the context of aging.

**Renal Complications**

As with other complications, flu-associated renal complications are worse in those with pre-existing risk factors, including obesity, chronic kidney disease, and increased age [211, 212]. Indeed, older adults are more likely to suffer from pre-existing renal impairment. Flu infection, even without severe complications such as rhabdomyolysis, can cause acute kidney injuries. Incidence of acute kidney injuries ranged from 18-66% in ICU admitted flu patients with the average age of 43 years and 13% of patients over 60 years [213-215]. Further, in the 2009
pandemic influenza A (H1N1) studies observed acute tubular necrosis (ATN), acute kidney injury was reported in up to 66% of critically ill adults (aged 25-50 years) and was associated with higher mortality rates [216-218]. These renal injuries are speculated to be due to decreased renal perfusion secondary to hypovolemia or a vasodilatory state of sepsis during severe flu infection. Importantly, renal function is regularly monitored in ICU admitted older adults for almost all infections. However, renal function and filtration declines with aging and the decrease of repair capacity leaves the organ vulnerable in older adults. In the context of future directions, there should be more research into the relation between kidney injury and flu infection so that more preventative action can be taken.

**Diminished immune responses with aging predispose older adults to common and uncommon influenza complications**

Diminished immune responses in older adults sets this population aside from others with pre-existing vulnerability to influenza infection and its complications. In addition to weakened and delayed immune responses, older adults often struggle with recovery post infection due to extenuating complications. Indeed, the dysregulation of immune responses and increased systemic inflammation contribute to multiorgan complications that negatively impact older adults. The most common complication in older adults is secondary bacterial infection, such as bacterial pneumonia. Indeed, secondary bacterial infection is implicated in the majority of flu-associated deaths in older adults. While commonly overlooked, uncommon complications, such as ARDS, often times follow pneumonia and can lead to advanced disease states. Extrapulmonary flu complications are also more common in older adults. The severity of flu-associated myocarditis spans a wide spectrum ranging from asymptomatic to severe heart disease, though the relation to age is not clear. Interestingly, skeletal muscle more distal to the
site of infection also is affected by flu infection with older adults more at risk for weakness and muscle atrophy, often resulting in declines in physical function. Surprisingly, even separate from these instances of flu virus in the intestine, there are microbiota altering events during lung flu infection, which shift T cell population frequencies and induce dysbiosis. Adverse neurological and central nervous system complications with flu, mainly seen in pediatric and older adults, have associations with demyelination, encephalopathy, seizures, and cognitive declines. Other commonly overlooked complications from systemic inflammation and multiorgan dysfunction include hepatic and renal systems which are already weakened in older adults from pre-existing complications. While vaccination reduces some of these common and uncommon complications, the negative impacts of flu infection are still evident in older adults. Thus, older adults need to be monitored more closely for flu-associated complications to prevent flu-associated morbidities and disabilities.

**Skeletal muscle homeostasis**

Humans by age 80 have an estimated 30-40% less of skeletal muscle fibers when compared to young adults [219]. Skeletal muscle controls voluntary motion, but is also the body’s natural amino acid reservoir, in which amino acids are incorporated into myofibrillar proteins and cellular organelles with an average daily skeletal muscle protein turnover rate of 1-2% [220, 221]. More importantly, to facilitate with wound healing, especially following traumatic injury/acute infection, the body requires a large and sustained supply of amino acids to heal properly [154, 155, 221]. The exact mechanism to sacrifice and degrade skeletal muscle for usable amino acid is unknown, but there have been several studies that have analyzed the egress of tagged amino acids from leg muscle to wound sites for repair/immune energy needs [220, 222, 223, 224, 225, 226, 227]. This process even occurs during fasting, which is an
inadvertent side-effect of influenza, thereby contributing to the demand for amino acid from skeletal muscle. On a clinical level, skeletal muscle injury can be observed by assessing serum creatine kinase levels, as well as serum myoglobin [154, 155, 221]. We hope to identify ways to reduce or stop this process to preserve skeletal muscle mass in aged individuals since their regenerative capabilities are weakened to the point where disability is guaranteed and there is no hope in completely regaining lost function/mass.

**Influenza vaccination**

The age-associated decline in immunity is partially the result of reduced production of high affinity neutralizing antibodies, which then increases susceptibility to pathogen invasion [228]. This is exacerbated by declines in T cell receptor repertoire diversity and the number of naïve T cells that are available to recognize a new pathogen. To improve upon these defects in the aged immune system, flu-vaccination is highly advised for older adults because of the added protection and improved efficacy/faster response [116, 229, 230]. Vaccination has the potential to prevent disability, irreparable damage, and even death in older adults. Nonneutralizing influenza vaccines have the capacity to protect the public against secondary bacterial diseases and reduce the pathogenicity of flu-infection when neutralizing vaccines are not available [230]. Chapter three of this thesis will evaluate vaccine-induced prior immunity and its effect on reducing flu-associated myopathy by comparing cellular immunity and humoral immunity.

**Goals of thesis**

The primary goal of this dissertation is to expand our understanding of how aging impacts various components of the immune response to influenza infection, with a focus on skeletal
Our hypothesis predicts that flu-induced systemic factors trigger skeletal muscle atrophy, which then impairs muscle function and its regenerative capacity in aged mice because of prolonged inflammation and inflammatory immune cell presence in the muscle. This research has laid the ground work for others to further characterize and test pathways/genes as mechanism(s) of flu-induced muscle degradation and dysfunction in aging.

In chapter two, “Aging augments the impact of influenza respiratory tract infection on mobility impairments, muscle-localized inflammation, and muscle atrophy,” we elucidate how aging impacts several components of the flu response and myopathy. We determine the molecular link between pulmonary infection and skeletal myopathy. We also assess the contribution of the aged skeletal muscle function and environment in the promotion of impaired immune response/healing during influenza infection.

In chapter three, “Vaccination mitigates influenza-induced muscular declines in aged mice,” our goal was to determine if prior immunity reduces flu-induced skeletal muscle decrements. We demonstrated that prior immunity, induced by vaccination, prevents muscle fast twitch fiber atrophy and consequently protects muscle functionality. This chapter is proof that despite decrease reduced vaccine efficacy with aging, there is still protection to muscle histology, function, and molecular signals.

In chapter four, “Pathway Analysis of aged skeletal muscle RNA-Sequencing reveals strong leukocyte activity during pulmonary influenza infection,” the overarching scientific premise is based on our published study that demonstrated increased muscle atrophy during influenza infection with aging. We hypothesize that lingering systemic inflammation in aged individuals during flu infection and increased muscle sensitivity to this inflammation contributes to increased muscle-localized inflammation, cellular infiltration, and muscle atrophy. In our mouse model of
flu infection, we find functional decrements in skeletal, as well as molecular aberrations such as upregulation of muscle inflammatory and atrophy gene expression and down regulation of positive muscle regulators. Our goal in this project is to identify mediators involved with age-related flu-induced myopathies that will help us to formulate mechanistic hypotheses about how this happens and how it can be prevented in order to keep older adults healthier longer.

REFERENCES


204. M.D. de Jong, B.V. Cam, P.T. Qui, V.M. Hien, T.T. Thanh, N.B. Hue, M. Beld, L.T. Phuong, T.H. Khanh, N.V.V. Chau, Fatal avian influenza A (H5N1) in a child presenting with


CHAPTER TWO

Aging augments the impact of influenza respiratory tract infection on mobility impairments, muscle-localized inflammation, and muscle atrophy

ABSTRACT

Although the influenza virus only infects the respiratory system, myalgias are commonly experienced during infection. In addition to a greater risk of hospitalization and death, older adults are more likely to develop disability following influenza infection; however, this relationship is understudied. We hypothesized that upon challenge with influenza infection, aging would be associated with functional impairments together with upregulation of skeletal muscle inflammatory and atrophy genes. Infected young and aged mice demonstrated decreased mobility and altered gait kinetics. These declines were more prominent in hind limbs and in aged mice. Skeletal muscle expression of genes involved in inflammation, as well as muscle atrophy and proteolysis, increased with influenza with an elevated and prolonged peak in aged mice. Infection also decreased expression of positive regulators of muscle mass and myogenesis components to a greater degree in aged mice. Gene expression correlated to percent body mass loss, although evidence did not support direct infection of muscle. Overall, influenza leads to mobility impairments with induction of inflammatory and muscle degradation genes and downregulation of positive regulators of muscle. These effects are augmented and prolonged with aging, providing a molecular link between influenza infection, decreased resilience and increased risk of disability in older adults.
INTRODUCTION

It is well established that immune function declines with aging. Immunosenescence of both the innate and adaptive immune systems result in increased susceptibility to infection, as well as increased severity of infection in older adults. Influenza (flu) tends to be particularly problematic in older adults with increased risk for serious complications and hospitalization. Approximately 90% of flu-related deaths occur in older adults [1], with influenza and pneumonia being the seventh leading cause of death among persons over 65 years old in the United States [2]. Even when death is avoided, older adults have increased risk of morbidity and disability from flu infection. Flu-related hospitalizations are associated with increased loss of independence [3] and long term declines in activities of daily living are observed post flu infection among nursing home residents [4]. Further, flu is among the leading causes of catastrophic disability and dramatic losses of activities of daily living in older adults [5]. While it is known that prolonged hospitalization of older adults is associated with decreased muscle mass and strength; flu infections, independent of hospitalization, have some degree of muscle involvement with myalgia among the common symptoms even in uncomplicated infections [6].

While myalgia is a common non-pulmonary symptom of flu infection, other myopathies are less commonly reported. Interestingly, despite increased clinical severity in older adults, most flu associated myopathies are reported in pediatrics [7, 8], though it is possible, and quite likely, that older adult myopathies are under reported and not the primary focus of care due to other more life-threatening complications. In pediatric populations the most common flu-associated myopathy is acute myositis, characterized by severe calf pain, difficulty walking, and altered gait that generally resolves on its own within 30 days, but more commonly within a week [7, 8]. Less frequently acute myositis has also been reported in both adults [9] and older adults [10]. In a range of ages, elevated circulating markers of muscle damage, such as creatine kinase (CK), myoglobin, and lactate dehydrogenase, have been reported during flu infection [7-13].
Additionally, during flu pandemics there have been cases of rhabdomyolysis reported [14-16] and muscle biopsies have confirmed atrophic/necrotic muscle fibers, though inflammatory cell infiltration seems less common [7, 17]. Furthermore, during the 2009 H1N1 flu pandemic, elevated serum CK was associated with worse flu outcomes (length of intensive care unit stay, increased pulmonary, kidney, and other non-pulmonary complications) [11].

Nevertheless, the flu virus demonstrates great specificity for pulmonary epithelial cells, with all evidence indicating that in all or nearly all cases active infection remains limited to the respiratory system [6]. Thus, while a wealth of literature indicates symptomatic or functional muscle involvement with pulmonary flu infection, it is unclear if these complications only occur in severe infections or if they are under reported and under studied in less severe infections. The limited research regarding flu-induced myopathy pathogenesis is controversial; direct viral infection of the muscle and immune-mediated cytokine storm induced muscle damage are among the top hypotheses. While some in vitro studies have shown that myoblasts and myotubes may be susceptible to infection and might produce live viral progeny [18-20], isolation of virus from muscle biopsies is rare [12, 21-23]. In vivo murine experiments showed that a non-permissive infection is possible in mature muscle fibers, though this is more likely with intramuscular inoculation [24-26], so the clinical relevance of these experiments remains entirely unclear.

While the pathogenesis of flu-associated myalgia and myopathy has yet to be determined, their clinical significance is apparent. Though flu-induced myopathies in pediatric cases are not long lasting conditions with permanent effects, it is possible that due to decreased resilience in older adults flu-induced myopathies may be prolonged and have lasting effects; leading to the increased disability and loss of independence observed post flu [3-5, 27]. As the aging population continues to grow, emphasis on extending healthspan and increasing resilience is necessary [28]. Flu and possibly other respiratory tract infections may represent an under
reported risk factor predisposing older adults to sarcopenia, frailty, and overall decreased resilience. Here, in a well-validated murine model of flu infection, we aimed to characterize flu effects on skeletal muscle, both from a functional and molecular perspective, in both young and aged mice. We hypothesized that during flu infection aging would be associated with diminished mobility and functional performance together with upregulation of skeletal muscle inflammatory and atrophy genes.

METHODS

Mice

Young (2.5-4 month old) C57BL/6 male mice were obtained from Charles River Laboratories and aged (19-22 month old) C57BL/6 male mice were obtained through the National Institute on Aging rodent colony. All mice were housed in a climate controlled environment with 12:12 light:dark cycle and fed standard rodent chow and water ad libitum. All procedures were approved by the University of Connecticut Medical School IACUC (protocol 100705) and carried out in accordance with these regulations. All mice underwent gross pathological examination at time of sacrifice and animals with obvious pathology were excluded from the study.

Viral Infection

Mice were anesthetized with isoflurane and intranasally inoculated with 50μl of 500 EID50 of influenza virus A/PR/8/34 (PR8). Mice were weighed daily to monitor infection progression. At time points indicated, whole lung tissue was homogenized and RNA was isolated via RNeasy Mini Kit (Qiagen Inc., Valencia, CA). RNA was reverse transcribed with iScript cDNA synthesis
Kit (Bio-Rad Laboratories, Inc., Hercules, CA) and flu viral copies were detected via reverse transcription quantitative PCR of flu acid polymerase (PA).

Voluntary Locomotor Activity

Spontaneous voluntary locomotor activity was measured via open field test at time points indicated. All tests were performed between 6-8am to control for diurnal variations. Following acclimation to the dim-lit testing room (at least 1 hour), mice were placed in the center of the photobeam activity system-open field (PAS-OF, 16”x16”x15” acrylic animal enclosure, San Diego Instruments, San Diego, CA) and their activity was recorded for 20 minutes. The first 5 minutes was excluded as this is generally considered to be exploratory behavior rather than general voluntary locomotor activity. The number of beam breaks per minute during the last 15 minutes was then used to assess voluntary locomotor activity.

Gait Analysis

Gait analysis was performed using the DigiGait instrument (Mouse Specifics, Inc, Quincy, MA) and software (DigiGait Imager 4.0.0 and DigiGait Analysis 11.5, Mouse Specifics, Inc). The DigiGait instrument consists of a clear treadmill with a high-speed camera mounted underneath that collects images at 147 frames per second for high resolution of postural temporal gait parameters. Mice run within a 2” wide acrylic running chamber at set speeds. The ventral plane videos are analyzed with the DigiGait software which identifies portions of the paw that are in contact with the treadmill belt to produce both postural and kinematic gait parameters. Mice were introduced to the DigiGait system at a low speed (10cm/sec) briefly (30 seconds) prior to the initial testing. Mice were allowed to acclimate to the dim-lit room for 1 hour prior to each
testing period and all tests were performed between 6-8am. Mice ran at the testing speed (16cm/sec) until approximately 5 seconds of consecutive walking was recorded and this video segment was analyzed via DigiGait software.

Gastrocnemius Reverse-Transcription Quantitative PCR (RT-qPCR)

At the time points indicated for gastrocnemius gene expression mice were fasted with the exception of water for 4-6 hours prior to sacrifice to minimize potential confounding results due to postprandial muscle protein synthesis. The gastrocnemius muscle was dissected and placed in RNAlater (Qiagen Inc.) overnight at 4⁰C. RNAlater was removed and gastrocnemius was frozen at 80⁰C until RNA extraction. The muscle was homogenized and RNA was extracted via RNeasy Fibrous Tissue Mini Kit (Qiagen Inc.). RNA quantity and quality were assessed with Nanodrop 2000c (Thermo Scientific, Waltham, MA) and was reverse transcribed via iScript Advanced cDNA synthesis Kit (Bio-Rad Laboratories, Inc.). RT-qPCR was performed using custom designed PCR plates with predesigned commercially available primers (Bio-Rad Laboratories, Inc.). Gene expression was calculated via a modified Pfaffl method utilizing multiple reference genes (RPS18 and TBP, which showed the least variability between conditions and thus suitable reference genes) and normalized to gene expression of young mice at Day 0 prior to infection to give comparable fold changes.

In Vitro Myoblast Culture and Infection

Harvested leg skeletal muscle (gastroc, vastus medialis, vastus lateralis, soleus, anterior tibialis) from uninfected young and aged mice was incubated in collagenase type IIA and dissociated in growth media (Dulbecco modified Eagle’s medium (D-MEM) supplemented with 20% fetal
bovine serum (FBS), 1% penicillin/streptomycin, 5ng/mL basic fibroblast growth factor (bFGF)) and grown on extracellular matrix-coated plates (extracellular matrix, Sigma-Aldrich Corp., St. Louis, MO). When myoblasts reached ~50% confluency, they were purified via plating on an uncoated Petri dish to remove any adherent fibroblasts. Myoblasts were then seeded at 150,000 cells per well in coated 6-well plates and allowed to grow to 50% confluency prior to infection. For infections, myoblasts were washed twice with phosphate buffered saline (PBS) and incubated for one hour with 0, 10, or 100 EID50 PR8 in D-MEM supplemented with 1% penicillin/streptomycin. Then myoblasts were washed twice with PBS and cultured with growth media for the remaining days. Culture media at time points indicated was analyzed for cytokine/chemokine concentrations via multiplex (25Plex Magnetic Bead Panel, EMD Millipore, Billerica, MA). Most cytokine/chemokines were below detectable limits (GCSF, GMCSF, IFN-γ, IL10, IL12p40, IL12p70, IL13, IL15, IL17, IL1β, IL1α, IL2, IL4, IL5, IL7, IL9, MIP1β, MIP1α, MIP2, RANTES, TNF-α). Total RNA was extracted via TRIZol (Ambion, Life Technologies, Grand Island, NY) according to manufacturer’s recommendation. RNA was reverse transcribed and flu viral copies were detected via real-time quantitative PCR of flu PA.

Statistical Analysis

Weight loss, viral titers, functional performance, and log-transformed gastroc gene expression results were analyzed via 2-way ANOVA (age x time point) with Bonferroni posthoc corrections when indicated with significance set at p<0.05. Genes were considered differentially expressed if fold changes were > 2 and p<0.05. In vitro myoblast supernatant and RNA were analyzed via 3-way ANOVA (age x infection condition x time point) and 2-way ANOVA (age x infection condition), respectively, with Bonferroni posthoc corrections when indicated with significance set at p≤0.05. Univariate linear regression was used to compare the relationship between gastroc
gene expression and percentage weight loss independently for each variable that showed time effects with significance set at p<0.05. Step-wise multiple regression analysis was used to determine if multiple variables could better predict percent weight loss.

RESULTS

Following influenza infection aged mice have prolonged weight loss and elevated lung viral titers

Young and aged mice were infected intranasally with a sublethal dose (500 EID50) of influenza A/PR/8/34 (PR8). Percent weight loss following infection is more severe and prolonged in aged mice (Fig 1A). While young mice begin to gain body mass by 10 days post infection (DPI), aged mice do not recover as quickly and differences between young and aged mice exist on 10-15 DPI. In the aged groups, increased weight loss is accompanied by slower viral clearance (Fig 1B) as measured by influenza polymerase (PA) copies in whole lung tissue via RT-qPCR.

Although weight loss is a common marker used for pathogenicity in mice, it is rarely considered as a relevant outcome measure and mechanisms involved remain unknown.

Figure 1. Prolonged weight loss and elevated flu titers in aged mice during flu infection. Young and aged C57BL/6 mice were intranasally infected with 500 EID50 of PR8 influenza. (A) Weight loss was monitored throughout the infection and percent weight loss was calculated from day 0 prior to infection. Data shown as mean ± SEM. Two-way ANOVA with Bonferroni posthoc corrections showed significant weight loss (compared to day 0) on day 6 through 15 (not indicated in figure) and differences between young and aged mice at time points indicated (* = p<0.05). (B) On day 0, 3, 7, 11, and 15 whole lung tissue was harvested and RNA was isolated. Total number of copies of influenza PA was determined via
Influenza infection induces impairments in voluntary locomotor activity and gait parameters in young and aged mice

To assess functional decrements associated with flu infection, we examined both voluntary activity levels, as well as more sensitive postural and kinematic gait alterations. Decreased voluntary locomotor activity was evident by 3 DPI in both young and aged mice as assessed by beam breaks in the open field test (Fig 2A). Diminished activity persisted through 20 DPI. Aged mice had fewer beam breaks per minute than their young counterparts on 11 (77.9% fewer), 15 (67.5% fewer), and 20 DPI (63.1% fewer) indicating that flu-induced decreased voluntary locomotor activity is more pronounced and prolonged in aged mice.

More detailed analysis of walking patterns was performed using the DigiGait system, which employs ventral plane videography to assess both spatial and temporal indices of gait at a given speed. Preliminary studies determined that 16cm/s was a speed that both young and aged mice could complete without difficulty and have consistent gait patterns for analysis (data not shown). Throughout the course of flu infection, no significant changes in stride, swing, or stance duration were observed (data not shown); however, alterations in postural components and acceleration parameters existed. By 7 DPI mice reduced stance width of both the fore (Fig 2B) and hind (Fig 2E) limbs by 15% and 13%, respectively, compared to baseline. Interestingly, aged mice initially had a wider fore limb stance, but these differences were concealed later in the infection. More pronounced differences in flu-induced gait alterations between the young and aged mice were observed in the midline distance of the fore (Fig 2C) and hind limbs (Fig 2F) later in infection, with hind limb midline distance being 46% narrower in aged mice compared to young mice on 20 DPI. This indicates aged mice are reaching less from their center with every step, perhaps
due to increased muscle and joint pain limiting mobility. Additionally, later in the infection aged mice have increased stride length variability in the hind limbs compared to the young mice (Fig 2J). Gait symmetry index of the fore/hind limbs (Fig 2D) was increased in both young and aged mice at 11 DPI and 15 DPI as well, indicating a greater number of steps were taken with the fore limbs compared to the hind limbs.

Acceleration and deceleration measures were also affected by flu infection. The maximal rate of change of paw area contact during the braking phase (Max dA/dt), or how rapidly the mouse decelerates, is decreased in the fore (Fig 2H) and hind limbs (Fig 2K) later in the infection, though more pronounced in the hind limbs. Similarly, the maximal rate of change in paw area contact during the propulsion phase (Min dA/dt), or how rapidly the mouse propels itself into the next step, is decreased in the hind limbs by 7 DPI, but recovered by 20 DPI (Fig 2L). Both acceleration and deceleration are important parameters indicating the rate of force development, an important component of muscle health and quality. Interestingly, decreased Max dA/dt was still evident at 20 DPI.

Taken together, alterations in gait include decreased force development accompanied by decreased reaching distance and a narrower stance, more marked in the hind limbs and in aged mice. Diminished voluntary locomotor activity is prolonged in the aged mice as well. Functional impairments may be indicative of flu-induced muscle inflammation and damage, and that this is more severe and prolonged in aged mice.
Figure 2. Influenza infections induce functional decrements in both voluntary locomotor activity and gait kinematics that is more pronounced in the hind limbs and in the aged mice. Young and aged C57BL/6 mice were intranasally infected with 500 EID50 of PR8 influenza. On days 0, 3, 7, 11, 15, and 20 mice were tested for functional performance. (A) Spontaneous voluntary activity was assessed via the open field test on a photobeam activity system. Beam breaks were recorded as mice traveled at 16"x16" open field and locomotor activity was assessed as beam breaks per minute. Gait parameters were assessed utilizing DigiGait, a ventral plane videography treadmill system. Postural gait parameters (Stance width of the fore (B) and hind (E) limbs and midline distance of the fore (C) and hind (F) limbs) were altered during flu infection with more prominent results in the hind limbs of aged mice. Kinematic gait parameters were also altered with flu infection. Gait symmetry of the fore/hind limbs (D) was increased. Stride length variability of the fore limb (G) did not change, however the aged mice had increased stride length variability in the hind limbs later in the infection (J). Maximal rate of change of paw area contact during the breaking phase (Max dA/dt) and propulsion phase (Min dA/dt) is altered in the fore (H and I, respectively) and hind limbs (K and L, respectively) with more dramatic results in the hind limbs. All data analyzed via two-way ANOVA with Bonferroni posthoc corrections with effect of flu
infection over time (compared to day 0, p<0.05) indicated by brackets above data and differences between young and aged mice (p<0.05) at time points indicated by asterisk.

**Influenza induces altered inflammatory gene expression in skeletal muscle**

Since alterations in gait were primarily in hind limbs, the gastrocnemius (gastroc) muscle gene expression was further examined over the course of flu infection in young and aged mice. As hypothesized, inflammatory gene expression in the gastroc was altered over the course of the flu infection. By 7 DPI flu induced increased gene expression of interleukin (IL)-6 (IL6) and IL-6 receptor alpha (IL6RA) (Fig 3A and Fig 3B). IL6RA expression remained elevated by 3.6 fold in the aged mice at 11 DPI while the young mice returned to baseline expression levels. Aged gastroc had 2.7-fold increased expression of tumor necrosis factor (TNF) at baseline and these differences were intensified on 15 DPI with 6.7-fold greater expression of TNF in aged gastroc (Fig 2C). IL-6 and TNF are two key inflammatory mediators in skeletal muscle degeneration and repair, signaling through STAT3 and NFκB, respectively, that have vast effects on pro-inflammatory signaling, protein degradation, and atrophy gene induction (reviewed in [29]).

Additionally, a dramatic 43-fold increase in expression of chemokine (C-X-C Motif) Ligand 10 (CXCL10), a predominant player in T helper (Th) 1 responses that recruits immune cells, particularly T lymphocytes expressing its receptor CXCR3, into tissue, was observed in the aged gastroc at 11 DPI (Fig 3D). Prolonged and exaggerated levels of CXCL10 would increase immune cell recruitment and muscle inflammation, and potentially impair muscle regeneration processes.

Taking these results collectively, flu induced lingering inflammation in the aged muscle. As it is known that these inflammatory mediators signal through NFκB and other pathways that induce muscle atrophy and protein degradation, we next examined the expression of genes involved in these processes.
Figure 3. Influenza infection induces muscle-localized inflammatory gene expression in the gastrocnemius that is prolonged and elevated in aged mice. Young and aged C57BL/6 mice were intranasally infected with 500 EID50 of PR8 influenza. At day 0, 3, 7, 11, and 15, mice were fasted for 4-6 hours prior to sacrifice and gastrocnemius muscle was harvested and RNA was isolated. Gene expression was analyzed via RT-qPCR and normalized to reference genes and expression of young mice at day 0 to indicate fold changes. Influenza induced increased expression of IL6 (A) and IL6RA (B). Increased TNF (C) and CXCL10 (D) expression was observed in the aged mice. All data was log-transformed and analyzed via two-way ANOVA with Bonferroni posthoc corrections with effect of flu infection over time (compared to day 0, p<0.05) indicated by brackets above data and differences between young and aged mice (p<0.05) at time points indicated by asterisk.

*Increased expression of protein degradation and muscle atrophy genes post influenza infection is prolonged in aged mice*

Although there are many pathways involved in protein degradation and atrophy, we focused on the ubiquitin proteasome pathway, primarily the muscle-specific E3 ubiquitin ligases atrogin-1 (also known as muscle atrophy F-box (MAFbx)) and muscle RING finger 1 (MuRF1), as the majority of literature to date shows increased atrogin-1 and/or MuRF1 expression at some point during almost all conditions of muscle wasting and atrophy [30]. As part of the ubiquitin proteasome pathway, atrogin-1 and MuRF1 control the ubiquitination and therefore degradation
of specific target proteins in response to key signals. Key initiators of these pathways include inflammatory cytokines, as well as myostatin, glucocorticoids, FoxO transcription factors, and others. Following flu infection, we observed increased gastroc gene expression of both myostatin (MSTN) and FOXO1 at 7 DPI (Fig 4C and 4D), by 2.0- and 2.3-fold change, respectively. FOXO1 remained elevated at 11 DPI, while MSTN elevation was only transient. Interestingly, FOXO1 was elevated in the young compared to aged mice at baseline and 3 DPI.

Corresponding with elevated cytokines, myostatin, and FOXO1 expression, both ATROGIN1 and MuRF1 gene expression were elevated with flu infection at 7 DPI by 2.1 and 5.3-fold, respectively. Moreover, in accordance with the prolonged inflammation, aged mice ATROGIN1 and MuRF1 expression remained elevated at 11 DPI (Fig 4A and Fig 4B). While in young mice ATROGIN1 and MuRF1 expression was already decreased to below baseline levels (-2.6 and -2.3-fold, respectively) at this time point, in aged mice expression remained elevated 2.3 and 2.5-fold, respectively. Interestingly, ATROGIN1 was downregulated by 15 DPI, perhaps indicating an attempt to limit muscle degradation and begin repair processes. Baseline expression of MuRF1 was decreased in aged mice, while no significant baseline differences existed in ATROGIN1 expression. The influence of atrogin-1 and MuRF1 mRNA and protein expression on solely age-related muscle loss has yet to be resolved; some murine studies agree with our findings and show baseline suppression [31], while others have shown increased expression [32], and human studies have shown no differences [33, 34]. Nonetheless, induction of atrogin-1 and MuRF1 in response to flu infection indicates increased ubiquitination and proteolysis within the muscle. Furthermore, expression of two ubiquitin proteasome encoding genes, ubiquitin B (UBB) and ubiquitin C (UBC), was also increased with flu infection with a 2.6- and 3.6-fold increase at 7 DPI (Fig 4E and Fig 4F). These increases were more dramatic and prolonged in aged mice. In young mice UBC expression peaked at 7 DPI, while aged mice continued increasing expression to a staggering 9.8-fold increase at 11 DPI. The increased levels of UBB
and UBC, as well as ATROGIN1 and MuRF-1 indicate a catabolic environment of increased proteolysis, promoting muscle atrophy.

Figure 4. Influenza infection induced gastrocnemius expression of components of the ubiquitin proteasome pathway that is more dramatic in aged mice. Young and aged C57BL/6 mice were intranasally infected with 500 EID50 of PR8 influenza. At day 0, 3, 7, 11, and 15, mice were fasted for 4-6 hours prior to sacrifice and gastrocnemius muscle was harvested and RNA was isolated. Gene expression was analyzed via RT-qPCR and normalized to reference genes and expression of young mice at day 0 to indicate fold changes. Influenza induced increased skeletal muscle expression of negative muscle regulators (Myostatin (MSTN, C) and Forkhead box protein O1 (FOXO1, D)), as well as ubiquitin proteasome components (Atrogin1 (A), MuRF1 (B), Ubiquitin B (UBB, E), and Ubiquitin C (UBC, F)). All data was log-transformed and analyzed via two-way ANOVA with Bonferroni posthoc corrections with effect of flu infection over time (compared to day 0, p<0.05) indicated by brackets above data and differences between young and aged mice (p<0.05) at time points indicated by asterisk.

Influenza infection decreases expression of positive regulators of muscle growth

Increased atrogin1/MuRF-1 pathway components, as well as increased inflammatory cytokines, are associated with diminished muscle growth; however, following injury or atrophy muscle generally has a remarkable capacity to regenerate and repair. Muscle regeneration through myogenesis is regulated at many key steps by myogenic regulatory factors (MRFs), myocyte enhancer binding factors 2 (Mef2), and other growth factors. Thus, we next examined the gene expression of positive regulators of muscle growth following flu infection to determine if repair
processes are suppressed during this time and/or if muscle regeneration follows after atrophic responses with flu. At 7 DPI when atrophy and ubiquitin genes are upregulated, gastroc *IGF1* expression is reduced in both young and aged mice (Fig 5A). This was transient in the young mice, but remained downregulated in the aged mice through 11 DPI. During flu infection gastroc *PAX7*, a marker of satellite cells which are critical for adding myonuclei and regenerating muscle tissue, expression is decreased at 7 DPI in both young and aged mice, but to a greater degree in aged mice, less than one-fold reduction compared to ~3.5-fold reduction in the aged (Fig 5D). Similarly, at this time the expression of both *MYOD1* and *MYOG*, key MRFs, was decreased to a greater degree, with approximately 7- and 2-fold reductions, respectively, in the aged mice (Fig 5E and 5F). *MEF2C*, which acts in concert with the MRFs to control DNA binding and transcriptional regulation, was also suppressed at this time point and remained suppressed at 11 DPI in the aged mice (Fig 5B).

In summary, flu suppressed positive regulators of muscle mass and regeneration with concurrent increases in negative regulators. Additionally, these responses were greater and/or prolonged in the aged mice.
Figure 5. Influenza infection reduced gastrocnemius expression of positive regulators of muscle mass and myogenic regulatory factors to a greater degree in aged mice. Young and aged C57BL/6 mice were intranasally infected with 500 EID50 of PR8 influenza. At day 0, 3, 7, 11, and 15, mice were fasted for 4-6 hours prior to sacrifice and gastrocnemius muscle was harvested and RNA was isolated. Gene expression was analyzed via RT-qPCR and normalized to reference genes and expression of young mice at day 0 to indicate fold changes. Influenza reduced skeletal muscle expression of insulin-like growth factor 1 (IGF1, A), myocyte enhancer binding factor 2C (MEF2C, B), paired box protein 7 (PAX7, C), myogenic differentiation 1 (MYOD1, D), and myogenin (MYOG, E). All data was log-transformed and analyzed via two-way ANOVA with Bonferroni posthoc corrections with effect of flu infection over time (compared to day 0, p<0.05) indicated by brackets above data and differences between young and aged mice (p<0.05) at time points indicated by asterisk.

*Flu induced weight loss is correlated with gastroc gene expression of ubiquitin proteasome components*

In order to determine if flu-induced weight loss was associated with muscle degradation, we performed correlation analysis on all variables that exhibited a time effect over the course of the flu infection. Indeed, multiple variables had significant correlations and age interactions were evident (Fig 6). In both young and aged mice FOXO1 (Fig 6A), IL6RA (Fig 6B), UBB (Fig 6C), UBC (Fig 6D), and MuRF1 (Fig 6E) were significantly correlated with percent body mass where UBC accounted for the greatest variation with R2 = 0.487 and 0.541 in young and aged mice, respectively (Fig 6D). Interestingly, a significant correlation was observed with IGF1 expression in young mice that was not evident in aged mice (Fig 6G), and the opposite was observed with ATROGIN1 expression (Fig 6F).

Further, all variables that had any significant correlations per age group were placed in a step-wise multiple regression analysis to determine if multiple variables could account for greater variability. In young mice UBC, IGF1, and MuRF1 expression accounted for approximately 71% of variability seen in body mass changes (adjusted R2= 0.714, p=0.007), with additional variables not adding significantly to the model. In contrast, in aged mice UBC expression accounted for approximately 58% of the variability in body mass (adjusted R2= 0.576, p<0.001) with no other variables tested (ATROGIN1, MuRF1, IL6RA, UBB, and FOXO1) adding significantly to the model.
Figure 6. Influenza induced weight loss is correlated with gastrocnemius gene expression of ubiquitin proteasome pathway components. Percent weight loss at time of sacrifice and corresponding gastrocnemius gene expression was analyzed via univariate linear regression for all genes that showed significant time effects. Young and aged mice were analyzed separately to determine if relationships vary with age. FOXO1 (A), IL6RA (B), UBB (C), UBC (D), MURF1 (E), ATROGIN1 (F), and IGF1 (G) were significantly correlated with percent body mass in either young or aged, or both (Young (Y) and aged (A) mice regression analysis p and R² values indicated to right of graph, bolded if significant (p<0.05)), while no relationship was seen with percent body mass and expression of IL6, TNF, CXCL10, MEF2C, PAX7, MYOD1, and MYOG (data not shown).

Negligible viral copies detected in the gastrocnemius post influenza infection in vivo

Since flu-induced weight loss was correlated with gastroc gene expression, we next examined a potential mechanism; direct infection of the skeletal muscle in vivo. RNA harvested from the gastroc was probed for the flu PA similar to as performed on whole lung tissue. Though rare case studies have identified virus particles in muscle biopsies of influenza infected humans with myalgia, this highly unusual finding may be limited to critically ill and preterminal cases [12, 21-23]. Indeed, flu PA was not detectable in the majority of gastroc tissue and the few samples that had detectable levels of flu PA had only negligible levels (Fig 7A). Since there was an upregulation of muscle degradation genes in all mice by day 7 post infection, the mechanism of direct infection occurring during a natural infection is not supported by these results.
Figure 7. The pathogenesis of influenza-induced myopathies is likely not direct infection of skeletal muscle as viral copies are not seen in the gastrocnemius muscle. Young and aged C57BL/6 mice were intranasally infected with 500 EID50 of PR8 influenza. (A) On day 0, 3, 7, 11, and 15 whole gastrocnemius was harvested and RNA was isolated. Total number of copies of influenza PA was determined via RT-qPCR with a positive control used (infected mice lung tissue). Uninfected young and aged murine myoblasts were incubated with 0, 10, or 100 EID50 PR8 influenza for one hour and then were cultured in growth media. Myoblast supernatant was analyzed for chemokine/cytokines via multiplex assay. Detectable cytokines were analyzed by 3-way ANOVA (age x infection condition x time point). While a significant age effect was observed (p<0.05), there was no effect of infection and no interaction of infection and time for myoblast secretion of IL6 (C), CXCL10 (D), CXCL1 (E), and CCL2 (F). At 96 hr post infection, total RNA from the myoblast culture was extracted and total number of copies of influenza PA was determined via RT-qPCR. No viral copies were present in in vitro myoblast cultures (B).

In vitro myoblasts do not harbor active influenza infection

To further investigate the possibility that direct infection of skeletal muscle could occur, we harvested leg skeletal muscle (gastroc, vastus medialis, vastus lateralis, soleus, and anterior tibialis) of uninfected young and aged mice. Myoblasts were harvested, grown, purified, and replated prior to infection. Myoblasts were incubated for 1 hour with 0 EID50, 10 EID50, or 100 EID50 PR8. Supernatants were collected at 24, 48, and 72 hours post infection and cytokine/chemokine concentration were determined via multiplex (Fig 7C, 7D, 7E, and 7F). IL-6 secretion was significantly greater from the aged myoblasts, (age effect: p<0.001), but was not affected by infection or infection over time (infection effect: p=0.600, infection*time interaction effect: p=0.583, Fig 7C). The same pattern was observed for CXCL10 (age: p<0.001, infection:
p=0.609, infection*time: p =0.684, Fig 7D), CXCL1 (age: p<0.001, infection: p=0.865, infection*time: p =0.121, Fig 7E), and CCL2 (age: p<0.001, infection: p=0.921, infection*time: p =0.263, Fig 7F). Thus, cytokine secretion was affected by age, but not by flu infection.

Myoblasts were harvested and RNA was extracted at 96 hours post infection to probe for flu PA copies as previously done. Though some studies [18-20], show that myoblasts can be infected by influenza in vitro, our results indicate that both young and aged myoblasts are not susceptible to a productive flu infection (Fig 7B).

**DISCUSSION**

Studies addressing the pathophysiology of flu infection typically focus on respiratory and immune systems, while those seeking to understand aging-related declines in mobility performance emphasize muscle biology and relevant neural systems. Nevertheless, it has become clear that systems-based approaches are essential to both aging research and clinical care of older adults since a failure to think more broadly fails to consider crosscutting biological themes and motifs in aging and also ignores crucial bidirectional signals between different systems and tissues. With these considerations in mind, we have investigated the potential clinical significance of flu-associated myalgia and myopathies in relation to flu-induced disability in older adults. Indeed, we have shown that flu infection induces both functional decrements and upregulation of muscle inflammation and atrophy gene expression that is more pronounced with aging, indicating the impact of flu infection on muscle may directly predispose older adults for catastrophic disability and sarcopenia. Additionally, the functional alterations observed during flu infection may increase risk of falls and other musculoskeletal injuries. Thus, here we have identified flu infection as a previously unrecognized, but potentially targetable, inducer of muscle atrophy potentially leading to decreased resilience in older adults.
Aged mice have more severe and prolonged weight loss, as well as increased lung viral titers and delayed viral clearance following sublethal flu infection [35]. Similarly, voluntary locomotor activity is decreased with flu infection, and this reduction is prolonged in aged mice. However, since it is possible that voluntary activity could be diminished due to general flu-induced malaise, we analyzed gait patterns to assess more specific flu-induced alterations in functional performance. We showed that aged mice initially had a wider fore limb stance compared to young mice, but this was decreased with flu. It is known that older adults generally increase stance width to increased stability [36, 37], though this relationship is not yet established in mice. Midline distance was also decreased with flu infection and this was more dramatic in the hind limbs of the aged mice. Narrower steps with flu infection likely lead to decreased balance, potentially leading to increased risk for injury, as narrower stride width is associated with increased fall risk in older adult humans [36]. Stride length variability and gait symmetry index also increased with flu infection and is more prominent in the aged mice. Indeed, gait variability is also a predisposing factor for falls in older adults [38, 39]. Additionally, we showed decrements in acceleration and deceleration parameters with flu infection in both young and aged mice. Declines in the rate of force development and power output are evident in older adults, and more importantly, are strong predictors of functional status and falling risk [40, 41]. Thus, many flu-induced functional alterations could be particularly problematic for already at-risk older adults.

Since functional alterations were primarily in the hind limbs, we examined the gastroc, a large mixed fiber type muscle, for flu induced alterations in gene expression. Importantly, we demonstrated that flu-induced functional decrements were associated with increased inflammatory and atrophy gene expression. Both young and aged gastroc had increased expression of IL6 and IL6RA by 7 DPI, while IL6RA expression remained elevated at 11 DPI only in aged mice. Additionally, TNF expression was only increased in the aged mice. Though
the relationship between IL-6 and muscle inflammation and regeneration is not completely clear, higher expression of IL-6 and TNF in older adult skeletal muscle is associated with decreased muscular strength [42]. Further, it has been demonstrated that older adult humans have elevated expression of inflammatory mediators, particularly IL-6 and TNF-α, and dysregulated signaling responses that lead to an increased inflammatory milieu and impaired myogenesis [43]. This low grade inflammation with aging also impairs postprandial muscle protein synthesis [44]. In addition to the common inflammatory mediators in muscle, CXCL10 expression was dramatically increased only in the aged mice. While CXCL10 is predominantly associated with a Th1 response, it has been recently identified in inflammatory myopathies [45, 46], and secretion of CXCL10 from human fetal skeletal muscle cells is induced by treatment with either interferon (IFN)-γ or TNF-α [45]. The dramatic increase in CXCL10 expression in the aged mice contributes to exaggerated and prolonged gastroc inflammation. Thus, our results agree with previous research utilizing chemical [47] or exercise injury [43] and suggests that flu induces muscle inflammation in the aged that is heightened and prolonged potentially leading to further muscle damage and diminished regeneration.

Indeed, lingering inflammation in the aged was accompanied by increased and prolonged expression of atrophy and protein degradation genes. At 7 DPI both young and aged mice gastroc had increased expression of ATROGIN1 and MuRF1; however, at 11 DPI while young mice downregulated expression, aged mice atrophy gene expression remained elevated. Similarly, both UBB and UBC expression was significantly higher in aged mice at 11 DPI. Proteins targeted for degradation that lead to muscle atrophy by atrogin-1 include myogenic regulatory factor MyoD and eukaryotic translation initiation factor 3 subunit f (eIF3-f), while MuRF1 preferentially targets myosin heavy chains and other myofibrillar proteins, though these targets are likely not exclusive to either ligase [30]. Further, in many instances, UBC acts in concert with atrogin1 and MuRF-1 through FOXO dependent pathways [48] suggesting that the
increased FOXO1 expression may tie together these proteolytic pathways in our flu induced muscle atrophy model. Moreover, increased UBC expression has been one of the most prominent mRNA increases seen in multiple muscle wasting disorders [49], so it is not surprising that UBC expression was the highest correlated gene with weight loss. Indeed, multiple atrophy and degradation genes were correlated with flu-induced weight loss, suggesting flu-induced weight loss is at least partly due to muscle degradation and atrophy. Interestingly, IGF1 was negatively correlated with weight loss in young mice only. Also, in the step-wise multiple regression model, the addition of IGF1 accounted for greater explanation of variability in weight loss in the young mice; however, this was not observed in the aged mice, suggesting anabolic signals are not strong contributors to percent weight loss and recovery in aged mice. Indeed, this lack of relationship observed with IGF1 expression in the aged mice is likely attributable to anabolic resistance, a more recent concept described as the diminished response in aged muscle to many anabolic stimuli including branched chain amino acids and exercise [50, 51].

The observed suppression of positive regulators of muscle mass further tips the protein synthesis and protein degradation balance; and these were greater suppressed and prolonged in the aged mice. IGF1 and MEF2C suppression was prolonged to 11 DPI in the aged mice, and peak suppression of PAX7, MYOD1, and MYOG was greater in aged mice compared to young mice. Certainly, IGF1 has been of particular interest in aging research over the years, and low circulating levels of IGF1, particularly in combination with elevated IL-6, have been associated with decreased muscle strength and increased prevalence of sarcopenia [52]. Thus, flu induces these unfavorable responses in muscle tissue itself, predisposing the aged muscle to sarcopenic conditions. Surprisingly, no upregulation of positive regulators was evident during our time course, suggesting muscle mass may not be recovered.
Together our results suggest that flu induces functional decrements, as well as muscle inflammation, proteolysis, and atrophy, and that these changes are augmented and prolonged with aging. The pathogenesis behind this effect still remains unknown. Despite some studies suggesting direct infection of muscle cells [18-20], we showed no viral copies in gastrocnemius muscle in vivo throughout the infection. Additionally, myoblasts were not susceptible to a productive infection in vitro. Desdouits et al. [19] reported that human myoblasts were less susceptible to flu infection than myotubes and response was variable among donors and flu strain, though many reports regarding productivity of infection in myoblasts and myotubes are conflicting [18-20, 53]. Further, it is important to note that mature muscle fibers are much different than myoblasts and myotubes, where these immature muscle cells exist only transiently. Satellite cells are only present in great quantities in early postnatal development and decrease dramatically in adulthood, accounting for 30-35% then 2-7%, respectively, of sublaminal nuclei on myofibers [54]. Supporting this, Nevalainen et al. [53] showed that mature muscle fibers do not produce viral progeny, though a non-permissive infection occurs. Indeed, in vivo studies have shown that a non-permissive infection may occur in the skeletal muscle [25], though this seems more likely when intramuscular flu infection models are used [24, 26]. Since we performed intranasal infections, similar to the natural route of infection in humans, it is unlikely this would occur; however, it is still possible that this non-permissive infection leads to viral copies below our detectible limit and direct viral infection, or perhaps the presence of viral particles, may contribute to muscle degradation. Collectively, the lack of in vivo evidence to suggest that mature muscle fibers are susceptible to infection and that this would actually occur during a natural flu infection leads us to conclude further research is necessary to determine the mechanism behind flu-induced muscle inflammation and degradation.

In summary, this chapter and its manuscript is the first to identify in a controlled experiment setting flu-induced muscle inflammation and atrophy as well as functional impairment. Further,
these effects are prolonged with aging, providing a molecular link to flu infection and disability in older adults, together with some initial insights into the mechanism which may underlie aging-related declines in resilience. We have demonstrated that key inflammatory signals, and key ubiquitin proteasome components, both atrogin1 and MuRF1, as well as ubiquitin B and ubiquitin C, are upregulated. As it is known that muscle repair is diminished with aging, it is likely these muscle losses are not easily recoverable. Thus, future research may be able to target these pathways to prevent flu-induced atrophy and potential loss of quality of life in older adults.

ACKNOWLEDGEMENTS

Work was supported by National Institutes of Health (NIH)-National Institute of Aging (NIA) P01 grant AG02160 (L. Haynes). S. Pan was supported by American Federation of Aging Research (AFAR) Medical Student Training in Aging Research (MSTAR) program. G. Kuchel is the Citicorp Chair in Geriatrics and Gerontology.

The authors also thank April Masters, M.S., Erica Lorenzo, Sandra Jastrzebski, and Judy Kalinowski for their assistance with experiments.

REFERENCES


5. Ferrucci L, Guralnik JM, Pahor M, Corti MC and Havlik RJ. Hospital diagnoses, Medicare charges, and nursing home admissions in the year when older persons become severely disabled. JAMA. 1997; 277(9):728-734.


72


CHAPTER THREE

Vaccination mitigates influenza-induced muscular declines in aged mice

ABSTRACT

Influenza (flu) infection increases the risk for disability, as well as falls and broken bones in older adults. In murine models, flu causes mobility and strength impairments with induction of inflammatory and muscle degradation genes that are more pronounced and prolonged with aging. As the aging population grows, ways to protect older adults from disability are increasingly important. Here, we examined if vaccination could reduce flu-induced muscle decrements with aging. Young and aged mice were vaccinated with recombinant flu nucleoprotein (NP), infected with flu virus, and muscle parameters were measured following infection. We determined that vaccination provides almost complete protection from functional decrements, muscle gene expressions alterations, and morphological damage in young mice and partial protection from these muscular declines in aged mice. Similarly, vaccination improved protection from lung localized and systemic inflammation in aged mice. Despite documented decreased vaccine efficacy with aging, vaccination still provided partial protection to aged mice and represents a potential strategy to prevent flu-induced disability with aging. These initial findings provide translational insight on ways to reduce flu-induced disability in the aging population.
INTRODUCTION

Influenza (Flu) and pneumonia are the seventh leading cause of death among older adults in the US with 90% of all flu-related deaths occurring in late life [1]. Equally compelling from a clinical and public health perspective, but less well understood, is the relationship between flu infection and disability. Older adults have a greatly increased risk of both progressive and catastrophic disability following flu infection [2, 3], including a greater risk of falls and broken bones [4]. Nevertheless, mechanisms by which flu infection contributes to this risk of disability remain unknown and unexplored, resulting in missed opportunities for the discovery of interventions designed to prolong function and independence in late life. Flu infections, even in uncomplicated cases, have some degree of muscle involvement with myalgia being a common symptom [5]. We previously reported that flu infection, which by its very nature is limited to pulmonary epithelial cells, results in mobility and strength impairments and increased markers of muscle atrophy in a well-established murine model of infection. Moreover, these effects are more pronounced and prolonged with aging, providing a molecular link between flu infection and disability in older adults [6]. The pathogenesis of this interaction is unknown and little information exists detailing the systemic impact of flu infection and how it changes with aging. Here we investigate if prior immunity can prevent the muscular ailment. Our goal is to provide translationally relevant insight on potential ways to protect older adults from flu-induced functional decrements and muscle atrophy.

The average flu-related mortality is between 20k-40k people annually [7]. Flu vaccination substantially reduces mortality in humans [7]. Indeed, a 1% increase in total vaccination rate would result in an estimated 800 fewer deaths [7]. Through decades of research, it is clear that vaccination improves the humoral and cellular functions of the immune system in response to flu. Inactivated and recombinant flu vaccines induce robust antibody and CD4+ T cell responses. Neutralizing antibodies work to prevent infection, while non-neutralizing antibodies
facilitate viral clearance via mechanisms such as antibody-dependent cell-mediated cytotoxicity [8]. This results in faster clearance of flu and less severe illness. Previous vaccination studies from our lab [9] emphasized the two-pronged protection of flu nucleoprotein (NP) vaccination with antibody and lung-homing T cell effector generation to provide non-neutralizing protection. Though general vaccination efficacy is reduced with aging, inflammation and lung viral copies were reduced in both young and aged mice with NP vaccination.

While many benefits of vaccination have been previously reported, the effects of vaccination on muscle function and overall muscle quality, specifically in the more vulnerable aged population has not been explored. Indeed, muscle integrity and quality are key components when considering recovery, resilience, and prevention of physical disability in the aging population. Here, we investigated how non-neutralizing immunity induced by vaccination with NP impacts the muscular decrements observed during flu infection. NP vaccination induces a protective heterosubtypic antibody response in young mice [10, 11], and reduces lung inflammation and susceptibility to secondary bacterial infection following primary flu infection [12]. Previously we showed that NP vaccination of aged mice protected them from death following flu infection, but did not protect from flu-induced weight loss [9]. Thus, we hypothesized that NP vaccination would mediate protection in muscle through reduction in flu-induced inflammation.

Skeletal muscle is a complex tissue consisting of many different cell types, as well as different muscle fibers. The four main fiber types in murine skeletal muscle are based on different muscle myosin heavy chains (MyHC). MyHC I fibers are considered slow twitch fibers while, MyHC IIA, IIX, and IIB fibers are fast twitch fibers [13, 14]. Fiber areas and muscle cross sectional areas in skeletal muscles of mice differ with fiber sizes increasing in the order type I < IIA < IIX < IIB [13]. Typical mouse skeletal muscle is 77% MyHC IIB [14]. With aging, type IIB and IIX fibers have impaired regeneration capacities in response to immobilization atrophy [15]. Translationally, fast twitch fibers control balance, strength, and numerous other necessary functions in humans and
rodents. In human aging, there is marked muscle atrophy, along with a decrease in total number of fibers with preferential loss of type II muscle fibers [17]. Indeed, the change in composition to greater slower-contracting isoforms leads to symptoms consistent with denervation [16]. In addition to the quantitative loss, there is also a qualitative decline in muscle in terms of specific force (force generation normalized for muscle cross-sectional area), dysfunctional proteins, and other age-associated deficiencies [18, 19, 20, 21]. Typically, fast-twitch muscle fibers are more vulnerable to degeneration and more sensitive to inflammation [22, 23, 24]. Importantly, the decline in muscle strength from type II fiber atrophy and loss can lead to sarcopenia, which increases risk of loss of activities of daily living. Since clinically there is an increase in loss of activities of daily living following flu infection and type II fibers are more sensitive to age-related loss and inflammatory stimuli, we hypothesize that flu will most severely affect type II fibers.

In this report, we examine the impact of NP vaccination on muscular function and quality in a preclinical model of flu infection with aging. As we have previously shown, NP vaccination leads to reduced inflammatory mediators in the bronchoalveolar lavage (BAL), high titers of NP-specific antibody, and improved lung viral clearance [9]. Here, we show that vaccination was able to preserve the function of young and aged mouse grip strength, gait kinematics, and voluntary mobility parameters, as well as reduce inflammation within the skeletal muscle and prevent morphological disruption and type II muscle atrophy. Thus, our results point to a novel finding that despite reduced vaccine efficacy with aging, vaccination provides protection from flu-induced muscular impairments highlighting the clinical necessity for flu vaccination with aging.
METHODS

Mice. Young (2.5-4-month-old) C57BL/6 male mice were purchased from Jackson Laboratories or obtained from the National Institute on Aging. Aged (19-22-month-old) C57BL/6 male mice were obtained from the National Institute on Aging rodent colony. All mice were housed in a climate controlled environment with 12:12 light:dark cycle and fed standard rodent chow and water ad libitum. All mice were cared for in accordance with the recommendations in the Guide for the Care and use of Laboratory Animals of the National Institutes of Health. All procedures were approved by the University of Connecticut Medical School IACUC, protocol number 100705. Recumbent mice and mice that lost more than 30% body weight were considered moribund and euthanized. All mice underwent gross pathological examination at time of sacrifice and animals with obvious pathology were excluded from the study.

Viral infection. Mice were anesthetized with isoflurane and intranasally inoculated with 50 ul of 500 EID_{50} of influenza virus A/PR/8/34 (PR8). Mice were weighed daily to monitor infection progression. At time points indicated, whole lung tissue was homogenized and RNA was isolated via TRIzol/chloroform extraction per manufacturer’s protocol (Ambion by Life Technologies, Naugatuck, CT and Sigma Aldrich, Natick, MA, respectively). RNA was reverse transcribed with iScript cDNA synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA) and flu viral copies were detected via reverse transcription quantification PCR of flu acid polymerase (PA) as previously described (58).

Vaccination. Recombinant A/PR/8/ influenza nucleoprotein (NP) was generated by the Protein Expression Core at UConn Health. Immunizations were prepared using NP protein and Imject Alum (Thermo Scientific) at 1:1. Injections were administered intraperitoneal at a concentration of 30ug NP in 100uL per mouse. Control mice were given 100uL PBS. Previous studies in our laboratory showed no differences between PBS control and PBS/alum control [9]. Mice received
one dose of vaccination or control at 30 days and a second dose at 20 days prior to influenza infection [10].

**Antibody Titers.** 96-well plates were coated with recombinant A/PR/8/34 influenza NP (generated by UConn Health Protein Expression Core). Serum samples were serial diluted (1:10 to 1:1x10⁸). Anti-NP IgG titers in serum were the determined using anti-mouse IgG-HRP (Southern Biotech, Birmingham, Alabama) and o-phenylenediamine (Sigma, Natick, Massachusetts) buffered in hydrogen peroxide. Samples were read in duplicate (490nm, Bio-Rad iMark microplate reader, Hercules, California). Antibody titers were determined by the last serum dilution with an optical density above background.

**Gastrocnemius Reverse-Transcription quantitative PCR (RT-qPCR).** At the time points indicated for gastrocnemius gene expression, mice were fasted with the exception of water for 4-6 hours prior to sacrifice to minimize potential confounding results due to postprandial muscle protein synthesis. The gastrocnemius muscle was dissected and placed in RNAlater (Qiagen Inc., Germantown, MD) overnight at 4°C. RNAlater was removed and gastrocnemius was frozen at -80°C until RNA extraction. The muscle was homogenized and RNA was extracted via TRIzol/chloroform extraction per manufacturer’s instructions (Ambion by Life Technologies and Sigma Aldrich respectively). RNA quantity and quality were assessed with Nanodrop 2000c (Thermo Scientific, Waltham, MA) and was reverse transcribed via iScript Advanced cDNA synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA). RT-qPCR was performed using predesigned commercially available primers (Bio-Rad Laboratories, Inc.). Gene expression was calculated via a modified Pfaffl method utilizing multiple reference genes (RPS18 and TBP, which showed the least variability between conditions and thus suitable reference genes as previously shown (6)) and normalized to the unvaccinated uninfected young mice to give comparable changes.
Multiplex Protein Analysis. Bronchiolar lavage fluid (BAL) was collected by flushing lungs with 1 ml PBS. Supernatant was collected after centrifugation and assayed for cytokine and chemokine content. Similarly, blood was collected via cardiac puncture, allowed to clot at room temperature, and the resultant serum was assayed for cytokine and chemokine content. Gastrocnemius muscle was dissected from each mouse and homogenized in Tissue Protein Extraction Reagent (Thermo Scientific, Bedford, MA)) supplemented with 5mM EDTA (Invitrogen, Carlsbad, CA) and Protease/Phosphatase Inhibitor Cocktail (Thermo Scientific)). Debris was removed via centrifugation and total protein content was determined using a Pierce™ BCA Protein Assay Kit (Thermo Scientific).

Voluntary locomotor activity. Spontaneous voluntary locomotor activity was measured via open field test at time points indicated. All tests were performed between 7-9am to control for diurnal variations. Following acclimation to the dim-lit testing room (at least 1 hour), mice were placed in the center of the photobeam activity system-open field (PAS-OF, 16”x16”x15” acrylic animal enclosure, San Diego Instruments, San Diego, CA) and their activity was recorded for 20 minutes. The first 5 minutes was excluded as this is generally considered to be exploratory behavior rather than general voluntary locomotor activity. The number of beam breaks per minute during the last 15 minutes was then used to assess voluntary locomotor activity.

Grip strength. Grip strength was determined by using a grip strength meter BIOSEB In Vivo Research Instruments, Pinellas Park, FL). Briefly, mice were permitted to grab onto a T-shaped-bar and pulled horizontally by the tail until they released their grip. The force (grams) read by the force meter at the release of the mouse’s grasp was averaged by their mass (grams) on that day and compared to their pre-infection results. The same researcher performed all testing to minimize variability.
**Gait analysis.** Gait analysis was performed using the DigiGait instrument (Mouse Specifics, Inc. Quincy, MA) and software (DigiGait Imager 4.0.0 and DigiGait Analysis 11.5, Mouse Specifics, Inc). The DigiGait instrument consists of a clear treadmill with a high-speed camera mounted underneath that collects images at 147 frames per second for high resolution of postural temporal gait parameters. Mice run within a 2-inch-wide acrylic running chamber at set speeds. The ventral plane videos are analyzed with the DigiGait software which identifies portions of the paw that are in contact with the treadmill belt to produce both postural and kinematic gait parameters. Mice were introduced to the DigiGait system at a low speed (10cm/sec) briefly (30 seconds) prior to the initial testing. Mice were allowed to acclimate to the dim-lit room for 1 hour prior to each testing period and all tests were performed between 8-10 am. Mice ran at the testing speed (16cm/sec, as previously optimized (6)) until approximately 5 seconds of consecutive walking was recorded and this video segment was analyzed via DigiGait software.

**Histology.** The gastrocnemius muscle was carefully dissected and blotted on a Kim Wipe before being placed in a cryomold, embedded in Optimal Cutting Temperature (OCT ThermoScientific Inc., Waltham, MA) and frozen in liquid nitrogen cooled isopentane. Samples were stored at -80°Celsius freezer until sectioning. 10um thick muscle cross sections were mounted on charged slides (Superfrost Plus Glass Slides, ThermoScientific Inc., Waltham, MA) for histological analyses. Standard Hematoxylin and eosin (H&E) staining was performed and imaged at 20X. H&E images were blindly scored by multiple evaluators utilizing a rubric adapted from the literature (33, 34). The rubric was based on two criteria, 1) infiltration of nuclei, and 2) architecture disruption and tissue damage. Each criterion was independently scored from 0-3 and combined for a total score that ranged 0-6.

**Fiber Type Staining.** Primary antibodies for muscle fiber types were acquired from the Developmental Studies Hybridoma Bank (DSHB) at the University of Iowa: myosin heavy chain type I (DSHB, BA-D5), myosin heavy chain type IIA (DSHB, SC-71, and myosin heavy chain
type IIB (DSHB, BF-F3). Secondary antibodies Alexa Fluor 647 (Invitrogen Carlsbad, CA), Alexa Fluor 488 (Life Technologies St. Petersburg, FL), and Alexa Fluor 555 (Life Technologies St. Petersburg, FL) were utilized, respectively. Nuclei were stained with Hoechst 33342 (Life Technologies St. Petersburg, FL). Stained slides were imaged via confocal microscopy (Zeiss LSM 880, Peabody, MA) Cross sectional area was quantified using ImageJ (NIH, Bethesda, MD).

**Statistical analysis.** All data analyzed via two-way (treatment x age) ANOVA with Bonferroni post hoc corrections. Significant differences comparing (*=p<0.05) to baseline (NoVax/NoFlu) age-matched controls, comparing (#=p<0.05) to unvaccinated infected (NoVax/Flu) age-matched controls, and comparing (brackets=p<0.05) between groups/ages within a condition.

**RESULTS**

*Prior vaccination prevents body mass loss and accelerates viral clearance during flu infection.* Utilizing established methods from our lab [9] and others [10, 11], young (2.5-4 months) and aged (19-22 months) mice were vaccinated with NP/alum (Vax) or PBS control (NoVax) and infected with a sublethal dose of PR8 influenza virus (Fig S1). In unvaccinated mice, infection led to greater weight loss (Fig1A) and higher flu copy number in the lung (Fig1B) in both young and aged mice when compared to vaccinated groups. Young vaccinated mice were protected from weight loss and had accelerated viral clearance, while aged vaccinated mice only had partial protection. At 9 days post infection (DPI), the vaccinated aged mice follow similar trends as unvaccinated young mice. We have previously shown that aged mice suffer greater and prolonged weight loss compared to young mice [6]. Similarly, young mice are more resilient than aged mice with vaccination. Despite similar peak virus levels in the lung at 7 DPI, vaccinated young and aged mice clear the virus faster than their unvaccinated controls (Fig1B), though
differences between young and aged mice are still evident. Importantly, vaccination induced increased serum anti-NP IgG antibodies (Fig1C), supporting the efficacy of the vaccination. Overall, the NP/Alum vaccination hastens viral clearance while reducing body mass losses in both young and aged mice.

A) Weight Loss

B) Viral Quantification

C) Anti-NP Antibody
Figure 1. The NP/Alum vaccination hastens viral clearance while reducing body mass loss. Young and aged C57BL/6 mice were vaccinated with NP/Alum or PBS before being intranasally infected with 500 EID₅₀ of PR8 influenza. A) The percent body mass loss graphed over the course of the experiment and bar graph of day 9 post infection differences (n=10-15/group). B) Influenza acid polymerase (PA) copy number determined by RTqPCR of mouse lungs at given time points (n=5-6/group). C) IgG titer for influenza nucleoprotein assayed via ELISA of mouse serum (n=5-6/group). Data analyzed via two-way ANOVA with Bonferroni post hoc corrections comparing to NoVax/NoFlu age-matched controls (*=p<0.05), comparing to NoVax/Flu age-matched controls (#=p<0.05), and comparing between day post infection/ages (brackets=p<0.05).

Prior vaccination protects muscular function during flu infection. As we have shown previously [6], flu infection without prior vaccination leads to functional decrements in voluntary locomotor activity, grip strength, and gait kinematics. Importantly, prior vaccination prevents these decrements in young mice and either prevents or reduces these decrements in aged mice. Postural gait parameters were assessed utilizing DigiGait at 7 DPI. Hind midline distance decreased with infection in both young and aged unvaccinated mice, while this decrease was prevented with vaccination only in young mice (Fig2A). Conversely, fore limb midline distance was only decreased in unvaccinated aged mice and vaccination prevented this decrease (Fig S2A). Thus, mice have a narrower hind stance during flu infection and vaccination was not able to prevent this narrowing in aged mice, however, the narrowing of the fore limb stance was protected with vaccination in aged mice. Hind and fore stride length increased with infection in aged unvaccinated mice, while this increase was prevented with vaccination in young and aged mice (Fig2B & Fig S2B). Kinematic gait parameters in the hind and fore limbs such as stride frequency (Fig2C & Fig S2C), time propelling (Fig2D & Fig S2D), stance time (Fig2E & Fig S2E), time in stride (Fig2F & Fig S2F), time in swing (Fig2G & Fig S2G) also followed similar trends in aged mice. In contrast, young mice experienced no kinematic gait changes in their hind (Fig2C-G) or fore (Fig S2C-G) limbs during infection. Importantly, aged mice had increased time in the propel, stance, stride, and swing phases during infection, which was only partially protected with vaccination as some decrements were still evident in aged vaccinated mice (Fig2C-G). Overall, these results suggest that flu infection leads to a narrower stance and
slower gait movements in aged mice and that vaccination preserved baseline gait parameters and protected aged mice from many flu-induced changes.

Similarly, vaccination significantly protected both young and aged mice from decreased voluntary activity at 8 DPI, assessed by open field, compared to their unvaccinated counterparts (Fig2H). Infection without prior immunity led to an approximate 50% decrease of their initial voluntary activity by 8 DPI. Vaccinated mice were protected from this decrease, however aged vaccinated mice did not perform as well as their young vaccinated counterparts. Additionally, grip strength, as measured by a grip strength meter and normalized to pre-infection levels, was preserved in vaccinated mice, while unvaccinated mice had decreased strength (Fig2I) in both young and aged groups.
Figure 2. Vaccination against influenza reduced flu-induced functional decrements in voluntary locomotor activity, grip strength, and gait kinematics. Young and aged C57BL/6 mice were vaccinated with NP/Alum or PBS before being intranasally infected with 500 EID$_{50}$ of PR8 influenza. Mice were acclimated to testing prior to infection and tested for functional performance at designated time points. A-G) Gait parameters were assessed utilizing DigiGait 7 days post infection (n=8-15/group). H) Spontaneous voluntary activity was assessed via the open field test at 8 days post infection (n=10-15/group). I) Grip strength was determined by using a grip strength meter at day 8 post infection (n=10-15/group). Data analyzed via two-way ANOVA with Bonferroni post hoc corrections comparing to NoVax/NoFlu age-matched controls (*=p<0.05), comparing to NoVax/Flu age-matched controls (#=p<0.05), and comparing between ages within a condition (brackets=p<0.05).

Vaccination mitigated alterations in cytokine/chemokine inflammatory milieu. Proinflammatory cytokine and chemokines (IFN-γ, IL-6, and CXCL10) were assessed in the BAL from young and aged mice post infection and they followed similar trends (Fig3A-C and Fig3F-H) with increased levels by 7 DPI in unvaccinated infected mice. Young vaccinated mice only showed modestly
increased levels of proinflammatory signals during infection and returned to baseline levels by 9 DPI. Vaccination did not mitigate the peak levels of inflammation in aged cohorts but did hasten the resolution of inflammation by 9 DPI. In sum, vaccination prevented prolonged inflammation in aged BAL, but did not reduce peak inflammation at 7 DPI. Type 2 cytokines (IL-4 and IL-10) are generally considered anti-inflammatory and counteract inflammatory cytokines (Fig3D-E & Fig3l-J) [25, 26]. IL-4 increased with infection, peaking at 7 DPI, and was higher in vaccinated mice compared to their unvaccinated age-matched counterparts (Fig3D & Fig3I). Interestingly, only aged vaccinated mice still had elevated IL-4 at 9 DPI (Fig3l). IL-10 peaked in all groups at 7 DPI and returned to close to baseline levels by 9 DPI (Fig3E & Fig3J). Interestingly, vaccination in aged mice led to significantly increased in IL-10 at 7 DPI, compared to the unvaccinated aged mice (Fig3J), while vaccinated young mice had lower IL-10 levels at 7DPI compared to unvaccinated young mice (Fig3E). It is likely the reduced inflammation in the young vaccinated mice did not require strong anti-inflammatory responses at that time point. In totality, vaccination reduced peak inflammation and limited prolonged inflammation in the BAL of young and aged mice while also promoting IL-4 and IL-10 production in aged mice.

Systemic inflammation, as measured by serum cytokines and chemokines, followed a similar trend as in the BAL. Flu infection led to increased serum IFN-γ, IL-6, and CXCL10 at 7 DPI in all groups (Fig3K-M & Fig3P-R). Vaccination reduced serum IFN-γ levels at 7 DPI in both young and aged compared to unvaccinated controls. Conversely, serum IL-6 was elevated by 5 DPI in all groups (Fig3L & Fig3Q). Vaccinated mice began to decrease levels of IL-6 by 7 DPI, while unvaccinated young mice continued to increase at 7 DPI before returning to baseline levels (Fig3L) and unvaccinated aged mice levels remained elevated at 9 DPI (Fig3Q). Serum CXCL10 increased with infection in all groups (Fig3M & Fig3R), with only minor increases in the young vaccinated mice. Conversely, aged vaccinated mice followed similar trends to the unvaccinated young mice with increases at 7 DPI that returned to near baseline levels by 9 DPI.
Similar to IL-6, serum CXCL10 continued to rise in unvaccinated aged mice with peak levels seen at 9 DPI. Thus, the unvaccinated aged group had prolonged IL-6 and CXCL10 elevation at 9 DPI compared to other groups. Prior vaccination resulted in increased serum IL-4 at 7 and 9 DPI in young mice only (Fig3N). Serum IL-10 was increased at 7 and 9 DPI in young vaccinated mice (Fig3O), but only at 9 DPI in aged vaccinated mice (Fig3T). Importantly, serum IL-4 and IL-10 did not change in both young (Fig3N-O) and aged (Fig3S-T) unvaccinated mice, suggesting vaccination speeds up systemic anti-inflammatory signals. In sum, vaccination reduced prolonged systemic inflammation and provided modest increases in anti-inflammatory signals in aged mice. It is known that muscle atrophy can be triggered by external stress signals and overall inflammation status of an individual. Thus, we hypothesized that by limiting systemic flu-induced inflammation with vaccination we could reduce the negative impact of flu on muscle.

Figure 3. Vaccination reduces flu-induced cytokines and chemokines in the BAL and Serum. Young and aged C57BL/6 mice were vaccinated with NP/Alum or PBS before being intranasally infected with 500 EID₅₀ of PR8 influenza. A-J Bronchoalveolar Lavage (BAL) was collected from mice at time of sacrifice as described in methods. The BAL was then analyzed via Millipore chemokine/cytokine Multiplex.
25-plex for the analytes (n=16-21). Serum was collected from mice at the time of sacrifice as described in methods. The serum was later analyzed via Millipore chemokine/cytokine Multiplex 25-plex for the analytes listed (n=16-21). Data analyzed via two-way ANOVA with Bonferroni post hoc corrections comparing to NoVax/NoFlu age-matched controls (*=p<0.05) and comparing to NoVax/Flu age-matched controls (#=p<0.05).

Vaccination mitigates negative skeletal muscle gene and protein expression changes. To determine if vaccination-related reduction in BAL and serum inflammation would be associated with reduced negative changes in skeletal muscle, we first examined muscle gene and protein expression. We have previously shown that flu infection results in dramatic increases in muscle atrophy genes [6]. Here, we found that vaccination mitigates flu-induced changes in skeletal muscle gene expression (Fig4A-J) and protein expression in gastrocnemius (Fig4K-T).

Vaccination in both young (Fig4A) and aged (Fig4F) mice prevents MuRF-1 expression (considered a major regulator of skeletal muscle atrophy). Similarly, Ubiquitin C (UBC) upregulation is reduced with vaccination in young mice (Fig4B), with minor upregulation evident in aged vaccinated mice at 9 DPI (Fig4G). Conversely, unvaccinated aged mice show dramatic upregulation at 9 DPI (Fig4G). This suggests that vaccination completely protects young mice from muscle atrophy gene expression changes, while only partially protecting aged mice. Insulin Growth Factor-1 (IGF-1), a positive regulator of the mTOR pathway that promotes muscle hypertrophy and suppresses atrophy gene expression [27, 28], was surprisingly not statistically significantly decreased, but was trending significance in unvaccinated infected groups 7 DPI (young NoVax/Flu p=0.08 and aged NoVax/Flu p=0.09) (Fig4D and Fig4I). The aged vaccinated mice had an increase at 9 DPI (Fig4D and Fig4I). It is possible that a decrease in IGF-1 was missed in the unvaccinated groups during the experimental time course. It is also possible that an increase in IGF-1 was not observed in the young vaccinated mice due to decreased damage and need for any muscle repair. Myoblast determination protein 1 (MyoD), a critical factor regulating satellite cell proliferation and muscle repair, was downregulated in young and aged unvaccinated mice at 7 DPI (Fig4E and Fig4J), but remained downregulated at 9 DPI only in
unvaccinated aged mice compared to their aged vaccinated counterparts (Fig4J). Conversely, vaccination protected both young and aged mice from downregulation of MyoD (Fig4E and Fig4J). Thus, vaccination promoted positive muscle regulators of both young and aged mice at later time points, suggesting accelerated healing from flu-induced muscle damage.

Since it is known that the inflammatory milieu of skeletal muscle affects muscle atrophy and muscle repair pathways [6], we next examined how vaccination impacts the flu-induced cytokine and chemokine protein expression in the gastrocnemius muscle. Not surprisingly, baseline differences existed in multiple cytokines and chemokines [29, 30, 31, 32]. IFN-γ only significantly increased in unvaccinated aged mice at 7 DPI and unvaccinated young mice at 9 DPI, while vaccinated groups are protected from this change (Fig4K and Fig4P). Conversely, IL-6 changes are not vaccination dependent. Young mice have increased IL-6 at 9DPI (Fig4L), and aged mice have increased IL-6 at 7 DPI irrespective of vaccination status (Fig4Q). CXCL10, a chemokine for recruiting type 1 inflammatory cells, remained unchanged in young unvaccinated and vaccinated mice (Fig4M). Conversely, CXCL10 is elevated at 7 DPI in both unvaccinated and vaccinated aged mice, but to a greater degree in the unvaccinated mice (Fig4R). By 9 DPI, only unvaccinated aged mice have elevated CXCL10, suggesting vaccination prevented prolonged CXCL10 expression (Fig4R). In totality, since IL-6 actions are pleiotropic in skeletal muscle, it is hard to distinguish the pathology of the discordant increases with age. Importantly, however, IL-6 in the aged muscle is increased at the same time as IFN-γ and CXCL10. This suggests a more inflammatory environment. Conversely in vaccinated young mice, there is no simultaneous IFN-γ or CXCL10 increase, possibly suggesting an appropriate signal for myogenesis. On the anti-inflammatory side, IL-4 and IL-10 were both increased in aged mice at baseline (NoVax/NoFlu) compared to young mice (NoVax/NoFlu) (Fig4S-T). IL-4 increased steadily in both unvaccinated and vaccinated aged mice and was elevated at 7 DPI and 9 DPI with greater levels in the vaccinated aged group (Fig4S). In contrast, young mice did not exhibit
any changes in IL-4 irrespective of vaccination (Fig4N). IL-10 was increased in all groups except the unvaccinated aged mice at 9 DPI (Fig4O and Fig4T). It is likely that the lower levels of inflammatory signals in young mice did not require a robust anti-inflammatory response as evident by the low levels of IL-4 (Fig4N). Furthermore, this is corroborated by the gene expression changes at this time showing that young mice have decreased muscle atrophy gene expression when compared to aged mice with or without vaccination. In totality, vaccination prevents muscle atrophy gene expression changes in young mice and reduces muscle atrophy gene expression in aged mice, which is accompanied by similar changes in the inflammatory milieu including reduced pro-inflammatory signals and increased anti-inflammatory signals in the aged vaccinated mice.

Figure 4. Vaccination reduces flu-induced skeletal muscle gene expression and protein expression in gastrocnemius. Young and aged C57BL/6 mice were vaccinated with NP/Alum or PBS before being intranasally infected with 500 EID_{50} of PR8 influenza. A-J) Vaccination reduced changes in gastrocnemius muscle gene expression (n=10-19). K-T) Vaccination altered the kinetics of muscle cytokine, chemokine, and myokine levels (n=4-6). Data analyzed via two-way ANOVA with Bonferroni post hoc corrections comparing to NoVax/NoFlu age-matched controls (*=p<0.05) and comparing to NoVax/Flu age-matched controls (#=p<0.05).
Vaccination reduces muscle morphological changes and fast twitch fiber atrophy. To determine if the changes in gene expression and inflammatory milieu resulted in detectable changes in muscle morphology and architecture, we investigated gastrocnemius histology. Cross-sections of the gastrocnemius muscle of unvaccinated and vaccinated infected mice at 9 DPI were hematoxylin and eosin (H&E) stained and blindly scored on multiple histological parameters adapted from the literature (33, 34). Flu infection increased architectural damage and cellular infiltration in young and aged unvaccinated mice (Fig5A-B). Importantly, vaccination mitigated these changes in both age groups (Fig5A-B & Fig S3A-B). Similarly, total H&E scores showed pronounced protection from morphological changes with vaccination (Fig5B). Further immunohistochemistry revealed that flu infection induced fiber specific atrophy, where type IIB fibers were the only ones detrimentally affected during infection (Fig5C-D & Fig S3C). Type IIB fibers lose one third of their cross-sectional area by 9 DPI in both unvaccinated groups (Fig5C & Fig5D), while vaccination prevented these losses. Type I, IIA, and IIX muscle fibers were not severely impacted during flu infection. This agrees with the general consensus that MyHC IIB is more sensitive to inflammation and other stressors [22, 23, 24].
Figure 5. Vaccination reduces muscle morphological changes and fast twitch fiber atrophy. Young and aged C57BL/6 mice were vaccinated with NP/Alum or PBS before being intranasally infected with 500 EID_{50} of PR8 influenza. Mice were sacrificed at 9 days post infection and gastrocnemius muscle was harvested for histological analyses. A) Represented Hematoxylin and Eosin (H&E) staining of gastrocnemius skeletal muscle from uninfected (left), unvaccinated/infected (middle), and vaccinated/infected (right) 9 DPI aged mice. Scale bars are 100 μm. B) Quantification (detailed in methods) of muscle morphological changes (n=3-5). C) Muscle cross-sectional area via immunofluorescent staining from uninfected (left), unvaccinated/infected (middle), and vaccinated/infected (right) 9 DPI aged mice. Colors: myosin heavy chain I (red), myosin heavy chain IIA (green), myosin heavy chain IIB (yellow), myosin heavy chain IIX (no stain), nuclei (cyan). Scale bars are 100 μm. D) Quantification of fiber type cross-sectional area (n=3-5). Data analyzed via two-way ANOVA with Bonferroni post hoc corrections comparing age-matched counterparts (*=p<0.05).
DISCUSSION

With age, the immune response to flu infection diminishes resulting in slower viral clearance and increased lung inflammation [9]. Indeed, unvaccinated aged mice follow this pattern in our study. Aged mice are typically slower to clear virus and exhibit higher and prolonged inflammatory cytokines in the BAL and serum when compared to young. This lingering inflammation in response to flu is associated with dramatic changes in mouse mobility, gait kinematics, strength, and other functional parameters. Interestingly, vaccination in aged mice reduced weight loss, but did not prevent it as in young mice. Vaccination also resulted in faster viral clearance and improved anti-IgG antibody titers in both young and aged mice. In older adults, flu infection increases the susceptibility to secondary infection and other flu complications [35]. Thus, ways to better control viral infection, especially in older adults, are a clinically important goal. Here, we show that vaccination not only controls viral loads in the lung, but also prevents flu-induced muscle dysfunction, both on a functional and molecular level. We believe that the impact of flu infection on muscle may directly predispose older adults for catastrophic disability and sarcopenia as well as increase risk of falls and other musculoskeletal injuries. Thus, we have identified vaccination as a proactive protective preventative measure with the potential to increase resilience in older adults.

We previously demonstrated that flu infection leads to prolonged muscular deficits, both functionally and molecularly, with more pronounced changes in aged mice compared to young mice [6]. This provided a molecular link for the increased risk of disability in older adults following a flu infection seen clinically. Here, we investigated the clinical significance of prior immunity to protect against flu-induced muscle decrements. Indeed, we have shown that vaccination mitigates functional losses. Importantly, vaccination reduces the decrements in voluntary mobility, grip strength, and gait kinetics observed with flu infection in aged mice.
Vaccination also protected young and aged mice from increased and prolonged inflammation systemically. Some level of inflammation is necessary to clear flu virus from the lungs. In fact, without IL-6, mice die from flu infection [36, 37]. Similarly, without adaptive immune trafficking to the lung via ligands such as CXCL10, there is not proper viral clearance [38]. We emphasize here that prolonged inflammatory responses exacerbate the damage to skeletal muscle. IFN-γ is primarily produced by immune cells and has been known to trigger CXCL10 expression [38, 39, 40]. The production of these inflammatory cytokines and chemokines were not completely prevented in our vaccinated aged mice, but their prolonged elevation was mitigated. Vaccinated groups also had higher levels of IL-4 and IL-10 in the BAL, serum, and skeletal muscle. While serum IL-4 and IL-10 play a role in IgG antibody responses [41], they also act to suppress the systemic inflammation seen during peak infection. In fact, we see increases in IL-4 and IL-10 in the BAL and serum at 7 and 9 DPI as viral titers are diminishing and the lung is polarized towards type-2 regenerative response. These factors are also crucial to skeletal muscle regeneration. While IL-6, IL-1β, and TNF-α have all been associated with skeletal muscle atrophy [42, 43, 44, 45] and contribute to a catabolic environment which over time reduces muscle function in humans and rodents, IL-4 and IL-10 have crucial roles in muscle repair and regeneration. IL-4 is a molecular signal that controls myoblast fusion with myotubes. Indeed, muscle cells lacking IL-4 signaling/receptors form normally, but are reduced in size and myonuclear number [46]. Absence of IL-10 is associated with elevated IL-6, IL-1β, and TNF-α expression in response to lipopolysaccharide (LPS) in skeletal muscle, with aging further exacerbating these responses [47]. Similarly, both IL-4 and IL-10 trigger changes in macrophage phenotype that promote muscle growth and regeneration [48, 49]. In fact, muscle growth and regeneration are greatly slowed by loss of IL-10 [49]. Our research confirms that flu infection induces a pro-inflammatory environment in the muscle and that vaccination can increase the anti-inflammatory milieu with IL-4 and IL-10.
This more anti-inflammatory environment generated upon vaccination corresponded with both improved muscle gene expression and preserved muscle morphology. Fast twitch muscle fibers (mainly MyHC IIB) are most sensitive to inflammation [22, 23, 24] and we found that they were significantly decreased in size by 9 DPI in unvaccinated young and aged mice. Similarly, histology scores showed poor architecture and increased cellularity at this time point in unvaccinated mice. Importantly, these same mice had increased expression of the atrophy genes Murf-1 and UBC, as well as pronounced functional deficits. Vaccination reduced or prevented all of these declines with preserved type IIB fiber cross sectional area and morphology, as well as reduced expression of atrophy genes and less impairment of muscle function. Indeed, there was no visible difference between naïve controls and vaccinated mice of both age groups. Additionally, we observed upregulated skeletal muscle IGF1 in vaccinated aged mice at 9 DPI. In vitro, IGF-1 stimulation of C2C12 myotubes increased mouse type IIB MyHC mRNA, suggesting IGF-1 has a role in MyHC IIB gene expression [50]. Further, overexpression of IGF-1 for 9 months in extensor digitorum longus (EDL) muscles (primarily composed of type IIB fibers) prevented muscle atrophy and loss of type IIB fibers in aged mice [51]. This suggests vaccination is also promoting positive muscle signals to prevent loss of function. Our research clearly highlights the association between inflammatory milieu, muscle gene expression, muscle atrophy, and muscle function.

As previously mentioned, we anticipated the fiber-specific cross-sectional area changes due to the increased sensitivity to stressors in type II fibers. Translationally, fast twitch fibers control balance, strength, and numerous other necessary functions in humans and rodents. It is possible that the type II fiber atrophy explains the narrowing of stance in infected mice and potentially the increased fall risk in humans due to both weakness and lack of balance. In long-stay nursing home residents, influenza-like illnesses are associated with a 13% average increase in hip fracture hospitalization risk [4]. The corresponding observations during acute
illness focused on unsteady gait and dizziness, which also included a greater risk of falls and broken bones [4]. The loss of fast twitch fibers in our mice might suggest a potential mechanism connecting flu-induced fast twitch fiber loss with fall risk and frailty. Importantly, the decrease in mouse fast twitch fiber cross sectional area was prevented by vaccination in both young and aged mice. Correspondingly, function was preserved in vaccinated mice as well. We also saw increased cellularity in the H&E of unvaccinated mice. In other animal models, cachexia was caused by CD8+ T cells during anti-viral responses [52] and some suggest the possibility of autoimmune reactivity [53, 54, 55, 56, 57]. Future research is necessary to confirm the identity and function of these infiltrating cells during flu infection as it is possible these cells could be a potential target for therapeutics.

In summary, this chapter is the first to identify in a controlled experiment setting flu-induced muscle inflammation and atrophy mitigation by vaccination/prior immunity. We demonstrated that prior immunity, induced by vaccination, prevents muscle fast twitch fiber atrophy and consequently protects muscle functionality. We also determined that vaccine-induced protection is not NP antibody mediated (Fig S4) and more research is necessary to determine if protection is solely mediated via a T cell dependent mechanism. As disability is one of the major complications of flu infection in older adults, the goal of this study was to determine if prior non-neutralizing immunity could provide protection to skeletal muscle. We determined that vaccination indeed could prevent or reduce muscle decrements due to flu infection despite reduced vaccine efficacy with aging. Thus, flu vaccination is still an essential part of protecting older adults from flu-induced disability. Future research may be able to identify specific pathways and therapeutics to fully prevent flu-induced atrophy and potential loss of quality of life in older adults.
ACKNOWLEDGEMENTS

This work was supported by National Institutes of Health/National Institute on Aging grants AG021600 and AG060389 (to L.H.). This research was partially conducted while Jenna Bartley was a Glenn/AFAR Postdoctoral Fellow.

The primary monoclonal antibodies for myosin heavy chain type I (BA-D5), IIA (SC-71), and IIB (BF-F3), developed by S. Schiaffino at the University of Padova, was obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242.

The authors would like to thank Sandra Jastrzebski and Darcy Ahern for their assistance with experimental assays and manuscript preparation.

AUTHOR CONTRIBUTIONS

Spencer R. Keilich (SRK), Erica C. Lorenzo (ECL), Jenna M. Bartley (JMB), and Laura Haynes (LH) conceived and designed the experiments. SRK, ECL, Andrew G. Harrison (AGH), and Blake L. Torrance (BLT) carried out experiments. SRK and ECL analyzed data. SRK led data interpretation and manuscript preparation. JMB and LH supervised the project and assisted with data interpretation and acquisition of funding. All authors discussed the results and contributed to the final manuscript.
REFERENCES


3. L. Ferrucci, J.M. Guralnik, M. Pahor, M.C. Corti, R.J. Havlik, Hospital diagnoses, Medicare charges, and nursing home admissions in the year when older persons become severely disabled, Jama, 277 (1997) 728-734.


27. J.P. White, Control of skeletal muscle cell growth and size through adhesion GPCRs, Adhesion G Protein-coupled Receptors, Springer2016, pp. 299-308.

28. Y. Zou, Y. Dong, Q. Meng, Y. Zhao, N. Li, Incorporation of a skeletal muscle-specific enhancer in the regulatory region of Igf1 upregulates IGF1 expression and induces skeletal muscle hypertrophy, Scientific reports, 8 (2018) 2781.


48. N. Makita, Y. Hizukuri, K. Yamashiro, M. Murakawa, Y. Hayashi, IL-10 enhances the phenotype of M2 macrophages induced by IL-4 and confers the ability to increase eosinophil migration, International immunology, 27 (2014) 131-141.


Supplemental Figure 0. The Graphical Abstract made with biorender.com.
Supplemental Figure 1. Experimental plan for the experiments described. The timeline for our experiments depicting when mice were vaccinated, infected, and sacrificed. Figure made with biorender.com.
Supplemental Figure 2. Vaccination to influenza reduced flu-induced functional decrements in voluntary locomotor activity, grip strength, and gait kinematics. Young and aged C57BL/6 mice were vaccinated with NP/Alum or PBS before being intranasally infected with 500 EID$_{50}$ of PR8 influenza. Mice were acclimated to testing prior to infection and tested for functional performance at designated time points. A-G) Gait parameters were assessed utilizing DigiGait, a ventral plane videography treadmill system 7 days post infection (n=8-15). G) Spontaneous voluntary activity was assessed via the open field test at 8 days post infection (n=10-15/group). I) Grip strength was determined by using a grip strength meter at day 8 post infection (n=10-15/group). Data analyzed via two-way ANOVA with Bonferroni post hoc corrections comparing to NoVax/NoFlu age-matched controls (*=p<0.05), comparing to NoVax/Flu age-matched controls (#=p<0.05), and comparing between ages within a condition (brackets=p<0.05).
Supplemental Figure 3. Vaccination preserves architecture and nuclear infiltration of young and aged skeletal muscle H&E’s. A) The H&E’s are representative for each group at each time point analyzed. Scale bars are 100 μm. B) Blindly scored H&E summaries across each timepoint (n=3-5). C) Muscle cross-sectional area via immunofluorescent staining from uninfected (left), unvaccinated/infected (middle), and vaccinated/infected (right) 9 DPI young mice. Colors: myosin heavy chain I (red), myosin heavy chain IIA (green), myosin heavy chain IIB (yellow), myosin heavy chain IIX (no stain), nuclei (cyan). Scale bars are 100 μm. Data analyzed via two-way ANOVA with Bonferroni post hoc corrections comparing to NoVax/NoFlu age-matched controls (*=p<0.05), comparing to NoVax/Flu age-matched controls (#=p<0.05).
Supplemental Figure 4. Musculoskeletal protection is not NP-antibody mediated in aged mice. Aged C57BL/6 mice were given NP antibody delivered IP or isotype antibody controls before being intranasally infected with 500 EID50 of PR8 influenza. 

A) Treatment and isotype control mice each lost equivalent amounts of body mass during flu infection. 

B) There were no differences in high scoring H&E scores between groups (NP antibody left and isotype control right).

C) There was no difference in the myosin heavy chain type IIB fibers (yellow stained) between groups (NP antibody left and isotype control right). 

D) The adoptive antibodies were not protective against flu-induced gastrocnemius gene expression alterations at 9 DPI. All data analyzed via two-way ANOVA with Bonferroni post hoc corrections with effect of adoptive antibody treatment (compared to age matched isotype control, p<0.05) indicated by asterisks above data set.
CHAPTER FOUR
RNA sequencing of aged skeletal muscle reveals strong leukocyte activity during pulmonary influenza infection

ABSTRACT
Influenza (flu) infection leads to loss of independence in older adults. Despite no direct viral infection of skeletal muscle, there were alterations in the muscle inflammatory milieu and muscle atrophy, with more pronounced and prolonged deficits with aging. To identify potential mechanisms of flu-induced muscle atrophy, we employed a multidisciplinary systems-based approach to examine the relationship between immune responses, flu-induced inflammation, skeletal muscle tissue morphology, muscle function, and full muscle transcriptomics. Our integrated approach will test the hypothesis that flu-induced systemic factors lead to muscle atrophy, while also generating additional unbiased hypotheses based on transcriptomics and statistical modeling to determine the driving mechanisms behind flu-induced muscle atrophy. Understanding the mechanisms responsible will then be able to guide preventative and therapeutic measures. Furthermore, this study has significant potential to discover high impact translational findings that will improve care for flu-infected older adults and help them maintain their independence later in life.
INTRODUCTION

Influenza (flu) is the seventh leading cause of death among older adults in the US with 90% of flu-related deaths occurring in late life [1]. Equally compelling from a clinical and public health perspective, but less well understood, is the relationship between flu infection and disability. Older adults have a greatly increased risk of both progressive and catastrophic disability following flu infection [2, 3]. Nevertheless, mechanisms by which flu infection contributes to disability remain unknown and unexplored, resulting in missed opportunities for the discovery of interventions designed to prolong function and independence in late life. Even in uncomplicated cases, flu infections have some degree of muscle involvement with myalgia being a common symptom [4]. The studies in this chapter are based on premises from our published work, which showed for the first time that flu infection, which by its nature is limited to pulmonary epithelial cells [4], results in mobility impairments, muscle inflammation, and increased markers of muscle atrophy. Moreover, these effects are more pronounced and prolonged with aging, thus directly predisposing older adults to disability following flu infection [5]. However, the pathogenesis for this interaction is unknown and little information exists detailing the systematic impact of flu infection and how this changes with aging. Here, we aim to define specific mechanism(s) and pathways involved in flu-induced muscle atrophy with aging and will lay the groundwork for development of preventative measures and therapeutic treatments. Thus, this study has significant potential to discover high impact translational findings that will improve care for flu-infected older adults.

Our studies have revealed a molecular link between flu infection and disability [5]. Even though flu remains a respiratory infection, it exerts distant effects on skeletal muscle in terms of functional decline, inflammation and atrophy gene expression; these effects are more severe and prolonged with aging. The mechanism behind these outcomes is unclear and warrants further investigation. There is no evidence of direct viral infection [5], thus we believe systemic
signals from flu infection likely trigger the cascade of muscle-localized inflammation and atrophy. Importantly, this supports the notion that other infections that cause systemic inflammation to a similar extent will also result in muscle loss with aging. Thus, this chapter will delve into mechanisms behind not only flu-induced muscle atrophy, but also other infection and inflammation induced myopathies. Indeed, the clinical significance of this thesis transcends flu-infected older adults and will provide a working framework for other infection-induced disabilities and diminished resilience with aging. We hypothesize that exaggerated flu-induced systemic inflammation, as well as muscle sensitivity to this inflammation, contributes to increased flu-induced muscle loss with aging.

mRNA sequencing (mRNA-seq) is a breakthrough technology that provides quantitative and sensitive detection of gene expression levels with a broad dynamic range [6], which was demonstrated by Han and colleagues [7, 8]. Indeed, there are many studies which use sequencing technologies to investigate skeletal muscle gene changes. A systems biology approach revealed a link between systemic cytokines and skeletal muscle energy metabolism in a rodent smoking model and human COPD [9]. This is similar to our hypothesis that lung infection damage and inflammation is contributing to the myopathy and functional changes. This same group, Davidsen and colleagues, reported that several catabolic pathways were upregulated and that “hub genes” Cxcl10 and Cntf were at the center of most pathways. In fact, elevated levels of pro-inflammatory cytokines have previously been linked to skeletal muscle wasting [9, 10]. Additionally, several sources even associate marked reduction in body weight with lung damage and attempt to correspond it to changes in skeletal muscle metabolism [11, 12, 13]. In these studies, the soleus muscle was more directly affected by oxidative stress and hypoxia because it is oxidative muscle with high Type I fiber content; lung damage from smoking causes less effect in the gastrocnemius muscle which is mixed Type II composition that is predominantly glycolytic [9].
Similar studies showed that inflammatory signaling interrupted skeletal muscle development, resulting in fibrosis. IFN-γ mediates disruption of energy expenditure-related gene expression by repressing Sirtuin 1 (SIRT1) transcription in skeletal muscle cells [14]. SIRT1 activity loss in muscle disrupts gene expression, development, and regeneration as well as premature exit of cell cycle [15]. Mice also upregulate Fibroblast Growth Factor (FGF-6) after injury to restore damaged muscle and without FGF-6, mice develop fibrosis and myotube degeneration [16]. Conversely, overexpression of Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) in aging muscle enhances a subset of young-like molecular patterns [17]. Importantly, myostatin directly regulates skeletal muscle fibrosis [18] as well as Kruppel Like Factor 10 (KLF10) gene expression [19]. KLF10 moderates the fibrotic effects of TGF-β signaling in chronically damaged regenerating muscle [19]. Collagen, type I, alpha 1 (Col1a1) and fibronectin gene expression and protein deposition were increased in KLF10/-/- mice, as well as increased SMAD2, 3, 4, &7 with downstream effects that increased cardiomyopathy, fibrosis, and impaired regeneration [19].

Adult tissue repair and regeneration require activation of resident stem/progenitor cells that can self-renew and generate differentiated progeny. The regenerative capacity of skeletal muscle relies on the muscle satellite cells and their interplay with different cell types within the niche [20]. We hypothesize that aged mice have greater populations of inflammatory immune cells infiltrating and residing in their skeletal muscle post flu infection, which contribute to the delayed muscle regeneration and disruption. High-dimensional single-cell sequencing reveals novel skeletal muscle resident cell populations of macrophages, neutrophils, B cells (Ptprc, Ms4a1, CD19, CD22), T cells (CD4, CD8a, CD3), and CD45+ cells; using single-cell RNA-seq and mass cytometry to identify functional populations, characterize their gene signatures, and determined key markers [20].
We are the first to use a systems-based approach with aging mice and flu infection. It is imperative to take a multidisciplinary approach to understand how aging and immunological challenge (flu infection) impact skeletal muscle health. By concurrently examining immunological parameters, systemic factors (blood cytokines and chemokines), muscle function, muscle gene expression, and muscle histology, we can delve more deeply into understanding the mechanism(s) of flu-induced muscle atrophy. This is the first unbiased kinetic profile of skeletal muscle transcriptome during flu infection. Poly-A RNA-sequencing (RNA-seq) has never been performed on skeletal muscle in the context of flu infection, let alone, flu infection and aging. Importantly, the mechanism(s) behind the clinical association between flu infection and future disability, as well as our findings of flu-induced muscle atrophy are not clear. Flu infection, and most likely other infections, represent major stressors that decrease resilience and result in increased disability in the older adults. Thus, understanding these mechanisms is essential to developing prevention and treatment methods to avert disability and maintain independence in old age.

**MATERIALS AND METHODS**

**Mice.** Young (2.5-4-month-old) C57BL/6 male mice were purchased from Jackson Laboratories or obtained from the National Institute on Aging. Aged (19-22-month-old) C57BL/6 male mice were obtained from the National Institute on Aging rodent colony. All mice were housed in a climate controlled environment with 12:12 light:dark cycle and fed standard rodent chow and water ad libitum. All mice were cared for in accordance with the recommendations in the Guide for the Care and use of Laboratory Animals of the National Institutes of Health. All procedures were approved by the UConn Health IACUC. Recumbent mice and mice that lost more than 30% body weight during the course of influenza infection were considered moribund and...
euthanized. All mice underwent gross pathological examination at time of sacrifice and animals with obvious pathology were excluded from the study.

**Viral infection.** Mice were anesthetized with isoflurane and intranasally inoculated with 50 ul of 500 EID₅₀ of influenza virus A/PR/8/34 (PR8). Mice were weighed daily to monitor infection progression. At the indicated time points post-infection, whole lung tissue was homogenized and RNA was isolated via TRIzol/chloroform extraction per manufacturer’s protocol (Ambion by Life Technologies, Naugatuck, CT and Sigma Aldrich, Natick, MA, respectively). RNA was reverse transcribed with iScript cDNA synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA) and flu viral copies were detected via reverse transcription quantification PCR of flu acid polymerase (PA) as previously described [21].

**Cardiac perfusion protocol.** Immediately following CO₂ euthanasia, the majority of blood was removed via cardiac puncture. The remaining blood was flushed from the animal through standard cardiac perfusion. Briefly, a 60 mL syringe containing cold 1xPBS equipped with polyethylene tubing was used to perfuse the animal via perfusion pump (WPI SP100I syringe pump) at a rate of 47.82 mL/hr. The right atrium was clipped, and a 25g needle attached to the polyethylene tubing was inserted at the apex of the base of the heart. Each animal was perfused with a minimum of 20 mL 1xPBS until liver began to clear and blood flowing from the right atrium was no longer visible.

**Gastrocnemius RNA.** To examine gastrocnemius gene expression, mice were fasted with the exception of water for 4-6 hours prior to sacrifice in order to minimize potential confounding results due to postprandial muscle protein synthesis. The gastrocnemius muscle was dissected and placed in RNAlater (Qiagen Inc., Germantown, MD) overnight at 4°C. RNAlater was removed and the muscle was frozen at -80°C until RNA extraction. All RNA isolations were performed by the same individual to minimize variability. The muscle was homogenized and
RNA was extracted via miRNeasy extraction per manufacturer’s instructions (Qiagen Inc., Germantown, MD). RNA quantity and quality were assessed with Nanodrop 2000c (Thermo Scientific, Waltham, MA).

**Library preparation and high-throughput sequencing and quantification of gene expression.**

Total RNA was checked on a BioAnalyzer (Agilent). mRNA-seq libraries were structured according to the standard Illumina TruSeq Stranded mRNA protocol. Paired-end 151-bp sequencing was performed on an Illumina HiSeq 2000 (Shelton, CT). Sequencing reads were trimmed with trimmomatic [22] to remove the universal Illumina adaptor and uniquely mapped to the UCSC mouse reference genome (mm9) and a custom splice junction library in parallel using hisat2/2.1.0 [23, 24]. Genes were considered not expressed if they had zero reads in all technical and biological replicates. Mapped reads in were counted using HTSeq and then differential expression was performed with DESeq2. We continued our analysis with samples that contained at least 36 million mapped reads, which excluded samples from analysis for lack of genome reads (samples under 9 million reads included SRK017: 74,75,76,77,78,79 and SRK018: 67).

**To perform GO analysis.** GO analysis was performed via DAVID [25] gene functional classification tool. This tool classifies large gene lists into functional related gene groups based on representation, which can be interpreted as a cluster of biologically relevant regulatory networks or pathways. In our analysis, we considered only GO categories with a false discovery rate < 0.05 and pathways with adjusted P < 0.05, which we summarize in Supplemental Tables 1 & 2. Our analysis generated a significant pool of novel, zone-specific genes that were excluded from GO and pathway analysis because of missing annotations.

**RT-qPCR.** RNA was reverse transcribed via iScript Advanced cDNA synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA). RT-qPCR was performed using predesigned commercially
available primers (Bio-Rad Laboratories, Inc.). Gene expression was calculated via a modified Pfaffl method utilizing multiple reference genes (RPS18 and TBP, which showed the least variability between conditions and thus suitable reference genes as previously shown (6)) and normalized to the uninfected young mice.

**Multiplex Protein Analysis.** Bronchiolar lavage fluid (BAL) was collected by flushing lungs with 1 ml PBS. Supernatant was collected after centrifugation and assayed for cytokine and chemokine content. Similarly, blood was collected via cardiac puncture, allowed to clot at room temperature, and the resultant serum was assayed for cytokine and chemokine content. Gastrocnemius muscle was dissected from each mouse and homogenized in Tissue Protein Extraction Reagent (Thermo Scientific, Bedford, MA) supplemented with 5mM EDTA (Invitrogen, Carlsbad, CA) and Protease/Phosphatase Inhibitor Cocktail (Thermo Scientific)). Debris was removed via centrifugation and total protein content was determined using a Pierce™ BCA Protein Assay Kit (Thermo Scientific).

**Voluntary locomotor activity.** Spontaneous voluntary locomotor activity was measured via open field test at time points indicated. All tests were performed between 7-9am to control for diurnal variations. Following acclimation to the dim-lit testing room (at least 1 hour), mice were placed in the center of the photobeam activity system-open field (PAS-OF, 16”x16”x15” acrylic animal enclosure, San Diego Instruments, San Diego, CA) and their activity was recorded for 20 minutes. The first 5 minutes was excluded as this is generally considered to be exploratory behavior rather than general voluntary locomotor activity. The number of beam breaks per minute during the last 15 minutes was then used to assess voluntary locomotor activity.

**Grip strength.** Grip strength was determined by using a grip strength meter BIOSEB (In Vivo Research Instruments, Pinellas Park, FL). Briefly, mice were permitted to grab onto a T-shaped-bar and pulled horizontally by the tail until they released their grip. The force (grams) read by
the force meter at the release of the mouse’s grasp was averaged by their mass (grams) on that
day and compared to their pre-infection results. The same researcher performed all testing to
minimize variability.

Gait analysis. Gait analysis was performed using the DigiGait instrument (Mouse Specifics, Inc.
Quincy, MA) and software (DigiGait Imager 4.0.0 and DigiGait Analysis 11.5, Mouse Specifics,
Inc). The DigiGait instrument consists of a clear treadmill with a high-speed camera mounted
underneath that collects images at 147 frames per second for high resolution of postural
temporal gait parameters. Mice run within a 2-inch-wide acrylic running chamber at set speeds.
The ventral plane videos are analyzed with the DigiGait software which identifies portions of the
paw that are in contact with the treadmill belt to produce both postural and kinematic gait
parameters. Mice were introduced to the DigiGait system at a low speed (10cm/sec) briefly (30
seconds) prior to the initial testing. Mice were allowed to acclimate to the dim-lit room for 1 hour
prior to each testing period and all tests were performed between 8-10 am. Mice ran at the
testing speed (16cm/sec, as previously optimized (6)) until approximately 5 seconds of
consecutive walking was recorded and this video segment was analyzed via DigiGait software.

Histology. The gastrocnemius muscle was carefully dissected and blotted on a Kim Wipe before
being placed in a cryomold, embedded in Optimal Cutting Temperature (OCT ThermoScientific
Inc., Waltham, MA) and frozen in liquid nitrogen cooled isopentane. Samples were stored in the
freezer at -80° Celsius until sectioned. Ten-micron thick muscle cross sections were mounted
on charged slides (Superfrost Plus Glass Slides, ThermoScientific Inc., Waltham, MA) for
histological analyses. Serial sectioned (10um) muscle was fixed in ice-cold acetone for 3
minutes and stained with antibodies against laminin, CD45, CD3, CD31, and Lyve1 (Table 1
below). Sections were blocked for 30 minutes with 2% goat serum, stained over night at 4°C in a
humidified chamber with primary antibody cocktail, washed in PBS and then stained for 2 hours
at room temperature with secondary antibodies. Slides were mounted with Immu-Mount
(Thermo Scientific), cover slipped and sealed. Images were acquired using the Zeiss 880 laser scanning microscope (Carl Zeiss; air objective 20× Plan-Apochromat with NA 0.5). For analysis, four z-stack (4-0.5um steps) images per mouse were acquired of the skeletal muscle cross-section with the 20x objective. Volume and disconnection of laminin and vessels were quantified using the isosurface tool in Imaris 8.1 software (BRITPLANE). Laminin was used to detect the basement membrane of the skeletal muscle. CD45 and CD3 were used to detect leukocytes and T lymphocytes, respectively; while CD31 and Lyve1 were used to detect blood vessels and lymphatic vessels, respectively. Images were acquired on a Zeiss LSM880 confocal microscope and analyzed via Imaris software. Intramuscular leukocytes were identified by CD45+Lyve1-CD31- within laminin borders. Intramuscular T cells were identified by CD3+CD45+ Lyve1- CD31- within laminin borders.

**Table 1. Antibodies used for phenotyping infiltrating cells in skeletal muscle by immunofluorescence.**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Fluorochrome</th>
<th>Clone</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Laminin, Polyclonal Laminin (primary)</td>
<td>Unconjugated</td>
<td>LAMA1</td>
<td>Novus Biologicals™</td>
</tr>
<tr>
<td>Donkey anti-rabbit IgG Laminin (secondary)</td>
<td>BV510</td>
<td>Poly4064</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD3</td>
<td>AF647</td>
<td>17A2</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD45</td>
<td>PE</td>
<td>30-F11</td>
<td>Biolegend</td>
</tr>
<tr>
<td>Lyve1</td>
<td>AlexaFluor488</td>
<td>ALY7</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD31</td>
<td>BV421</td>
<td>MEC13.3</td>
<td>BD</td>
</tr>
</tbody>
</table>

**Statistical analysis.** All data analyzed via two-way (DPI x age) ANOVA with Bonferroni post hoc corrections. Significant differences comparing to baseline (0 DPI) age-matched controls (*=p<0.05), and comparing between groups/ages within a condition (brackets=p<0.05). RNA-seq data was analyzed as noted above. Several contrast comparisons were made from the DESeq2 results at an adjust P-value < 0.05: contrasting Young 0 DPI to every other Young DPI, Contrasting Aged 0 DPI to every other Aged DPI, and Contrasting Young and Aged at the same DPI. Heatmaps were created based off genes with log2FoldChange > 2 or log2FoldChange < -2.
with an adjusted P-value < 1e-2. The hierarchal clustering was performed with Spearman method for gene expression association analysis. Volcano plots were given a cutoff of P-value = 5e-2 and 2 log2 fold change. Gene ontology pathway analysis with DAVID used q-value cutoff = 5e-2 and required genes to have at least a log2 fold change with and adjusted p-value 1e-2.

RESULTS

Aged mice have increased weight loss and increased flu PA copies in the lung during infection, simultaneously with prolonged functional deficit. This data, from a new cohort of mice, matches our previously shown data in chapter 2 [5] and chapter 3 [unpublished Keilich et al]. Aged mice have greater and prolonged weight loss during flu infection when compared to their young counterparts (Fig1A) and aged mice plateau at 10% loss from their starting body weight. Aged mice also display delayed viral clearance compared to young counterparts on days 9 and 11 post infection (Fig1B). Functionally, aged mice have greater deficits at 11 days post infection (DPI) than their young counterparts when measuring grip strength (Fig1C). Open field testing, which measures the voluntary motion of mice, demonstrated that young mice were more mobile than aged mice at pre-infection baseline trials and across all other testing time points except 7 DPI where they performed equally (Fig1D). Due to the fact that young and aged mice had different starting points in DigiGait analysis parameters, we decided to draw comparisons to each mouse’s “pre-infection” test so that each mouse is compared to its own baseline results 2 days prior to infection denoted -2 DPI (Fig1E-I). Aged mice had significantly decreased stance width at 5 of the 8 time points (Fig1E) and did not return to pre-infection stance, whereas their young counterparts were insignificantly different from their pre-infection stance width after 15 days post infection. The time in “stance” with the paw on the ground was increased and prolonged in aged mice, but not in young mice (Fig1F). Similar trends were seen in the aged
mouse time in “swing” with the paw in the air and time in “stride” (Fig1G-H). Young and aged mice followed similar trends for time in the “propel” stage of stepping (Fig1I). These data are further verified by forelimb data (data not shown).

Figure 1. Aged mice have increased weight loss and increased flu PA copies in the lung during infection, simultaneously with prolonged functional deficit. Young and aged C57BL/6 mice were intranasally infected with 500 EID$_{50}$ of PR8 influenza. A) The percent body mass loss graphed over the course of the experiment. B) Influenza acid polymerase (PA) copy number determined by RTqPCR of mouse lungs at given time points. C) Grip strength was determined by using a grip strength meter. D) Spontaneous voluntary activity was assessed via the open field test. E-I) Gait parameters were assessed
utilizing DigiGait. Data analyzed via two-way ANOVA with Bonferroni post hoc corrections comparing young and aged mice (brackets=p<0.05) and comparing to age-matched pre-infection (*=p<0.05).

**Inflammation is elevated and prolonged with aging in BAL and serum.** Bronchoalveolar lavage (BAL) was collected from mice at the time of sacrifice. Granulocyte-colony stimulating factor (GCSF) is a glycoprotein that simulates the bone marrow to produce granulocytes (primarily neutrophils) and stem cells and release them into the blood stream. Interleukin 1 alpha (IL-1α) is responsible for production of inflammation as well as the promotion of fever and/or sepsis. Aged mice had significantly more GCSF and IL-1α in the BAL compared to young mice on 5, 7, and 9 DPI (Fig 2A & 2D). Granulocyte-macrophage colony-stimulating factor (GMCSF) is a monomeric glycoprotein secreted by macrophages, T cells, mast cells, natural killer cells, endothelial cells and fibroblasts that functions to stimulate/produce neutrophils, eosinophils, and basophils. Aged mice had significantly more GMCSF than young mice in the BAL on 7, 9, and 11 DPI (Fig 2B). Interferon gamma (IFN-γ) is a cytokine that is critical for innate and adaptive immunity against viral and some bacterial infections. IFN-γ is capable of activating the JAK-STAT pathway on cells and is an important activator of macrophages and inducer of class II major histocompatibility complex (MHC) molecule expression. IFN-γ was only different from in aged mice 7 DPI (Fig 2C). In BAL and serum, interleukin 6 (IL-6) promptly and transiently is produced to contribute to host defense through stimulation of acute phage responses, hematopoiesis, and immune reactions. IL-6 is very pleiotropic and has different effects in different environments. CXCL10 is also known as interferon gamma-induced protein 10 which is considered to be a proinflammatory chemokine for inflammatory cell types carrying the CXCR5 receptor. IL-6 and CXCL10 were both significantly upregulated in aged mice compare to young counterparts on 7 and 9 DPI (Fig 2E & 2F). Tumor necrosis factor alpha (TNF-α) is a cell signaling protein involved in systemic inflammation and is one of the cytokines that make up the acute phase reaction. The cytokine that had the greatest age difference was TNF-α, which reported higher levels in aged mice on 5, 7, 9, and 15 DPI when compared to young (Fig 2G). Interestingly, serum levels
of GCSF had opposite trends in young and aged mice (Fig 2H), where young mice were significantly decreased on days 9 and 11 when compared to young 0 DPI. Aged mice were only significantly increased compared to young 9 DPI (Fig 2H). Serum GMCSF was higher in aged mice compared to young 9, 15, and 20 DPI (Fig 2I). Serum IFN-γ and IL-1α did not have age differences at measured time points. Serum IL-6 and TNF-α had age differences on 9 DPI but TNF-α did not have any increase in young mice (Fig 2L and 2N). Serum CXCL10 peaked in both young and aged mice 7 DPI, but was higher in young mice 5 DPI and higher aged mice 9 and 11 DPI (Fig 2M). Overall, young mice resolved inflammation more quickly than aged cohorts and aged mice had increased levels of inflammatory cytokines compared to young mice.

Figure 2. Inflammation is elevated and prolonged with aging in BAL and serum. Young and aged C57BL/6 mice were intranasally infected with 500 EID$_{50}$ of PR8 influenza. A-G) Bronchoalveolar Lavage (BAL) was collected from mice at time of sacrifice as described in methods. The BAL was then analyzed via Millipore chemokine/cytokine Multiplex 25-plex for the analytes. H-N) Serum was collected from mice
at the time of sacrifice as described in methods. The serum was later analyzed via Millipore chemokine/cytokine Multiplex 25-plex for the analytes listed. Data analyzed via two-way ANOVA with Bonferroni post hoc corrections comparing young and aged mice (brackets=p<0.05), comparing to age-matched 0 DPI (*=p<0.05).

Determination of age and DPI specific differences in gene expression using RNA sequencing.

The RNA extraction and library preparation were performed as stated in the methods section with samples from two rounds of the same experiment run together. Mapped sample reads were analyzed with DESeq2 with comparisons between age groups and days post infection. The data then underwent principal component analysis (PCA), which is a statistical procedure that uses orthogonal transformation to convert a set of observations of possibly correlated into a set of values of linearly uncorrelated variables called principal components. These principal components are plotted in PCA plot (Fig 3A), which shows that the overall gene expression profiles of the samples cluster primarily by infection (DPI), and then by age. This demonstrates that comparisons can be made between groups because the replicates of each group are similar to each other and distinct from other groups. Similarly, we observed similar gene changes in groups from different batches (Fig 3B), where mice had the same gene trends at the same ages and DPI. Indeed, when clustered in smaller groups (comparing ages at the same DPI or comparing DPI within age groups), mice are blindly clustered with other samples in their infection, age, and DPI groups (Fig 3C and data not shown). Overall, these are excellent indications that skeletal muscle global gene expression is changing with respect to time point post infection in an age-dependent manner.
Figure 3. Determination of age and DPI specific differences in global gene expression. We performed pair-end 151-bp polyA-selected sequencing was performed using an Illumina HiSeq 2000. A) Principal Component Analysis plot of all samples. B) Heatmap of all samples showing breakdown of meta-data groups and general whole sample gene changes. C) Clustered histogram comparing young (SRK017.01-SRK017.05 & and SRK018.01-SRK018.05) and aged samples (SRK017.06-SRK017.10 & and SRK018.06-SRK018.10) at 0 DPI.

Young and aged mice undergo different kinetics for inflammation and gene expression changes. Young and aged mice have significantly different gene expression at 0 DPI (Fig 3C and Fig 4A), with extra cellular matrix (ECM) genes such as Actc1 and Col1a1 downregulated in aged mice. At 9 DPI, aged mice have increased expression of chemotaxis genes compared to young mice with upregulated CXCL13, CXCL2, and CCL8 to name a few (Fig 4B). Additionally, aged mice have TGF-β superfamily genes, such as GDF5, upregulated in conjunction with continued downregulation of ECM genes (Fig 4B). These expression patterns continue on 11 DPI (Fig 4C) and as far out as 20 DPI (Fig 4C). Interestingly, we observed that aged mice had increased expression of inflammatory cytokines such as IL-6, IL-1β, and IFN-γ, as well as pro-inflammatory chemotaxis signals such as CXCL10, on 20 DPI (Fig 4D). Venn diagrams comparing gene expression of acute infection timepoints (Fig 4E and 4F) and resolving infection timepoints (Fig 4G and Fig 4H) displays that young and aged mice have similar overall changes in gene expression due to infection, but young mice see these changes at earlier time points than aged mice; and aged mice see continued expression long after these changes resolve in the young. All of the significant genes (adjusted p-value < 0.01) with significant fold changes (greater than +/- 1 log2 fold) were compiled and used for pathway analyses. Identifying significant pathways will highlight processes and genes that are “centric” or considered “hub genes” that are connected to multiple pathways in flu-induced muscle changes. Similar genes were upregulated in young and aged mice with infection, but the changes in aged mice occurred two days later than young.
Figure 4. Young and aged mice undergo different kinetics for inflammation and gene expression changes. A-D) Volcano plots comparing aged to young mouse expression at the given timepoint. We applied a cutoff for significance at $p = 0.05$ and twofold change in expression to determine differentially...
expressed genes. E-H) Venn diagrams shows the acute (E-F) and resolving (G-H) infection in young (E&G) and aged (F&H) mice after pairwise comparison to age-matched 0 DPI. We applied an adjusted-P-value < 0.05 and twofold change in expression to determine differentially expressed genes.

*Pathway analysis of aged skeletal muscle RNA- Sequencing reveals strong leukocyte activity during pulmonary influenza infection.* Pathway analysis of significantly upregulated and downregulated genes using DAVID gene ontology [25] revealed the differences in aged pathway expression compared to young pathway expression at given timepoints (Fig5). Graphed in Figure 5 are the top 20 significant pathways calculated, the full list of pathways and genes involved is in Supplemental Table 1 and Supplemental Table 2. Without infection, young mice are more adept at ECM organization and skeletal muscle development, which is shown in the pathway downregulation of aged mice at 0 DPI compared to young mice (Fig 5A). Interestingly, only aged mice displayed T cell aggregation, T cell activation, and other upregulated positive expression for leukocytes and lymphocytes (Fig 5B). These immune cell mediating pathways were most significant in the aged mice and continued across timepoints until about 30 DPI (Supplemental Table 1). Aged muscle underwent greater stress pathway upregulation with genes that triggered oxidative stress, apoptosis, and ubiquitin catabolic pathway results (Fig 5C). In addition, downregulation of pathways involved in sarcolemma, ECM organization, and regeneration were observed in aged mice 9 DPI (Fig 5C). Many of these catabolic upregulated pathways and downregulated regeneration pathways persisted in aged compared to young muscle out to 20 DPI (Fig 5D and Fig 5E). We observed downregulated pathways for axonogenesis, neuron development, and neuromuscular repair pathways 11 DPI that were unique to aged muscle (Fig 5D). Indeed, this GO pathway analysis highlights the aged muscle’s lack of regenerative capacity, degradation, and emphasis on prolonged immune cell chemotaxis and activation.
A) 0 DPI aged compared to young

Pathways Downregulated

B) 7 DPI aged compared to young

Pathways Upregulated

C) 9 DPI aged compared to young

Pathways Upregulated

D) 11 DPI aged compared to young

Pathways Upregulated

E) 20 DPI aged compared to young

Pathways Upregulated

Pathways Downregulated
Increased leukocyte and T cell infiltration in the skeletal muscle. Our GO pathway results highlighted several chemokine pathways as well as leukocyte and T cell markers which were specific to aged but not young mice expression. Upon further investigation, we probed muscle sections for CD45+ cells, which were Lyve-1- to ensure that they were not in the lymph vessels and CD31- to ensure that they were not in the blood vessels (Fig 6A-H). We found that aged muscle had the most significant increase in CD45+ cells on 15 DPI (Fig 6A). Following a similar pattern, CD3+ cells were on seen to increase in aged muscle 11 DPI and 15 DPI when compared to young mice (respectively 11 DPI and 15DPI) and aged 0 DPI. Thus, gene expression changes observed in the pathway analysis correspond to observed cell populations in skeletal muscle, suggesting that flu leads to pro-inflammatory cell infiltration in aged skeletal muscle that causes muscle atrophy and hinders muscle regeneration.
**Figure 6. Increased leukocyte and T cell infiltration in the skeletal muscle.** Young and aged C57BL/6 mice were intranasally infected with 500 EID$_{50}$ of PR8 influenza and sacrificed at day post infection (DPI) indicated. Serial sectioned (10um) muscle was stained with antibodies against laminin, CD45, CD3, CD31, and Lyve1. Laminin was used to detect the basement membrane of the skeletal muscle. CD45 and CD3 were used to detect leukocytes and T lymphocytes, respectively; while CD31 and Lyve1 were used to detect blood vessels and lymphatic vessels, respectively. Images were acquired on Zeiss LSM880 confocal microscope and analyzed via Imaris software. A) Quantification of intramuscular leukocytes were identified by CD45+Lyve1-CD31- within laminin borders. B) Quantification of intramuscular T cells were identified by CD3+CD45+Lyve1-CD31- within laminin borders. C-H) Representative images of young and aged muscle with CD3 (red), CD45 (yellow), Lyve-1 (green), CD31 (blue), and Laminin (purple). Data analyzed via two-way ANOVA with Bonferroni post hoc corrections comparing young and aged mice (brackets=p<0.05), comparing to age-matched 0 DPI (*=p<0.05).

**DISCUSSION**

This project is a multidisciplinary effort to determine the mechanisms at the interface of aging, flu infection, and muscle health. Our novel finding that pulmonary flu infection influences specific changes in skeletal muscle gene expression and function has culminated in this study [5]. Aging increases the negative impact of flu infection on skeletal muscle health [26]. Another observation is that flu-induced changes in skeletal muscle expression of inflammatory and atrophy genes were increased to a greater amplitude and duration in aged animals. Flu infection also decreased expression of positive regulators of muscle mass and myogenesis to a greater degree in aged mice. Additionally, aged mice had prolonged mobility and functional decrements. Flu-related disabilities in the older adults are an extremely urgent clinical problem, especially with an ever-increasing older adult population. Understanding the molecular mechanisms that link flu infection and disabilities could lead to novel therapeutic discoveries to decrease post-infection disability with aging.

Flu infection instigated inflammation in the BAL and serum of both young and aged mice that was more pronounced and prolonged with aging. Prior to autophagy and ubiquitin pathway activation, the first pathways to change expression in the skeletal muscle are upstream of the NF-κB pathway and inflammasome pathways. Both of these trigger inflammatory responses in
skeletal muscle in response to systemic inflammatory factors. In fact, our sequencing showed guanylate binding protein 5 (GBP5), activator of NLRP3 inflammasome assembly, was triggered through IFN-γ signaling. Circulation of IFN-γ, TNF-α, and IL-6 in the blood during peak infection precedes changes in skeletal muscle gene expression. Skeletal muscle is highly vascularized and contains a variety of mononuclear cells, including those in peripheral blood, as well as endothelial cells, fibroblasts, and myogenic satellite cells [27]. Therefore, cytokines in the blood have been shown to induce metabolic changes in skeletal muscle during lung trauma [9, 11, 12, 13]. Some of our observed cytokine influxes in the serum during flu-infection – IFN-γ, IL-1α, IL-6, TNF-α – are known to cause muscle atrophy and block muscle maturation/differentiation [10]. These cytokines act as the catalyst of change for skeletal muscle and we hypothesize that the changes we observe are triggered by mechanisms downstream of their signaling.

More specifically, similar studies showed that inflammatory signaling interrupted skeletal muscle development, resulting in fibrosis. Simultaneous IFN-γ and TNF expression lead to increases in pro-inflammatory chemokines such as CCL5, CXCL9 and CXCL10. IFN-γ also mediates disruption of energy expenditure-related gene expression by repressing Sirtuin 1 (SIRT1) transcription in skeletal muscle cells [14]. SIRT1 activity loss in muscles disrupts gene expression, development, and regeneration as well as premature exit of cell cycle [15]. Our sequencing revealed this decrease in SIRT1 in our aged cohorts compared to young at the same timepoints suggesting the impact of IFN-γ was more impactful in aged skeletal muscle.

Mice upregulate FGF-6 after injury to restore damaged muscle; but without FGF-6, mice develop fibrosis and myotube degeneration [16]. Compared to young mice, aged mice had downregulated expression of FGF-6 and similar downregulation myogenic signals. Importantly, myostatin directly regulates skeletal muscle fibrosis [18] as well as KLF10 gene expression [19]. KLF10 acts to inhibit TGF-β and its suppression increases the risk of fibrosis in aged muscle. Col1a1 and fibronectin gene expression and protein deposition were increased in KLF10−/−
mice, as well as increased SMAD2, 3, 4, &7 with downstream effects [19]. KLF10 moderates the fibrotic effects of TGF-β signaling in chronically damaged regenerating muscle [19]. The aged mice in our study follow similar trends during KLF10 downregulation, displaying increased SMAD gene expression and increased fibronectin gene expression. With these changes in expression, we see fibrosis staining in aged mouse skeletal muscle (chapter 3). Aged mice start to upregulate T cell chemotaxis and activation GO pathways as early as 7 DPI. One of the genes in these pathways is T cell specific GTPase 1 (Tgtp1), which is induced by IFN-γ. Moreover, the downstream effects of IFN-γ have multiple implications on many of these genes and their pathways.

Additionally, the other factors in the serum that we report on – GCSF, GMCSF, CXCL10 – all have the capacity to activate and stimulate white blood cell proliferation and trafficking. Taken together, this inflammatory milieu has been shown to not only directly impact skeletal muscle homeostasis, but also potential immune cells circulating in the blood that traffic into the muscle following muscle chemotaxis signaling (Fig 5 & 6). Here, we aimed to determine the mechanism of flu-induced muscle atrophy with aging. We hypothesized that exaggerated flu-induced systemic inflammation, as well as increased muscle sensitivity to this inflammation contributes to immune cell infiltration into the skeletal muscle and increased flu-induced muscle atrophy with aging. We found upregulation of chemokines such as CXCL10, which promotes immune cell infiltration, in young and aged skeletal muscle tissue with dramatically higher and prolonged expression only in the aged muscle. Correspondingly, immunohistochemistry of murine gastrocnemius muscle revealed higher numbers of leukocytes during flu infection in young and aged mice with a prolonged elevation in the aged mice. Interestingly, an influx of T lymphocytes only occurred in aged muscle later in infection (Fig 6A and Fig 6B). It is possible that infiltrating T cells are causing undue harm to the aged muscle potentially hindering muscle regeneration and functional recovery.
Indeed, it is likely T cell infiltration contributes to increased muscle damage and atrophy during flu infection with aging as well as other inflammatory leukocytes observed in our GO pathway results (Fig 5 and supplemental Table 1). Further analyses will determine the phenotype of these T cells and other leukocytes via flow cytometry or single cell RNA-Seq, as well as determining the etiology of the CXCL10/CXCR3 axis within skeletal muscle during flu. Understanding the molecular mechanisms that link flu infection and physical disabilities could lead to novel therapeutic discoveries to decrease post-infection disability with aging. In fact, other research into resident cell types of skeletal muscle has illuminated the existence of immune populations but chose to focus on other aspects of new progenitor cell populations [20]. Additionally, an alternate approach to examine these cells is to digest the muscle and perform flow cytometry as previously described [28, 29]. These leukocytes are necessary for skeletal muscle regeneration and signaling muscle precursor cell activation and differentiation, however, their prolonged presence and inflammation points to more fibrosis pathways.

In chapter 3, the infiltrating T cells have been identified in our microscopy by CD3+ cells and have not been further identified as CD4+ or CD8+ T cells. These cells would be easily identifiable via flow cytometry. The markers for CD4 and CD8 would quickly denote T cell subset, but activity could also be determined by expression of B cell leukemia/lymphoma 3 (BCL3), T cell specific GTPase 1 (Tgtp1), IL4rα, IL2rγ, V-set and immunoglobulin domain containing 4 (Vsig4) (all of which were observed in GO pathways for T cells in our experiment). Additionally, we can use granzyme staining to look for cytotoxic T cells, CD44hi cells as those being antigen experienced, CD69 in T cells following activation, downregulation of CD62L in activated T cells, and CD25 for regulatory T cells. Indeed, the literature supports that both CD4+ and CD8+ T cells are present in the skeletal muscle for various reasons. Diane Mathis’ group shows that CD8+ T cells have the potential aid in regeneration or cause inflammation to the environment. Similarly, subsets of CD4+ T cells secreting IFN-γ can cause harm to muscle
tissue repair, but CD4+ Tregs can limit IFN-γ production and quell inflammatory macrophages [30, 31, 32]. Similarly, single cell RNA-seq has shown the presence of both CD4+ and CD8+ T cells in skeletal muscle during homeostasis, but did not go into further detail [20].

To determine the role of infiltration and T cells during infection, we hope to use a skeletal muscle specific inducible knockout of CXCL10 mouse from Jackson Laboratory (B6.129S4-Cxcl10tm1Adl/J), which has impaired proliferation and IFN-gamma secretion following antigenic challenge. This mouse has reduced contact hypersensitivity compared to wildtype mice and are considered useful in studies of Th1-type inflammatory disease, chemokine biology, and T cell priming, proliferation, and trafficking. Through preventing T cell admission to the skeletal muscle, we would be able to tease out the role and impact of their presence in the muscle. If the T cells infiltrating the skeletal muscle were inflammatory, I expect to see improvement in the aged muscle histology, function, and decreases in inflammation. If these T cells were aiding in regeneration and dampening inflammation, then I would expect to see worsened pathology. However, our results suggest that these cells are pro-inflammatory and are responding to pro-inflammatory chemokines as well as potentially responsible for producing the pro-inflammatory cytokines (TNF, IFN-γ, IL-1β, and excessive IL-6) that remain elevated in aged mice past 20 DPI. Moreover, this conditional knockout model would determine if the difference in aged skeletal muscle recover is due to the infiltrating inflammatory cells. Through limiting the chemotaxis of inflammatory cells, which do not disappear in aging as they do in young mice, we hypothesize that aged skeletal muscle would not have prolonged inflammation or lasting disability.

Interestingly, there is a link between influenza and central and peripheral nervous system complications, which we highlight in chapter one of this thesis. Several events occur simultaneously in only aged cohorts of mice, which point to a new hypothesis that autoimmune reactive T cells are causing damage to neuromuscular junctions and demyelinating aged
skeletal muscle. While we do not directly examine aspects of nervous system damage, pain, or sensory reflex in our mice, several other key elements are all present. Our data shows mRNA and protein levels of CXCL10 to be upregulated in only aged mice, just before increased T cell presence via immunofluorescence. This increased cell traffic was observed at 9 and 11 DPI, which coincides with disrupted tissue architecture in H&E staining, fibrosis/collagen staining via Masson’s Trichrome, and decreased cross-sectional area of fast-twitch fibers (MyHC IIB atrophy). Further, our Gene Ontology (GO) pathway analysis showed that aged cohorts had downregulated axon development, downregulated axonogenesis, downregulated regulation of axonogenesis, increased regulation of apoptotic signaling pathways, extrinsic apoptotic signaling pathways, and more suggesting negative impacts to nervous system via external signals. In addition, aged cohorts exhibited GO pathway upregulation for T cell activation and leukocyte migration as early as 7 DPI, which lasted through 20 DPI with notable increases in inflammatory cytokines/chemokines in the muscle at 20 DPI. To confirm that this was not an affect of direct viral interaction, we performed flu RT-qPCR on skeletal muscle and found no evidence flu presence. Taken together with the prolonged loss of function in aged cohorts, which has been shown in the literature to be signs of denervation. Aged mice have significantly decreased abilities in grip strength and open field testing from 7-19 DPI (Fig 1). We also observed lasting alterations in gait kinematics of aged mice. We originally contributed these observations to loss of fast twitch fibers (chapter 3), but now have to consider the possibility that there is also denervation present. Based on this data, our hypothesis is that aged mice have upregulation of chemokines that call in autoreactive T cells, which causes the observed changes in gene expression and function because of the damage/disruption to neuromuscular junction and axons in the skeletal muscle tissue. More specifically, we observed inhibition of acetylcholinesterase (AChE) in aged muscle, which leads to accumulation of acetylcholine (Ach) in the synaptic cleft and results in impeded neurotransmission. Impeded neurotransmission such as this is observed in diseases such as myasthenia gravis, where the end plate potential
(EPP) fails to effectively activate the muscle fiber due to an autoimmune reaction against acetylcholine receptors, resulting in muscle weakness and fatigue [33, 34]. Impeded neurotransmission also causes destabilization of coordination and reduced muscle strength [35]. Normally, AchE terminates signal transduction at the neuromuscular junction by rapid hydrolysis of the Ach released into the synaptic cleft. Similarly, synaptotagmin IX (SYT9) is involved in Ca2+ dependent exocytosis of secretory vesicles through Ca2+ and phospholipid binding to the C2 domain and is also disrupted in aged muscle. Evidence of demyelination and autoimmunity comes from pathways associated with apolipoprotein D (ApoD), a hub gene in our analysis associated with neurological nerve injury especially related to the myelin sheath. According to the literature, muscle fibers continue to undergo atrophy after denervation, with collagen deposits forming in the endomysium and perimysium [36]. In our own studies, the gene expression we observed points to denervation occurring in aged cohorts simultaneously with collagen deposits described in our H&E and trichrome staining (chapter 3). As stated above, further studies are necessary to verify this hypothesis and these juxtaposed observations. However, the preliminary observations which point to this hypothesis cannot be ignored and if true have huge clinical ramifications.

Inflammaging is detrimental to many facets of the immune system and full body homeostasis. Using selective pharmaceutical/biochemical inhibitors to disrupt exorbitant and prolonged inflammation would theoretically restore/rejuvenate normal systems. As long as essential signaling pathways to influenza clearance are not disrupted, infection clearance will ideally continue as it normally would without blockers. For this reason, IL-6 cannot be targeted because it is essential to many systems including muscle homeostasis, stem cell activation, immune cell signaling, and most importantly in these circumstances, viral clearance [38]. Cyclooxygenase (COX)-1 and COX-2 are involved in pathways that are important in modulating immune responses, including body weight and temperature changes. COX-1 deficient mice have
enhanced inflammation and increased mortality with profound hypothermia and greater weight loss [38, 39]. COX-2 is associated with inflammatory responses and has increased expression with age-related and flu-induced manners; COX-2 specific inhibition attenuates inflammation and viral titers [38-47]. COX-2 upregulation leads to proinflammatory cytokines in the BAL and enhanced inflammation systemically; it also drives mice into hypothermic states and to lose weight with infection. Inhibiting COX-2 has proven to reduce mortality from infection, maintain weight with infection, and blunt inflammatory cytokine responses including G-CSF and TNFα [38-42]. Selective COX-2 inhibition with celecoxib reverses wasting and associated cachexia [45-47] and has even been given to geriatric hospital patients with acute inflammation from infectious disease to reduce muscle fatigue and improve mobility [45]. It is believed that COX-2 inhibition does not significantly modulate disease severity [39]. COX-2 along with TNFα and other proinflammatory cytokines were hyperinduced in epithelial cells and infected macrophages during flu infection [42]. In fact, COX-2 and TNFα have been shown to upregulate each other when once one is present. Inflammatory molecules, such as TNFα, increase in serum concentration with aging and activate NF-κB in differentiated skeletal muscle myotubes to induce protein degradation [48, 49]. Evidence supports TNFα receptor-mediated apoptotic events increase with age, especially in type II fiber-containing muscles [48]. By age 80 an estimated 30-40% of skeletal muscle fibers are lost [48]. TNFα blocking antibodies have been used in geriatric patients with rheumatoid arthritis and other inflammatory diseases to reduce inflammation and reduce muscle atrophy. TNFα exposure up-regulates Atrogin1/MuRF1 mRNA to mediate atrophy in myotubes in vitro within 2 hours of exposure [50, 51]. TNFα acts directly to stimulate loss of muscle protein, binding to the surface receptors to increase ROS activity with skeletal muscle fibers and upregulate NF-κB [49-52]. It is a catabolic cytokine which increases with age, especially in fast twitch fibers and in the serum [48]. TNFα-activated pathway negatively regulates satellite cells number and expression/differentiation in regenerating muscles [51]. Neutralizing antibodies against TNFα such as infliximab have been successful in
reducing systemic levels of TNFα and promote muscle regeneration [53, 54]. Infliximab is a monoclonal anti-TNFα antibody that binds specifically to TNFα with high affinity binding; it is chimeric composed of both human and mouse proteins and given as an intravenous infusion to humans [53]. It is our goal to halt the myopathy and damage done to muscle by inhibiting these known pathways for muscle degradation during influenza infection. Since we are not able to block major cytokines and inflammatory pathways without also impairing the host immune response, we chose COX-2 and TNFα to inhibit as they do not have huge roles in clearance of influenza virus. We hypothesize that by decreasing flu-inflammation we can improve the molecular and functional skeletal muscle pathways normally disrupted in aging/flu-infection. With inflammaging, it is more likely for the elderly to scar or develop fibrosis in tissues, rather than regenerate lost muscle; therefore, it is imperative to preserve muscle mass, limit lung damage, and reduce flu-associated pathologies.

COX-2 upregulation leads to increased vasodilation, vascular permeability, cytokine release, leukocyte migration, and pain. Inflammation from COX-2 originates in macrophages, fibroblasts, and endothelial cells. Our studies have shown TNFα expression as well as mRNA expression of ubiquitin proteasome degradation genes were elevated and prolonged in aged mice when compared to young during influenza infection [5]. In combination with exaggerated and prolonged chemokine expression CXCR3 and CXCL10, skeletal muscle had the potential for increase immune cell recruitment [5]. COX-2 expression in macrophages is elevated with aging, which increases production of a degradation inducing and T cell suppressor protein, prostaglandin E2 (PGE2) [43,55,56]. Since COX-2 is elevated with infection and age-association it contributes to age-related immune dysregulation. Infliximab treatment increased number of PAX7 positive cells in muscle, which were otherwise suppressed by increased TNFα expression [51]. These data give us a potential molecular link between influenza infection and disability. Changes in gene expression come from mechanism(s) which we hypothesize arise
from exaggerated and prolonged flu-induced inflammation, which skeletal muscle is sensitive to and begins to breakdown. Data supports that TNFα-/- mice were able to survive and clear influenza without problem [57] and that acute in vivo administration of TNFα has been shown to impair amino acid uptake by muscle [58]. The blockade of TNFα with infliximab does not impair plasma cell differentiation [59] and has shown faster decline of viral titers in patients [46]. Altogether, these data support the use of biochemical intervention with the goal of potential therapies for the elderly to alleviate their influenza-induced ailment/decline.

Preliminary data [43] reveals age-associated increase in skeletal muscle COX-2 expression. In the event that neither treatment is effective, we can assume that adding the drugs may have triggered other compensatory cytokine/chemokine pathways that might be mechanistically driving the breakdown of muscle fibers. It is possible that inhibition of TNFα and COX-2 needs to be administered locally to the site of infection (lung) by intranasal inoculation to reduce the flu-induced pathology [61]. There is an increased risk when blockading TNFα for secondary bacterial infection and less effective vaccination through T-cell mediated pathways. While this is not a concern for mice in our clean facility, it is very dangerous for elderly human populations, which is why it is advised to vaccinate for influenza and pneumonia before undergoing treatment for assurance of better vaccination [53]. A more dangerous result deals with the supply and demand for amino acids and energy to fight the infection. If the drugs work "too well", it will stop the flow of “rescue” amino acids from the skeletal muscle and there is no supply of amino acids to the lungs for repairing flu-damage, as well as less energy/resources for the immune response. In this circumstance, we would characterize which pathways are essential and cannot be stopped during the course of infection through process of elimination; but raises the question that if we supplied an additional source of amino acids (not from the skeletal muscle) if the problem would be remedied. An alternative to these therapies would be combining lower dose treatment of both anti-TNFα and COX-2 inhibition to block multiple pathways, but less
aggressively to reduce the observed exaggerated inflammation to average levels. The point of these experiments is to preserve muscle mass and maintain functionality in aging throughout infection. This is a critical step forward because these mechanisms could generate pointed therapies and development of preventative measures to ensure the maintenance of elderly muscle strength and function.

In summary, we have identified many pathways and genes changing during the response to flu, which illuminate potential mechanisms of aged skeletal muscle atrophy, disability, and impaired recovery. Overlapping genes in pathways, denoted as hub genes, can be considered potential mechanisms for flu-induced muscle atrophy and degradation. We identified a list of such genes, which serve as the potential future hypotheses for causality of skeletal muscle atrophy and degradation as well as hypotheses for the lingering effects in aged muscle (Table 2). These genes focus on several key aspects of skeletal muscle health and regeneration and are vital to finding therapeutic strategies for combating muscle disability and degradation in aging. We demonstrated the presence of T cell leukocytes in aged skeletal muscle, which are potentially responsible for inflammation, fibrosis, and even denervation in GO pathway analyses. Our proposed future experiments aim to target chemotaxis of inflammatory cells, directly inhibit inflammatory factors such as IFN-γ and TNF-α, and bolster protection of aged skeletal muscle. The studies in this thesis are the first to examine in depth how flu impacts skeletal muscle with aging and leads to muscle dysfunction and disability. While many questions remain, this research has laid the ground work for others to test pathways/genes as mechanism(s) of flu-induced muscle atrophy with aging as the first step of developing prevention and treatment options. Indeed, development of prophylactic and therapeutic treatments for flu-induced myopathies could save lives of countless older adults, prevent catastrophic disability, and increase overall resilience and healthspan.
AUTHOR CONTRIBUTIONS

Spencer R. Keilich (SRK), Darcy T. Ahern (DTA), Justin L. Cotney (JLC), Jenna M. Bartley (JMB), and Laura Haynes (LH) conceived and designed the experiments. SRK carried out experiments. SRK and DTA analyzed data. SRK led data interpretation and manuscript preparation. JMB and LH supervised the project and assisted with data interpretation and acquisition of funding. All authors discussed the results and contributed to the final manuscript.

ACKNOWLEDGMENTS

We thank Sandra Jastrzebski for her assistance with experimental assays. We acknowledge Darcy Ahern, Justin Cotney, and Vijender Singh for their mentorship and guidance with computational analysis and bioinformatics. We acknowledge Cara Hardy for her help with the profusion of mice for the RNA-sequencing experiment.

REFERENCES


3. Ferrucci L, Guralnik JM, Pahor M, Corti MC and Havlik RJ. Hospital diagnoses, Medicare charges, and nursing home admissions in the year when older persons become severely disabled. JAMA. 1997;277(9):728-734.


35. Shi, Yun, Maxim V. Ivannikov, Michael E. Walsh, Yuhong Liu, Yiqiang Zhang, Carlos A. Jaramillo, Gregory T. Macleod, and Holly Van Remmen. "The lack of CuZnSOD leads to impaired neurotransmitter release, neuromuscular junction destabilization and reduced muscle strength in mice." PLoS One 9, no. 6 (2014).


## Table 2. Target genes for future hypothesis testing

<table>
<thead>
<tr>
<th>Gene</th>
<th>Ensemble ID</th>
<th>Brief Description</th>
<th>Interpretation of Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>MURF-1 (TRIM63)</td>
<td>ENSMUSG 00000028834</td>
<td>tripartite motif-containing 63; E3 ubiquitin-protein ligase enzyme that upregulates during muscle atrophy</td>
<td>major substrate is myosin heavy chain, known to be upregulated during denervation, immobilization, and trauma</td>
</tr>
<tr>
<td>Atrogen-1 (fbxo32)</td>
<td>ENSMUSG 00000116076</td>
<td>F-box protein 32; one of the four subunits of the ubiquitin protein ligase complex, which is highly expressed during muscle atrophy</td>
<td>its upregulation signifies that skeletal muscle is undergoing atrophy</td>
</tr>
<tr>
<td>UBB</td>
<td>ENSMUSG 0000019505</td>
<td>ubiquitin-B; targets cellular proteins for degradation</td>
<td>upregulation indicates skeletal muscle is atrophying and degrading internal proteins via proteasome</td>
</tr>
<tr>
<td>UBC</td>
<td>ENSMUSG 0000008348</td>
<td>ubiquitin-C; associated with protein degradation, DNA repair, cell cycle regulation, kinase modification, endocytosis, and regulation of other cell pathways</td>
<td>upregulation indicates skeletal muscle is atrophying and degrading internal proteins via proteasome</td>
</tr>
<tr>
<td>NLRP3</td>
<td>ENSMUSG 0000032691</td>
<td>NLR family, pyrin domain containing 3; signals downstream to NF-κB and plays a role in regulating inflammation and apoptosis</td>
<td>Downregulated by ApoD; NLRP3 inflammasome plays role in muscle inflammation; potentially activated by RNA viral particles</td>
</tr>
<tr>
<td>CCR2</td>
<td>ENSMUSG 0000049103</td>
<td>chemokine (C-C motif) receptor 2; expressed on Th1 cells, Th17 cells, macrophages, and microglia</td>
<td>Binds to CCL2(MCP-1), CCL7(MCP-3), CCL8, CCL13(MCP-4), and CCL16 ligands</td>
</tr>
<tr>
<td>CCR5</td>
<td>ENSMUSG 0000079227</td>
<td>chemokine (C-C motif) receptor 5; expressed on macrophages, dendritic cells, and memory T cells</td>
<td>Binds to MIP1α, MIP1β, RANTES, MCP-2 ligands</td>
</tr>
<tr>
<td>CCL2</td>
<td>ENSMUSG 0000035385</td>
<td>chemokine (C-C motif) ligand 2; produced primarily by monocytes, macrophages, and dendritic cells</td>
<td>Chemotactic activity for monocytes and basophils with CCR2 and CCR4 receptors</td>
</tr>
<tr>
<td>CCL5</td>
<td>ENSMUSG 0000035042</td>
<td>chemokine (C-C motif) ligand 5; also known as RANTES; produced by macrophages, DCs, lymphocytes, and endothelial cells</td>
<td>Interacts with CCR3, CCR5, CCR1, and GPR75</td>
</tr>
<tr>
<td>CCL6</td>
<td>ENSMUSG 0000018927</td>
<td>chemokine (C-C motif) ligand 6; expressed in neutrophil and macrophages</td>
<td>CCL6 is the chemokine for CCR1 cells</td>
</tr>
<tr>
<td>CCL7</td>
<td>ENSMUSG 0000035373</td>
<td>chemokine (C-C motif) ligand 7; also known as MCP3, produced by macrophages</td>
<td>attracts monocytes and regulates macrophage function</td>
</tr>
<tr>
<td>CCL8</td>
<td>ENSMUSG 0000009185</td>
<td>chemokine (C-C motif) ligand 8; also known as MCP2, and binds with CCR1, CCR2B, CCR3, and CCR5</td>
<td>Chemotactic for and activates mast cells, eosinophils, basophils, monocytes, T cells,</td>
</tr>
<tr>
<td>Gene Symbol</td>
<td>ENSMUSG ID</td>
<td>Description</td>
<td>Function</td>
</tr>
<tr>
<td>-------------</td>
<td>------------</td>
<td>-------------</td>
<td>----------</td>
</tr>
<tr>
<td>CCL12</td>
<td>ENSMUSG 00000035352</td>
<td>chemokine (C-C motif) ligand 12; known as MCP-5</td>
<td>Attracts eosinophils, monocytes and lymphocytes</td>
</tr>
<tr>
<td>CCL17</td>
<td>ENSMUSG 0000031780</td>
<td>chemokine (C-C motif) ligand 17</td>
<td>Chemokine specifically binds and elicits chemotaxis in T cells and interacts with CCR4</td>
</tr>
<tr>
<td>Aif1</td>
<td>ENSMUSG 00000024397</td>
<td>allograft inflammatory factor 1; found in activated macrophages within inflammation</td>
<td>Responds to IFN-γ, IL-1β, and responsible for arterial thickening; decrease glucose secretion and impairs glucose elimination</td>
</tr>
<tr>
<td>IL7r</td>
<td>ENSMUSG 0000003882</td>
<td>interleukin 7 receptor; expressed on T cells, B cells, monocytes, and dendritic cells</td>
<td>Responds to IL-7</td>
</tr>
<tr>
<td>BCL3</td>
<td>ENSMUSG 0000053175</td>
<td>B cell leukemia/lymphoma 3; signals through NFκB</td>
<td>Pro-survival and pro-inflammatory gene expression in T cells</td>
</tr>
<tr>
<td>IL-1β</td>
<td>ENSMUSG 0000027398</td>
<td>interleukin 1, beta; highly proinflammatory cytokine produced by multiple cell types, epithelial cells, macrophages, dendritic cells, and B cells</td>
<td>Proinflammatory, part of the NLRP3 inflammasome process and contributes to pain/hypersensitivity</td>
</tr>
<tr>
<td>IL-6</td>
<td>ENSMUSG 0000025746</td>
<td>interleukin 6; stimulates satellite cell activation, can be secreted by macrophages in response to PAMPs or TLR activation; supports growth of B cells</td>
<td>IL-6's role as an anti-inflammatory myokine is mediated through its inhibitory effects on TNF-α and IL-1, and activation of IL-10 and IL-1ra; in muscle cells IL-6 triggers MAPK pathways</td>
</tr>
<tr>
<td>IL-6ra</td>
<td>ENSMUSG 0000027947</td>
<td>interleukin 6 receptor, alpha; CD126, related to signal transducer for several cytokines including LIF, oncostatin M, CNTF, IL-11, IL-27</td>
<td>Important to sensing inflammation and regulating response to inflammation; promotes survival of neurons and neuron outgrowth</td>
</tr>
<tr>
<td>Sparc</td>
<td>ENSMUSG 0000018593</td>
<td>secreted acidic cysteine rich glycoprotein; aka Osteonectin, induces apoptosis, glycoprotein that binds calcium</td>
<td>Cell matrix interactions and collagen binding are dependent on Sparc</td>
</tr>
<tr>
<td>CXCL2</td>
<td>ENSMUSG 0000058427</td>
<td>chemokine (C-X-C motif) ligand 2; MIP2-α, binds CXCR2</td>
<td>Secreted by monocytes and neutrophils at sites of inflammation</td>
</tr>
<tr>
<td>CXCL10</td>
<td>ENSMUSG 0000034855</td>
<td>chemokine (C-X-C motif) ligand 10; proinflammatory conditions secreted by leukocytes, activated neutrophils, eosinophils, monocytes, epithelial cells, endothelial cells, stromal cells, fibroblasts, and keratinocytes in response to IFN-γ</td>
<td>Binds to cells with CXCR3 and is a chemokine for T lymphocytes, NK cells, and pro-inflammatory cell types</td>
</tr>
<tr>
<td>Gene</td>
<td>Ensembl ID</td>
<td>Protein Name</td>
<td>Description</td>
</tr>
<tr>
<td>------</td>
<td>------------</td>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>CXCL13</td>
<td>ENSMUSG00000023078</td>
<td>Chemokine (C-X-C motif) ligand 13; secreted by follicular dendritic cells</td>
<td>Binds with CXCR5 and attracts B cells</td>
</tr>
<tr>
<td>Col1a1</td>
<td>ENSMUSG0000001506</td>
<td>Collagen, type I, alpha 1; fibril-forming collagen</td>
<td>Cross-links result in the formation of very strong mature type 1 collagen fibers</td>
</tr>
<tr>
<td>Col1a2</td>
<td>ENSMUSG00000029661</td>
<td>Collagen, type I, alpha 2; fibril-forming collagen</td>
<td>Cross-links result in the formation of very strong mature type 1 collagen fibers</td>
</tr>
<tr>
<td>KLF10</td>
<td>ENSMUSG00000037465</td>
<td>Kruppel-like factor 10; induced by TGF-β</td>
<td>Promotes TGF-β, apoptosis, and related to fibrosis</td>
</tr>
<tr>
<td>Act1</td>
<td>ENSMUSG00000068614</td>
<td>Actin, alpha, cardiac muscle 1; blood vessels and related to z-lines of the sarcomere</td>
<td>The skeletal muscle isoform is ACTA1, needed for growth and formation of muscle tissue</td>
</tr>
<tr>
<td>Fbn2</td>
<td>ENSMUSG00000024598</td>
<td>Fibrillin 2; elastic fibers found in connective tissue, secreted into ECM by fibroblasts</td>
<td>Microfibrils are composed end-to-end polymers of fibrillin</td>
</tr>
<tr>
<td>TLR9</td>
<td>ENSMUSG00000045322</td>
<td>Toll-like receptor 9; expressed on dendritic cells, macrophages, NK cells, and more</td>
<td>Binds to RNA viruses or particles</td>
</tr>
<tr>
<td>TNF</td>
<td>ENSMUSG00000024401</td>
<td>Tumor necrosis factor; systemic inflammation and involved in acute phase reaction, primarily produced by macrophages, CD4+ lymphocytes, NK cells, neutrophils, mast cells, eosinophils, and neurons</td>
<td>Causes apoptotic cell death, cachexia, inflammation, and can lead to sepsis</td>
</tr>
<tr>
<td>UBD</td>
<td>ENSMUSG0000035186</td>
<td>Ubiquitin-D</td>
<td>Upregulation of this gene may promote inflammation in chronic kidney disease; 26s proteasome degradation</td>
</tr>
<tr>
<td>Tgtp1</td>
<td>ENSMUSG00000078922</td>
<td>T cell specific GTPase 1; involved in innate cell-autonomous resistance to intracellular pathogens, induced by IFN-γ</td>
<td>T cells responding to IFN-γ</td>
</tr>
<tr>
<td>GBP5</td>
<td>ENSMUSG00000105504</td>
<td>Guanylate binding protein 5; activator of NLRP3 inflammasome assembly and has a role in innate immunity and inflammation</td>
<td>Pro-inflammatory response to IFN-γ and activation of the inflammasome</td>
</tr>
<tr>
<td>IL4ra</td>
<td>ENSMUSG00000030748</td>
<td>Interleukin 4 receptor, alpha; CD124, binds to IL-4 and IL-13 to regulate IgE antibody production in B cells. Promotes Th2 differentiation</td>
<td>T/B cells/lymphocytes in the muscle</td>
</tr>
<tr>
<td>IL2rg</td>
<td>ENSMUSG00000031304</td>
<td>Interleukin 2 receptor, gamma chain; CD132, surface of immature leukocytes/lymphocytes, directs the growth and maturation of T cells, B cells and NK cells</td>
<td>T/B/NK cells/lymphocytes in the muscle; these cells kill viruses, make antibodies and help regulate immune system</td>
</tr>
<tr>
<td>Gene</td>
<td>ENSEMBL ID</td>
<td>Description</td>
<td>Expression and Function</td>
</tr>
<tr>
<td>------</td>
<td>------------</td>
<td>-------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>Corol1a</td>
<td>ENSMUSG00000030707</td>
<td>Coronin, actin binding protein 1A; actin binding protein that interacts with microtubules and is associated with phagocytosis</td>
<td>Leukocyte markers; this will denote phagocytic inflammatory cells in the skeletal muscle</td>
</tr>
<tr>
<td>Vsig4</td>
<td>ENSMUSG00000044206</td>
<td>V-set and immunoglobulin domain containing 4; negative regulator of T cell responses; or a receptor for the C3b and iC3b complement components</td>
<td>Leukocyte markers; this denotes limitation and reduction of T cell responses</td>
</tr>
<tr>
<td>Adam8</td>
<td>ENSMUSG00000025473</td>
<td>A disintegrin and metallopeptidase domain 8; membrane-anchored proteins related to disintegrins used in muscle development</td>
<td>Leukocyte markers; may be involved in neurodegeneration, but also muscle development</td>
</tr>
<tr>
<td>Lgals3</td>
<td>ENSMUSG00000050335</td>
<td>Lectin, galactose binding, soluble 3; involved with cell-cell adhesion, cell-matrix interactions, macrophage activation, angiogenesis, metastasis, apoptosis</td>
<td>Granulocyte markers; a correlation between Lgals3 and fibrosis is found; high levels in cardiovascular disease; related to stroke</td>
</tr>
<tr>
<td>Itgam (CD11b)</td>
<td>ENSMUSG00000030786</td>
<td>Integrin alpha M; or CD11b, surface marker on leukocytes involved in innate immune system mediating inflammation via leukocyte adhesion and migration</td>
<td>Granulocyte markers; phagocytosis, cell-mediated cytotoxicity, chemotaxis, and cellular activation of inflammatory leukocytes</td>
</tr>
<tr>
<td>Fcgr3</td>
<td>ENSMUSG00000059498</td>
<td>Fc receptor, IgG, low affinity III; neutrophil marker and Fc receptor</td>
<td>Granulocyte markers; denotes neutrophil activity</td>
</tr>
<tr>
<td>Mstn</td>
<td>ENSMUSG00000026100</td>
<td>Myostatin; GDF-8; produced and released by myocytes that act on muscle cells' autocrine function to inhibit myogenesis</td>
<td>Associated with muscle wasting, muscular dystrophy, and muscle hypertrophy through Akt inhibition</td>
</tr>
<tr>
<td>Ddit4</td>
<td>ENSMUSG0000020108</td>
<td>DNA-damage-inducible transcript 4; negative regulator of mTOR involved in autophagy, upregulated in response to hypoxia DNA damage, or energy stress</td>
<td>Response to virus; this is a pro-autophagy response to damage in the cell</td>
</tr>
<tr>
<td>Nlrc5</td>
<td>ENSMUSG0000074151</td>
<td>NLR family, CARD domain containing 5; innate immunity to viruses and potentially by regulating interferon activity</td>
<td>Response to virus in T, NK, and NKT lymphocytes; pro-inflammatory &amp; inflammasome activity</td>
</tr>
<tr>
<td>Rnf216</td>
<td>ENSMUSG0000045078</td>
<td>E3 ubiquitin-protein ligase ring finger protein 216 interacts with serine/threonine protein kinase, receptor-interacting protein. Attaches ubiquitin</td>
<td>Response to virus with proteasome activity and ubiquitination of cell components, inhibition of TNF and IL1 induced NFκB activation</td>
</tr>
<tr>
<td>F7</td>
<td>ENSMUSG0000031443</td>
<td>Coagulation factor VII; one of the proteins that causes blood to clot in the coagulation cascade</td>
<td>Involved in pathways for regeneration/myo-differentiation</td>
</tr>
<tr>
<td>Gene</td>
<td>ENSMUSG</td>
<td>Function and Pathways</td>
<td></td>
</tr>
<tr>
<td>------</td>
<td>---------</td>
<td>-----------------------</td>
<td></td>
</tr>
<tr>
<td><strong>Mymk</strong></td>
<td>0000009214</td>
<td>myomaker, myoblast fusion factor; positive regulation of skeletal muscle hypertrophy, myoblast fusion involved in skeletal muscle regeneration</td>
<td>positive regulator of skeletal muscle regeneration/myo-differentiation</td>
</tr>
<tr>
<td><strong>Mustn1</strong></td>
<td>0000042485</td>
<td>musculoskeletal, embryonic nuclear protein 1; involved in wound healing, tissue regeneration</td>
<td>pan-musculoskeletal cell marker and regulatory gene in myogenic and chondrogenic lineages</td>
</tr>
<tr>
<td><strong>Mki67</strong></td>
<td>0000031004</td>
<td>antigen identified by monoclonal antibody Ki 67; evidence of cell proliferation</td>
<td>seen in regeneration/myo-differentiation</td>
</tr>
<tr>
<td><strong>ApoD</strong></td>
<td>0000022548</td>
<td>apolipoprotein D; associated with neurological nerve injury especially related to myelin sheath</td>
<td>involved in pathways for regeneration/myo-differentiation</td>
</tr>
<tr>
<td><strong>Cdk1</strong></td>
<td>0000019942</td>
<td>cyclin-dependent kinase 1 functions as serine/threonine kinase and is a key cell cycle regulator</td>
<td>associated with regeneration/myo-differentiation</td>
</tr>
<tr>
<td><strong>MyoG</strong></td>
<td>0000026459</td>
<td>myogenin; muscle specific basic helix loop helix transcription factor involved in the coordination of skeletal muscle development, myogenesis, and repair</td>
<td>centric in the regeneration/myo-differentiation of skeletal muscle</td>
</tr>
<tr>
<td><strong>Fn1</strong></td>
<td>0000026193</td>
<td>fibronectin 1 binds ECM proteins such as collagen, fibrin, and heparan sulfate proteoglycans</td>
<td>Altered fibronectin expression, degradation, and organization is associated with pathologies like fibrosis and cachexia</td>
</tr>
<tr>
<td><strong>SYT9</strong></td>
<td>0000062542</td>
<td>synaptotagmin IX; involved in Ca2+ dependent exocytosis of secretory vesicles through Ca2+ and phospholipid binding to the C2 domain</td>
<td>Neuron/axon/synapse pathways of neuromuscular junction</td>
</tr>
<tr>
<td><strong>Ache</strong></td>
<td>0000023328</td>
<td>acetylcholinesterase; terminates signal transduction at the neuromuscular junction by rapid hydrolysis of the acetylcholine released into the synaptic cleft</td>
<td>Neuron/axon/synapse at the neuromuscular junction; inhibition of AChE leads to accumulation of Ach in the synaptic cleft and results in impeded neurotransmission</td>
</tr>
</tbody>
</table>
CHAPTER FIVE

Discussion

The immune system has many intricate pieces that come together like an elegant mosaic. When all of the parts are fully operative, the system functions properly, protecting the host from disease. When one of the pieces malfunctions, compensatory mechanisms can often overcome the deficit. Occasionally, this is not the case and a singular problem can cause cascading downstream effects. In the case of the aged immune system, with many broken or declining parts, it becomes incredibly difficult to follow which are the mediators of the problem and which are simply the downstream consequence. Only by increasing our understanding of what changes are occurring in the aged immune system we can begin to dissect causality and consequences. This thesis, though primarily descriptive in nature, progresses our understanding of the aged immune system at the intersection of skeletal muscle biology and influenza infection.

We used C57BL/6 mice as our widely accepted biomedical research model of aging. Inbred laboratory mice have a much shorter lifespan of about 24 months [1] compared to the average United States citizen at 78.8 years [2]. Mice reach sexual maturity and are analogous to human adults around 8-12 weeks of age [3]. Mice are considered aged or “older adults” starting at roughly 18 months [4]. One problem that is becoming more widely considered and published in the immunology community with the laboratory mouse model is cleanliness and exposure to environmental antigens [5]. Our laboratory mice live in specific pathogen free (SPF) conditions and are thusly kept from pathogen exposure until purposeful experimental introduction. The lack of exposure to specific pathogens has been shown to alter the formation and function of the
immune system, which puts into question the comparability of SPF mice to human immune systems [6]. Chronic pathogens like cytomegalovirus (CMV) dramatically alter the immune system and are completely neglected in SPF mouse models while 50% of U.S. adults have CMV [7]. Currently, several groups are working to create mouse pathogen exposure cocktails and models to more adequately represent the human immune system [5, 6, 8]. These models could potentially be applied to the aging mouse model to enhance its clinical relevance.

The C57BL/6 mouse model is a powerful immunological tool because of the MHC Class I and Class II reagents and T cell receptor transgenic strains available to examine antigen-specific T cell responses [9], but it lacks genetic diversity. To overcome inbred models and recapitulate human genetic diversity, other mouse models can be employed such as diversity outbred mice, collaborative cross mice, and four-way cross mice [10-12]. Several of these are currently used in models of aging systems [4, 10] and collaborative cross mice were successfully used in a sequencing experiment to determine genetic variance of survival genes with Ebola virus [13]. Despite the shortcomings of inbred mouse models such as the C57BL/6 strain used pervasively in this thesis, it represents a widely accepted methodology and good starting point for studying the aged immune system. More complex models require further validation and development of reagents before they can become as useful tools for scientific studies.

We live in an aging world, where the burden on world healthcare is increasing rapidly with the increases in the vulnerable aging population. Older adults make up 90% of flu-related deaths and are at increased risk of both progressive and catastrophic disability following flu infection [14, 15, 16]. With these clinical needs in mind and the aforementioned models above, we set out to investigate the molecular mechanism of flu-induced muscle degradation and disability. In chapter two of this thesis, we established the molecular link between pulmonary infection and skeletal muscle. Equally important, we provided evidence that influenza RNA could not be detected in skeletal muscle via PCR nor were primary myoblasts from young or aged mice able
to be infected with our A/PR/8/34 (PR8) influenza virus \textit{in vitro} [17]. In summary, this chapter was the first to identify in a controlled experimental setting flu-induced muscle inflammation and atrophy alongside functional impairment. We have demonstrated that key inflammatory signals, and key ubiquitin proteasome components, both atrogin1 and MuRF1, as well as ubiquitin B and ubiquitin C, are upregulated. As it is known that muscle repair is diminished with aging, it is likely these muscle losses are not easily recoverable. Thus, laying the ground work for the next portion of my thesis project to target these pathways to prevent flu-induced atrophy and potential loss of quality of life in older adults.

The aim of chapter three was to determine if prior immunity reduces flu-induced skeletal muscle decrements. We demonstrated that prior non-neutralizing immunity, induced by vaccination with recombinant influenza nucleoprotein, prevents flu-induced muscle fast twitch fiber atrophy and consequently protects muscle functionality. We also determined that vaccine-induced protection is not mediated by antibody; however, more research is necessary to determine if protection is solely mediated via a T cell dependent mechanism. As disability is one of the major complications of flu infection in older adults, the goal of this study was to determine if prior non-neutralizing immunity could provide protection to skeletal muscle. We determined that vaccination indeed could prevent or reduce muscle decrements due to flu infection despite reduced vaccine efficacy with aging. Thus, flu vaccination is still an essential part of protecting older adults from flu-induced disability. Chapter three expanded on chapter two's discoveries in that similar parameters were tested with regard to muscle function, cytokine secretion, and muscle gene expression, but added in additional facets of immunohistochemistry. The immunohistochemistry highlighted the vulnerability of MyHC IIB fibers to inflammation during flu, especially in aging. Evidence confirms many inflammatory factors such as TNF-α receptor-mediated apoptotic events increase with age, especially in type II fiber-containing muscles [18].
This significant loss in fast twitch muscle fibers pointed to a measurable, functional and molecular change that can be targeted for therapeutic intervention.

Although the mechanism of influenza-induced myopathy is still to be determined, the research in this thesis has significantly progressed our understanding of many aspects of skeletal muscle decrement. We first determined that despite lack of direct flu infection, skeletal muscle still experienced atrophic gene expression which was prolonged and heightened in aged mice in chapter two. We determined, in chapter three, that the muscle specific atrophy observed in chapter two occurred primarily in MyHC type IIB muscle fibers. To complement this data, we confirmed that aged muscle had fibrosis formations at 9 DPI (via trichrome stain) and that aged muscle H&E’s revealed increased nuclei and architectural disruption across infection. These findings supported the hypothesis that the mechanism of muscle decrement could be from inflammatory cellular infiltration, which remains prolonged and elevated in aged mice. Further confirmation of this hypothesis came from our pathway analysis of skeletal muscle mRNA-Sequencing in chapter four, which highlighted chemotaxis of inflammatory cell types, activation of inflammatory cells, and secretory pathways specific to catabolic/inflammatory processes. These transcriptomic profiles were verified with immunofluorescence in young and aged skeletal muscle. We determined and quantified the presence of skeletal muscle T cells (CD3+ CD45+ Lyve1- CD31-) and leukocytes/lymphocytes (CD3- CD45+ Lyve1- CD31-), which showed that the only increases in these cell populations was in aged mice later in infection (11 and 15 DPI). This occurred concomitantly with disrupted architecture, fibrosis, and increased nuclei in aged muscle IHC staining. Taken together, there is much support for the theory that inflammatory cell types in the aged muscle are pervasively secreting inflammatory factors, which lead to more muscle damage and prevent the regeneration of aged muscle, and perpetuate the fibrosis and ECM dysregulation observed in our findings.
More experiments are necessary to determine functionally active cell populations within the skeletal muscle tissue. Other groups have attempted to identify new populations of cells in skeletal muscle via flow cytometry [19, 20], but this method is restricted by how many markers are used and biased by what markers are selected. Indeed, the best approach would be to use single cell RNA-sequencing of skeletal muscle tissue and cellular composition [21], which would result in infinitely more information. Neutrophils and macrophages are normally seen traversing inflamed skeletal muscle as a means of clearing debris and maintenance, but even regulatory T cell populations have been sited controlling inflammation [22]. Immune cell infiltration of muscle, despite lack of direct infection, occurs during flu infection because of the increase of chemotaxis receptors which attract/summon cells to the tissue [23, 24, 25, 26]. Muscle is particularly “sensitive” to changes in body homeostasis and it is known to secrete inflammatory mediators after exercise for repair/energy replenishment and is especially reactive to serum mediated inflammatory signaling [15, 17, 27, 28, 29, 30, 31]. This sensitivity may be an evolutionary mechanism for the release of amino acids from muscle breakdown to aid in reparation efforts elsewhere in the body. In fact, we hypothesize that influenza-induced weight loss is primarily due to skeletal muscle atrophy, but this was not confirmed via NMR/DEXA/MRI. The exact mechanisms involved are still unknown, however the morphological and molecular changes in muscle are evidence that mechanism(s) of cell infiltration, systemic inflammation, and/or flu-induced inflammation from lung infection [17, 24, 32, 33, 34].

Healthy aging is associated with an increased basal rate of muscle protein synthesis in women compared to men, and resistance to the anabolic effect of nutritional stimuli in both sexes [32]. There is sexual dimorphism in age-related skeletal muscle protein synthesis and metabolism responsible for age-related decline in muscle mass, which leaves women at a greater risk of disability than men. Average percent of muscle mass lost between the ages of 70 (W: 22%, M: 15%) and 90 (W: 81%, M: 57%) is much greater for women [35]. From an immunological
standpoint, women have more robust inflammatory responses to illness than men, but develop better antibody titers and long-term protection [36]. It is important to examine the sex-differences and model them appropriately, however, since we are still trying to discover the underlying mechanisms and pathways involved, we have matched our previous experiments and continued our research on male mice. It is well documented that there are sex differences in muscle responses to unloading [33, 37, 38] and hypertrophy [39, 40] and exercise-induced muscle damage [24, 41, 42]. These are likely due to endocrine differences, such as testosterone and estrogen, molecular signaling changes [32, 43] and other sex-related differences [34]. Clinically, sex differences in physical function and disability exist with aging, with higher disability rates in women [35, 44, 45]. Thus, the first set of transcriptomic analyses were performed on male mice for this thesis. Further experiments in the Haynes laboratory will be performed in female mice to determine relevant sex differences. This will be particularly useful to gain understanding of translationally relevant and clinically meaningful differences in resiliency and susceptibility to disability between sexes with aging.

The studies in this thesis are the first to examine in depth how influenza induces skeletal muscle degradation in aging, but many questions remain to be answered. Since skeletal muscle is still beginning to be appreciated for the role it plays in immune responses, it is not surprising that many aspects of myokines, muscle biology, and amino acid storage remain to be elucidated. This research has laid the ground work for others to further characterize and test pathways/genes as mechanism(s) of flu induced muscle degradation and dysfunction in aging. The findings in chapter 4 present multiple targets that could be used to enhance and protect skeletal muscle in older adults. This research has significantly progressed our understanding of the aged immune response to flu-induced myopathy. This thesis has contributed to our understanding of how specific fiber-types are more prone to atrophy during infection and how vaccination prevents protects aged muscle from this ailment. Foundationally, these studies have
culminated into a hypothesis generating transcriptomic project, which has painted a fully unbiased look into aged muscle processes during flu infection. The work following this thesis has the potential to develop future treatments that could help save lives of countless older adults and prevent catastrophic disability.

REFERENCES

3. Pinter, O., Beda, Z., Csaba, Z. & Gerendai, I. Differences in the onset of puberty in selected inbred mouse strains. (2007).


16. Ferrucci L, Guralnik JM, Pahor M, Corti MC and Havlik RJ. Hospital diagnoses, Medicare charges, and nursing home admissions in the year when older persons become severely disabled. JAMA. 1997; 277(9):728-734.


