Influenza Virus Infection: The Impact of Physical Activity on the Aging Immune System and Obesity-Associated Immune Impairments

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Influenza virus infection: The impact of physical activity on the aging immune system and obesity-associated immune impairments

by

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A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

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The role exercise plays in the modulation of the immune response has been a topic of interest to exercise immunologists for the last two to three decades. Some important issues that remain to be addressed include exercise-training induced adaptations of immune response, along with the effect of exercise “dose” on immune function. Results from multiple studies have determined that a high dose of exercise to exhaustion causes immune impairments and a poor infectious disease outcome in animal models. A moderate dose of exercise appears to improve disease outcome to infection. However, the mechanisms by which different doses of exercise affect infectious disease outcome remain to be clearly elucidated. In this dissertation, we used regular moderate exercise as our dose in comparison to no exercise (sedentary controls) to assess alterations in the immune response to influenza virus infection. Studies focused on the impact of moderate exercise training on 1) long-term primary antibody response and secondary antibody response to influenza challenge, 2) resident and recruited memory cell response in the lungs, 3) antigen-presenting cell (APC) function in young and old mice, and 4) immune response to influenza in obese mice. The primary topic of this dissertation was the effect of exercise on immunity. However, there was also an emphasis on the topic of aging and immunity. The results from many studies have shown that aging results in an immune-dysregulated state in the context of the immune response to influenza virus. In this dissertation, the effect of age on interferon-alpha related responses to influenza infection was evaluated.

The first exercise-immunity study (Chapter II) examined the long term effect of exercise on the memory response to influenza A virus. This study showed that levels of serum anti-influenza antibody were reduced in young treadmill-exercised mice. One mechanism that may
have been responsible for the exercise-associated reduction in antibody was a decrease in lung viral load, rather than impaired immune function. Support for this possibility include the finding that lung viral load is decreased with exercise at early time points p.i., and the finding that exercise did not reduce antibody response when virus was administered through an intraperitoneal route. These exercise-associated reductions in antibody, while not detrimental to the host, were also noted following a secondary lethal challenge with the same strain of influenza A virus. However, as mice were re-challenged with a lethal dose of influenza virus, they showed no clinical signs of illness indicating that the primary antibody response was sufficient to protect the mice from disease. Furthermore, the study showed that characteristics of the memory response were also altered with exercise. The non-dominant subtype of anti-influenza antibody (IgG1) was increased in the exercised mice after re-challenge. However, exercise training prior to the primary infection resulted in reduced numbers specific lung cell populations after rechallenge. The cell populations that were decreased included the influenza specific CD8+ granzyme B+ recruited cells as well as the influenza-specific CD8+ granzyme B+ resident cells. Taken together, our findings suggest that the reduction in CD8+ cells following secondary challenge was due to decreased viral load during the primary infection.

A subsequent study (Chapter III) evaluated the differential effect(s) of exercise on dendritic cell function in aged as compared to young mice infected with influenza virus. In this study, we determined the extent to which exercise may alter the innate or adaptive function of the pulmonary dendritic cells. The dendritic cell is important for recognition of foreign antigen in the respiratory tract and transportation of that antigen to secondary lymph nodes for the development of specific-immunity. The overall results showed that exercise altered dendritic cell function in both young and aged mice, however, the dendritic cell-associated immune
response were differentially affected by exercise in the young compared to aged. The conclusions from the dendritic cell study suggest that exercise can have a beneficial effect on various dendritic cells functions even though the effects might be different in a young compared to aged host.

Finally, the effect of exercise on the immune response to Influenza A virus (IAV) infection in diet-induced obese mice was evaluated in Chapter V of this dissertation. A higher incidence of viral and bacterial infections has been associated with obesity. Not surprisingly, during the 2009 Influenza pH1N1 outbreak a greater number of individuals with a higher BMI (>30) were hospitalized due to increased morbidity caused by the IAV infection. Studies using the diet-induce obese mouse model have demonstrated an impairment of immune response to IAV infection as compared to lean mice. The purpose of the last study in this dissertation was to determine whether exercise might improve immune responsiveness to IAV in obese mice (similar to our other findings in lean mice). The results showed that exercise improved specific immune responses in obese mice (enhanced immune cell infiltration, Type-I interferon production, influenza-specific CD8+ IFNγ cells numbers). The same immune measures have been shown to be dysregulated in diet-induce obese mice. Overall the findings suggest that exercise can “rescue” the obesity-related impairments of immune function, although the mechanisms appear to differ in lean as compared to obese mice.

In Chapter IV, the impact of age on immune response was examined. The immunomodulatory effect of interferon α (IFNα) in response to influenza virus infection was compared in the young and old. The role of IFNα had not been studied to a great extent in the elderly, and the results from our study demonstrate that IFNα appears to have different effects in
young and aged mice during IAV infection. The effect of anti-IFNα treatment had consistent effects in young and old mice with respect to a reduction in IFNα-associated gene expression and bronchoalveolar lavage fluid cytokine/chemokine concentration at day 4 p.i., decreased CD8+ cells in the lungs at day 8 p.i., but increased levels of serum anti-influenza IgG and IgG1 antibody at day 8 and 28 p.i.. One of the most interesting findings was that illness severity was reduced in the aged mice that had been treated with neutralizing anti-IFNα antibody whereas the young mice had increased illness severity to influenza virus infection when they were treated with neutralizing anti-IFNα antibody. Similarly, by day 10 p.i., aged mice treated with antibody had reduced viral loads in the lung whereas antibody-treated young mice had elevated viral loads. The concentration of specific cytokines and chemokines in the bronchoalveolar lavage fluid was altered by anti-IFNα treatment, but at day 8 and 10 p.i., the effects were different in young and old mice. Taken together, the findings from this study demonstrate for the first time that the immunomodulatory effects of IFNα differ in young and aged mice.
CHAPTER I

GENERAL INTRODUCTION

Organization of the Dissertation:

This dissertation consists of manuscripts that are being prepared for submission to peer-reviewed journals. The dissertation contains a total of six chapters including a general introduction, four research papers, and a general conclusion that discusses the overall findings from the dissertation, followed by acknowledgements. The references for each chapter, including Chapter I (Literature Review), are listed at the end of each chapter with figures following the references directly. The General Introduction (Chapter I) to the dissertation includes briefly the current understanding of influenza viruses and their impact on public health along with a brief description of the research objective of the dissertation. The Literature Review provides background information on the lifecycle of influenza A viruses, followed by a review of the host immune response to the virus, along with a review of aging immunology research and obesity-associated immune impairments that have only recently been identified. Finally, a brief summary of exercise immunology closes the review of literature. Chapters II, III and V are focused primarily in exercise immunology, whereas Chapter IV is focused on the differential immune responses to influenza virus in young and aged hosts. Chapter V specifically addresses obesity-associated immune impairments in influenza infection and whether those immune impairments can be corrected by implementing exercise as treatment.

This dissertation contains the experimental data and results obtained by the author during her Ph.D. study under the supervision of his major professor Dr. Marian L. Kohut at Iowa State University, Immunobiology Interdepartmental Program.
Introduction to the Dissertation:

Influenza virus is a negative sense, single stranded RNA virus known widely for the severe respiratory tract infection it can cause in humans. The aged population (> 65 years of age) is more susceptible to influenza infection than younger individuals. One reason for the increased susceptibility to infection in the aged is a decreased efficacy of the seasonal influenza vaccine which may provide only 17-53% protection against circulating strains in the elderly [1]. The elderly are considered an immune comprised group as a result of immunosenescence, and the annual influenza vaccine is recommended for this population. Comparatively younger individuals who receive the vaccine will have approximately 70-90% protection estimated by the Center for Disease Control (CDC) [1-3]. As the numbers of aged individuals in developed countries continues to increase worldwide, research to improve vaccine efficacy is expanding along with efforts to identify lifestyle interventions (dietary improvements, increasing physical activity) that can improve the immune response to vaccination, decrease the susceptibility to influenza infection, and reduce morbidity and mortality in the event that an individual was to become infected. Along with the natural process of aging, very recently obesity was identified as an independent risk factor for increased susceptibility to influenza infection. Obese individuals infected with the 2009 novel H1N1 virus had increased morbidity, mortality, and hospitalizations in comparison to non-obese individuals [4]. These results may have important public health implications given the increasing rates of obesity. From the year 1964-2004, the obese population, (defined as individuals with an estimated body mass index > 30 according to the World Health Organization) was estimated to have grown from 13% in 1964 to 32% by 2004 [5]. As of 2011, the WHO estimated 65% of adults over 20 years of age were overweight (BMI>25) worldwide [6]. These findings highlight the importance of studies involving intervention
strategies to reduce obesity, such as the consumption of a higher quality diet and increased regular physical activity to determine whether these strategies can improve the disease outcome to illnesses such as influenza.

Introduction to Influenza Viral Infection

The Orthomyxoviridae viruses are characterized as negative sense, single stranded RNA family of viruses. This family of viruses contains the less known thogotovirus and isavirus, along with the three genera of influenza viruses, types A, B and C. For the purpose of this review the primary focus will be on the influenza A viruses. Influenza A viruses are well known as the causative agent of a severe respiratory tract infection which induces a range of symptoms from no symptoms (individual maybe unaware of illness or viral shedding) to the more severe symptoms (fever, chills, rhinitis, malaise, among others) and viral shedding that can last for a period of one week and up to several months in some rare cases. Severe cases of infection may require hospitalization and can be fatal due primarily to damage caused by the immune response to the virus in the lung microenvironment. The immunopathology that occurs in the lungs during influenza A virus (IAV) infection may lead to reduced gas exchange and loss of lung function. Although influenza vaccine is available, as a result of the high mutation rate of influenza viruses, the task of determining the appropriate strains of influenza virus to include in the annual vaccine may be difficult. Cases of influenza are monitored worldwide, with virus isolated from patients as they present with influenza infection. The predominant isolates can then be evaluated by WHO experts who meet on an annual basis to identify and predict the specific influenza strains most likely to be circulating in specific populations in the following year [7-9]. Then, a yearly trivalent vaccine is developed that contains two influenza A virus strains and one influenza B
virus strain. For example, the seasonal influenza vaccine for 2010-2011 contained the novel H1N1 strain (A/California/7/09), also known as the pandemic 2009 H1N1, a H3N2 strain (A/Perth/16/2009) and an influenza B virus strain (B/Brisbane/60/2008) of virus [10]

**Antigenic Shift and Antigenic Drift: The Significance of this Phenomenon to Upcoming Influenza Viral Strains**

Influenza viruses are under constant evolutionary pressure due to the pool of hosts in which these zoonotic viruses reside. In April of 2009, the novel strain of influenza virus H1N1 emerged, causing a sudden pandemic. The quick development of a new influenza vaccine (2009 H1N1 vaccine), apart from the seasonal vaccination, was encouraged by World Health Organization (WHO) to aid in preventing the further spread of the virus [9]. Once the virus was isolated from infected patients, sequential analysis revealed that the 2009 virus was a reassorted virus from past avian, swine and human viruses. The novel H1N1 virus of 2009 derived its NA and M gene segments from the European avian-like origin H1N1 virus, with the HA, NP, and NS gene segments arising from the 1918 “classical swine” H1N1 virus, the PB2 and PA segments were derived from another avian influenza A virus source, and finally the PB1 gene segment was from a seasonal human H3N2 virus [11]. These findings provide evidence for how influenza viruses can mix and reassort in the various hosts leading to antigenic shift. In contrast, antigenic drift occurs when point mutations arise which alter the amino acid sequence of the HA structural components for example. As these point mutations accumulate, the obvious implications for the influenza susceptible host are that antibody might no longer neutralize an encountered virus via interaction with the HA structural protein [12]. These findings stress the importance of developing a protective annual vaccine that best fits the common circulating strains, but the
findings also highlight that we can never be thoroughly sure which strains will arise next, thus it’s important to research ways to alternatively enhance the host immune response. If possible, identifying broadly conserved epitopes would provide another means by which to combat the variability in the HA antigens of influenza viruses.

*Influenza A Virus (IAV) Lifecycle*

*Influenza Virus Structure.* How the complex structural components of influenza A viruses interact with the host becomes important to any research involving influenza viruses. The viral genome is segmented into 8 portions held within a viral core surrounded by a nuclear envelope [11]. Within this viral core, RNA processing proteins (PB1, PB2, and PA) are packaged along with the genetic material that codes for all the structural components (HA, NA, NP, M1, M2) and non-structural components (NS1 and NS2) of the virus. These ‘packages’ of viral components are called viral ribonucleoprotein complexes (vRNPs). The vRNPs work well to ensure that upon entry into the host cell the virus is ready to rapidly undergo translation (becoming positive sense genetic material) and begin production of its own proteins within the host cell. Along the outside of the viral core is a lipid membrane composed of M1, or matrix one protein, this makes up the viral capsule which anchors the hemagglutinin (HA) molecule into the viral core along with neuraminidase (NA) and matrix 2 proteins (M2) [13]. The HA and NA glycoproteins are discernible by electron microscopy on the surface of the virus and they are the primary target of the host immune response, including the target of neutralizing antibody which is the primarily responsible for the protection elicited by annual vaccination.

*Influenza Virus Attachment.* There are now 16 HA proteins that have been identified and nine structural variations of the NA proteins. The number that corresponds to the HA protein or
NA protein is how the strain distinctions are made. For example, the highly pathogenic avian virus strain, H5N1, expresses the hemagglutinin 5 protein and the neuraminidase 1 protein on its surface. In general the ratio of HA to NA proteins is 4:1 on the surface of the influenza A viruses, making the HA glycoprotein the dominant component of the external structure of the influenza A virus \([14]\). The HA glycoprotein of influenza virus interacts with the glycosylated sialic acid (SA) receptor on the host respiratory epithelial cells \([15]\). This interaction is what eventually gains the virus access to the host cytoplasm where it can deposit its genome and begin replication \([16-18]\).

SA residues are widely present on glycolipids and glycoproteins expressed on many cell types, including respiratory epithelial cells of humans, yet there are specific configurations of the SA residues (α-2,3 and α-2,6 linkage) that explain some of the lack of susceptibility of the human influenza strains to certain species and the lack of susceptibility of human hosts to the avian influenza virus for example \([19]\). Work by several groups has determined that the HA protein of human seasonal influenza A virus (IAV) preferentially recognizes the α-2,6 linkage which is expressed widely on the epithelium of the upper respiratory tract of humans, while the avian strains (H5N1) prefer the α-2,3 linkage for attachment This α-2,3 linkage is localized on ciliated cells of the lower respiratory tract in humans \([18, 20]\). Furthermore, the lower ciliated airway epithelial cell only accounts for a minor population of cells that make up the total airway epithelium. The second and much more dominant type of non-ciliated upper airway epithelial cell expresses the α-2,6 linkage instead and provides reason for the greater degree of susceptibility to the seasonal IAV in humans \([20, 21]\). The lower accessibility/availability of the α-2,3 ciliated cells might provide a cell specific explanation for why the highly virulent avian
influenza virus subtypes have been unable to effectively replicate and transmit from human to human.

**Influenza Virus Entry.** Once the virus has successfully attached to the respiratory epithelium, entry of the virus occurs through a pH-dependent fusion process. Influenza virus requires a low-pH environment in order to initiate fusion with the plasma membrane which then leads to the formation of the endosomal compartment. Eventual entry of the virus occurs by four possible pathways: clathrin-mediated endocytosis (CME), clathrin-independent endocytosis, the formation of calveolae, and finally through macropinocytosis [22-25]. However, the clathrin-mediated endocytic pathway seems to be the most commonly accepted form of viral entry utilized by influenza virus [26, 27]. This form of entry is characterized first by the congregation of several HA proteins interacting with glycoproteins, followed by the formation of clathrin-coated pits. The pit will eventually deepen into the plasma membrane and pull away into the inside of the cells [22, 28]. In this way, the virus is internalized within a clathrin-coated vesicle that will eventually uncoat and fuse with another more acidic endocytic vesicles. Within the fused endocytic vesicle, the acidic environment is further induce by M2, which serves as an ion channel pump, this increased acidity causes the fused HA proteins to undergo a conformational change leading to uncoating of the virus followed by release of the vRNPs into the cytosol of host cell [29, 30].

**Influenza Virus Replication.** Influenza virus is unique in comparison to other RNA viruses because it specifically requires the use of the nuclear machinery of the host for replication to occur. Once the virus has entered an endosomal compartment and uncoating occurs, the released vRNP complexes are actively taken up through nuclear pores. This process
is tightly regulated, the influenza genome is never found unattached from the nucleoproteins that make up the vRNP complex. Replication and transcription of viral RNA is thought to occur simultaneously within the host nucleus. A process called cap snatching initiates the transcription process as host mRNA caps are cleaved by PB2 and used by the viral polymerase as a primer for transcription. Transcription is terminated when a string of 5-7 uridines (U-track) are encountered and the addition of a polyadenylated (poly-A) tail to the newly synthesized viral mRNA is completed. The U-track, poly-A and cap are all necessary for stabilization and nuclear export of newly synthesized viral mRNA by the M1 protein. Viral mRNA has been shown to be synthesized in an early phase and a late phase. During the early, or first, phase of protein production, all eight genome segments are transcribed in equal amounts by the host cells, leading to an equal level of production of all of the viral proteins encoded in those segments. During the early second phase of transcription the NS1 and NP proteins are selectively produced, followed by a late phase in which NS1 production is reduced while HA, NA and M1 proteins begin to be up-regulated. M1 protein will bind to newly synthesized vRNPs and export them from the cell nucleus. Additionally, the NS2 protein is important for translocation of vRNPs and packaging of the viral components into a newly synthesized virion [13], which will not be fully formed until the virus begins to bud off from the host cell surface.

*Influenza Virus Release.* Virus assembly occurs around lipid rafts on the surface of influenza infected cells. The exact mechanism that leads to the viral budding, or release, is not fully understood, but seems to rely directly on the HA molecules association with the lipid raft. The NA protein will also associated with the lipid raft, but interestingly, the M2 molecule is excluded until after the virus has released from the surface of the infected cell. Viral budding has been shown to be specifically initiated by the HA molecule which induces a curvature on the
outside of the host cell leading to bud formation and subsequently the pinching off/release of a newly formed virion from the cell surface [31].

**Respiratory Epithelium and the Host Defense against IAV infection**

One of the first host defense mechanisms present in the lung environment is a thin layer of mucus that is in contact with the distal end of the cilia on the epithelial cells. The mucus layer is constantly being moved up and out of the respiratory tree as the cilia beat. In addition, this mucus layer is composed of various innate proteins (mucins, defensins, C-type lectins, collectins) some of which will be up regulated further by the respiratory cells if an infection or sufficient insult were to occur [32, 33]. These types of proteins are also involved in the first line of innate defense as they can bind and coat pathogens causing them to be neutralized. The mucus layer is also the site at which secretory IgA (s-IgA) will reside. If the host has been exposed to the virus previously, the mucosa-associated antibody can provide protection against the virus by opsonization leading to phagocytosis or neutralization which prevents the virus from infecting the respiratory epithelium [34].

When Influenza A virus (IAV) infection occurs, the respiratory epithelial cells will become infected with virus, and shortly thereafter, alveolar macrophages will also begin to phagocytosis the virus. This lung resident population of macrophages along with the respiratory epithelial cell will recognize intracellular single stranded viral RNA through intracellular innate immune receptors (PRR – Pattern Recognition Receptors). In particular, TLR-7 and RIG-I are recognized for their involvement in the early recognition of influenza virus and the subsequent Type-I interferon production that follows. Type-I interferons activate two pathways, the first being the interferon stimulated genes and second, the NF-κB pathway. Interferon stimulated
genes (ISG) produce an anti-viral state in surrounding cells, which are in close proximity to the infected cells and this limits the spread of the virus to some extent [21]. Meanwhile, the up regulation of the NK-κB pathway leads to the production of several proinflammatory cytokines and chemokines that will establish an inflammatory environment leading to cell activation in the surrounding area, and also create a chemokine gradient which will lead to cell infiltration of circulating innate cells and eventually T and B cells [35]. This finely orchestrated immune response, if successful, can control the viral infection. However, if the virus is not controlled, excessive virus replication and subsequent inflammation may occur, causing severe illness in the infected host. We will revisit this discussion further in the ‘contributions of immunopathology’ section of this review.

Type-I interferons (IFNα/β) are secreted very soon after IAV infection of the respiratory epithelium. IFNβ appears first and is produced by the epithelial cells and fibroblasts, followed shortly by the production of IFNα [36]. Along with fibroblasts, virally infected cells also produce these proteins leading to signaling through the JAK/STAT pathway, which then causes the up regulation of ISG. This family of ISG includes oligoadenylate synthetase (OAS), protein kinase R (PKR), and orthomyxovirus resistance GTPases (Mx proteins). Briefly, these anti-viral products limit viral replication by degrading any cellular RNA (OAS), including host RNA and viral RNA, interfering with transcription in the nucleus (Mx1- murine specific), altering post-translational activities (MxA – human specific) and shutting down cellular and viral protein synthesis (PKR) [37, 38]. Obviously, these functions are indispensible to the innate host response to influenza virus as they can completely limit viral protein production and replication in any cell in which they are up-regulated.
Innate cell recruitment is another aspect of the host immunity that is necessary to control the early replication and spread of IAV infection in the lungs. Alveolar macrophages are a resident population of cells that can become infected by the virus, but may also phagocytose dead cells and viral proteins thereby aiding in containment of the virus. NK cells are recruited to the site of viral infection and immediately begin killing infected cells through cellular cytotoxic activity in response to IFNα/β expression [39]. In addition to NK cells, neutrophils will also migrate to the site of infection and engulf cells opsonized with antibody [40]. Once these cells are engulfed by the neutrophil, the neutrophil may carry out a granulocytic and anti-microbial activity, leading to further containment of the virus. While the neutrophil may not be well recognized in the immune response to influenza, the plasmacytoid dendritic cell (pDC) has been implicated extensively in a successful immune response to IAV infection. The pDC produces massive amounts of IFNα, a protein whose function has already been highlighted in this review [41]. Furthermore, the conventional dendritic cell (cDC) also resides in the lung in an immature state. This type of dendritic cell will further expand/migrate into the lung environment after infection occurs. Even before infection, immature cDCs are constantly sampling (via phagocytosis) within the lung microenvironment. Once cDCs have sensed a pathogen (via PRRs), they will uptake that antigen, process it and begin to migrate towards a draining lymph node, and simultaneously take on a mature phenotype. This mature phenotype is characterized by the increased expression of MHC-class I and II molecules, and the co-stimulatory molecules CD80 and CD86 which are necessary for the activation of the adaptive immune response.

The adaptive immune response begins when a complex set of cell interactions occur. As antigen presenting cells (typically cDC) arrive in the lung draining lymph node, and are able to present antigen in the context of an MHC molecule, which will eventually be recognized by
cognate receptor on CD8+ or CD4+ T cells [41]. Influenza virus specific immunity relies on the activation of both CD8+ and CD4+ cells, especially since CD4+ T cell help delivered to B cells leads to the production of anti-influenza virus antibody necessary for the host to overcome this infection [17]. After activation, influenza-specific CD8+ cells will begin to clonally expand and return to the lung where they can fight the IAV infection as effector CD8+ cells (CTLs). CTLs have up-regulated IFNγ production (Type-II interferon) which will function to activate other immune cells (i.e. increased phagocytic activity by macrophages) and drive activated B cells to produce highly specific anti-influenza antibody. Additionally, these CTLs serve a cytolytic function by producing perforin and granzymes, which unlike the cytolytic activity of NK cells, is directed at influenza virus infected cells which are expressing cognate influenza peptides in the context of an MHC I molecule. CD4+ T cells are activated in a similar way by antigen presenting cells and are also capable of undergoing clonal expansion, however, they are not involved in cytolytic killing but instead are involved in the activation of influenza-specific B cells. The B cells eventually develop into antibody producing plasma cells which can produce high levels of neutralizing antibody against the influenza virus. These adaptive functions highlight the specificity of the adaptive immune response necessary to clear an influenza infection virus from the lungs.

Finally, a healing response after IAV infection is important in restoring the airway epithelium back to a functional state. Within the respiratory tract, pneumocyte hyperplasia typically occurs during IAV infection, along with edema and loss of the mucosal integrity. These changes all contribute to the illness and respiratory distress associated with the infection [33, 37]. However, approximately 4 days into IAV infection, just after peak viral replication has ceased (at approximately 3 days p.i.) and the adaptive immune response is expanding, the repair
of the lung epithelial cells and cells of the basement membrane will begin. During this repair process, anti-inflammatory cytokines (TGF-β and IL-10) will begin to arise in the cytokine milieu of the lung [42]. In addition, growth factors such as keratinocyte growth factor (KGF), hepatocyte growth factor (HGF) and fibroblast growth factor (FGF) will also be produced by the fibroblasts and cells of the basement membrane. It is these growth factors that will increasingly contribute to the cell differentiation and re-epithelialization of the lung [43].

**Contributions to Immunopathology: Cytokine Storm and Inflamm-aging**

The concept of the cytokine storm in the young and inflamm-aging in the aged have arisen in the literature and offer potential explanations for the development of life-threatening immunopathology that comes about when young hosts are infected with pandemic strains and when seasonal strains of influenza virus infect aged individuals [44, 45]. A paper written by Ma, et al (2011) graphed age versus mortality resulting from pneumonia and influenza virus-associated illnesses in the 1918 Spanish influenza epidemic and the 1915 (non-pandemic year) seasonal influenza deaths. The numbers of deaths during 1915 gave rise to a U-shaped curve, for higher numbers of deaths in the very young (less than 8 years of age) and very old (greater than 55 years of age). In contrast, during the 1918 pandemic, a W-shaped curve was observed which included a peak of higher death rates in younger (approximately 25 years of age) to middle-life individuals (approximately 40 years of age) (Fig. 1). It should be noted that the number of deaths in the very old were still relatively high during the 1918 pandemic year, but were not higher per 100,000 deaths than other years when seasonal influenza virus was circulating [46, 47].
The increased death rate in the young appears to be characteristic of pandemic influenza outbreaks and is thought to be related to the hypercytokinemia (cytokine storm) phenomenon. For example, the highly pathogenic avian influenza virus (H5N1) strain, which had briefly arisen in Asia during 1997 and again in 2003, is an example of highly pathogenic virus thought to induce extreme immune pathology in younger populations due to a cytokine storm [48, 49]. The first outbreak of H5N1 occurred in 1997 in a poultry market in Hong Kong resulting in approximately 18 human cases of infection. Since 2003, over 380 cases of H5N1 virus infections have been reported [50], resulting in approximately 60% mortality primarily in individuals 10-19 years of age [17, 51]. Mortality was associated with high viral load, alveolar damage, lung leukocyte and lymphocyte infiltration, and increased levels of various pro-inflammatory cytokine and chemokines (IL-6, IP-10, IFNγ, TNFα, MIP-1α, MIP-1β, and MCP-1) in lung tissue. Other findings in animal models also suggest that the pro-inflammatory mediators play a role in influenza-induced pathogenesis and mortality [52-56]. When severe immunopathology occurs, capillary leakage may occur, filling the alveoli with bodily fluid. This may lead to insufficient oxygen/CO₂ exchange and eventual lung failure [57]. Additionally, this highly pathogenic strain of virus can be detected systemically in severe cases, due to the lack of tissue tropism associated with H5N1 virus strains. Most influenza viruses rely on a specific protease, or trypsin-like molecule, produced in the respiratory tract to release from the respiratory epithelium after viral replication, however, the highly pathogenic H5N1 does not appear to require this protease to cleave off of the respiratory epithelium. It appears one limitation that this highly-pathogenic virus has been unable to overcome is its inability to transmit among humans. However, this virus remains present and poses a potential threat to individuals worldwide as it is constantly mixing and reassorting among birds [11, 14, 48].
Therefore, the concept of the cytokine storm may partially explain higher mortality rates among young individuals infected with H5N1 strains of IAV [58, 59].

In elderly hosts, a low level of systemic inflammation has been documented, although it is not clear whether this may contribute to a greater degree of immunopathology in aged hosts, leading to poor disease outcome to influenza virus and other infections (i.e. CMV) [60]. Unlike the cytokine storm which is induced upon infection, inflamm-aging is defined by a chronic systemic production of various acute phase proteins (IL-6, TNFα, CRP) that are present systemically in the individual prior to infection [45, 61]. Thus, once infection occurs, it is possible that the higher baseline level acute phase proteins or pro-inflammatory cytokines might contribute to greater immunopathology. A recent review published by Franceschi et al, coined the term inflamm-aging and examined the implications from an evolutionary perspective. The review points out that as medical technology has advanced leading to improvements in overall health, lifespan on average is becoming longer in individuals since the last century [62]. As average lifespan has lengthened, elderly individuals with sufficient immune responses early in life have survived longer, and have been exposed to increased antigenic stimulation as they have aged, leading to the development of inflamm-aging. Along with the inflamm-aging process are the development of chronic inflammatory conditions that present in later life; artherosclerosis, type-II diabetes, Alzheimers disease, osteoporosis, etc [45, 61]. There appears to be a select group of older individuals reaching 100 years of age that have a reduced level of systemic inflammation. According to the inflamm-aging theory, it is possible that these individuals were more susceptible to infection during their reproductive years because they did not develop more potent immune responses resulting in tissue damage. However assuming that these individuals survived infections, in spite of a reduced “immune activation” to pathogens, they escaped the
damaging effects of inflammation over a lifetime of exposure to multiple pathogens. As a result, they are considered to be in a state of healthy aging due to anti-inflammatory-aging. This is an interesting theory that may also explain why the aged host is more susceptible influenza-induced mortality.

Finally, the concept of ‘original antigenic sin’ may influence immune response to influenza virus infection in young versus older populations. The concept might explain why mortality rates don’t seem to differ significantly in pandemic versus non-pandemic years in the elderly. Often times when pandemic strains emerge, characteristics of these new strains contain similar structural components from viruses that arose many years before in previous pandemics or just during the seasonal influenza cycle. For example, the 2009 H1N1 virus emerged and quickly it was shown that this new virus shared some homology with past H1N1 viruses. Since elderly individuals, but not younger humans, might have been exposed to a similar virus early in life, they most likely had some memory for the component of the virus shared between the new strain and past strain. The concept of ‘original antigenic sin’ is important because it suggests that regardless of the subsequent strain that an individual encounters later in life, it is the first influenza virus infection or strain that they encounter in life to which they develop a dominant anti-influenza antibody response. The HA molecule on the surface of the virus is the main target for anti-influenza antibody, and aged individuals may have some protective memory from past exposures [46]. However, aged individuals will not develop highly specific antibody upon subsequent vaccination with new strains of the virus. Upon each subsequent exposure to influenza virus infection, they will only further develop more of that one type of HA-specific antibody which would only be protective if the host encounters a virus expressing the same HA molecule as the previously encountered strain. This is the main problem highlighted by the
concept of ‘original antigen sin’ in the aged host and it shows the importance of vaccination strategies that lead to development of memory T cell responses, which will target conserved structural components among influenza virus strains, in addition to antibody. Balancing protective immunity between T cell memory and sufficient antibody would lead to better protection in the aged host as well as the young.

**Immunosenescence: The Aging Immune System**

As the natural process of aging occurs, a gradual decline in immunity occurs, which has been characterized through research over the past two to three decades. With this declining immunity comes an increased susceptibility to infectious diseases and decreased vaccine efficacy in comparison to younger adults. In addition, during seasonal influenza infection, young individuals tend to recover more quickly from infections whereas elderly individuals tend to have a delayed immune response leading to a extend time to recovery [63]. Multiple aspects of the immune response might be impaired in elderly populations including innate and adaptive (cell-mediated and humoral) response. One major change that impacts immune response in elderly adults is thymic involution, which begins during puberty in humans. Thymic involution is thought to contribute to the progressively reduced number of naïve T cells entering systemic circulation. It has been suggested that over time as one encounters various infections and develops a memory response to various antigens, there will be fewer naïve T cells available upon encounter to new pathogens. However, knowing that very few naïve T cells (20-200 antigen-specific T cells) are initially necessary to develop specific immunity to a particular pathogen [64], perhaps the numbers of naïve T cells are less of an indicator of an age-associated dysfunction in comparison to the limited T cell repertoire available in the aged individual when
compared to a more broad repertoire available in a younger host. In aged populations, a greater portion of already committed memory T cell make up the circulating T cell pool and “take up space” in the peripheral T cell compartments that could otherwise be occupied by naïve T cells [65-67]. Although thymic involution may not be the only age-related impairment that impacts immune response, it is certainly a contributing factor that has been recognized for quite a long time.

**Adaptive and Humoral Immune Impairments with Aging**

The adaptive immune response has received the greatest attention in aging immunology research, as early studies showed impairments of CD4+ and CD8+ T cell proliferation and cytokine production. Murasko, et al. 1987 examined the age-related loss in mitogen stimulated proliferative responses for every ten year increment of age in humans, and found a decline with every ~10 year increase of age. Beyond 60 years of age, one or more of the mitogens tested failed to induce a response in every individual examined, and there were significant decreases in responsiveness (more than 40% less in comparison to the 20 - 29 age group for PHA (phytohemagglutinin) and Con A (concanavalin A) mitogens. The importance of this study was that it was one of the first studies to report a broad age-related proliferative impairment in peripheral blood mononuclear cells (PBMC) isolated from aged humans. Future work by this group and others in the field of aging immunology has gradually uncovered cell specific functions that have led to declined immunity with aging [68].

Helper CD4+ T cells have been reported to function at a reduced level with aging [69-71], and in particular, reduced IL-2 production by CD4+ T cells has been well documented in both human and mouse studies [72-75]. In one study conducted by Haynes, et al. (1999), the
results showed that aged CD4+ helper T cells isolated from transgenic mice produced less IL-2, proliferated less, and had reduced effector function in response to cognate antigen. Not surprisingly, when exogenous IL-2 was added to *in vitro* culture, expansion and effector function were restored, and the cells expressed a more activated phenotype. These results and others show the importance of IL-2 to the development of a functional immune response involving CD4+ helper cells [72, 76]. Interleukin-2 was first known to immunologists as TCGF, or T cell growth factor. As its early title would suggest, it is an important proliferative and activating cytokine, now classically known to be involved in the development of the Th-1 response. IL-2 may play a role in viral infection by inducing IFNγ production primarily by CD8+ cells [74, 77]. IL-2 production by CD4+ cells may also help to activate B cells leading to the eventual development of a humoral response characterized by the production of neutralizing antibody [67]. During viral infection, IL-2 production by CD4+ helper cell is important for both CD8+ and B cell responses [78].

Although many studies have evaluated the age-related effects on IL-2 production in CD4+ cells, the results from studies examining Th1 versus Th2 (reduced IL-2 by aged host causes Th2 > Th1) cell phenotypes is still a topic of debate. More recently the impact of age on the Th17 and Treg cell function has been examined [79, 80]. Studies in aged mice and humans have shown a greater propensity of CD4+ naïve cells to differentiate into the Th17 effector phenotype in comparison to young. With advancing age, naïve CD4+ cells have also been shown to be insensitive to IL-12 (for Th1 development) and IL-4 (Th2) yet still maintain their ability to respond to TGFβ and IL-23 which would induce the Th17 phenotype. This change could explain why Th17 effectors are found to be increased in aged mice and in human peripheral blood [81]. Th17 cells have been implicated in age-associated autoimmunity [82],
and Th17 cells could have a similar role in inducing the low-level chronic inflammatory condition associated with inflamm-aging. This seems relevant especially if this cell composed a higher proportion of the CD4+ T cells in the systemic T cell population [78, 83]. As mentioned above, the T regulatory (Treg) cells have received attention in aging immunology, and have been shown to be present at higher percentages in the peripheral blood of aged uninfected animals [79, 84, 85]. In aging humans, T regulatory cells are found to be increased in healthy aged donors and older asthmatic patients [86, 87]. As the name suggests, T regulatory cells serve an important regulatory function to the immune system upon infection, and are important in maintaining self-tolerance in the absence of infection [88, 89]. Treg cells may also suppress the immune response to respiratory viral infections [90], and have been shown to suppress IFNγ production in Leishmania major infection, contributing to a chronic infectious state in elderly individuals infected with this pathogen [91]. The complete loss of the T regulatory cell phenotype from the T cell population leads to an aggressive lymphoproliferative disorder called scurvy in mice, and IPEX syndrome in humans. Humans who have this genetic condition do not live to adulthood due to immunopathology in various organs (kidneys, liver) of the body that come from the loss of this regulatory cell subset [92]. One interesting theory regarding aging and T regulatory cells suggests that the greater expression of CD25 (IL-2Rα) on these cells along with a greater number of T regulatory cells results in greater consumption of the IL-2 that is produced, leaving less IL-2 available for other cells. [93]. Also, Williams-Bey et al (2011) observed a basal increase in T regulatory cells in aged mice prior to influenza infection in comparison to young mice. After infection, the levels of T regulatory cells were found to increase to a greater extent in the aged mice whereas young mice maintained the same levels of T regulatory cells throughout infection. The same investigators observed an equal level of
suppression induced by the T regulatory cells regardless of whether the host was aged or young. Therefore, if cell suppressive function of T regulatory cells in the aged is equivalent to the young, yet represents a higher percentage of the T cell population, it may account for the reduced immune response to influenza infection found in this study as well as other studies involving influenza infection in aged hosts [79].

As with the CD4+ T cell, there are quite a few studies that have shown defects in the CD8+ T cell population with aging. A very early study by Bender et al. (1991) showed impaired cytotoxic killing by CD8+ T cells in aged mice in comparison to young mice after influenza virus infection. Also, the age-related delay in viral clearance and prolonged infection has been correlated with the reduced killing capacity by these cytotoxic T cells [94, 95]. In addition to cytotoxic killing function of CD8+ T effectors, a portion of IFNγ production comes from these CD8+ T cells in response to viral infection [96]. IFNγ serves to activate other immune cells such as macrophages (increased phagocytosis) and is important for driving a Th-1 cytokine-induced isotype switch (IgG2a) in B cells. In aged individuals, results have been mixed with respect to IFNγ production by CD8+ T cells. Some studies have shown that IFNγ production upon antigenic stimulation was not different between young and aged humans [74], while other studies have shown a decreased expression of IFNγ by CD8+ T cells and reduced CD8+ T cell proliferation in influenza virus infected mice [90, 97]. These types of findings along with results demonstrating that CD8+CD28+ T cells are reduced in the host T cell repertoire of an aged host suggests that the intrinsic capability of these cells to respond to antigenic stimulation is at least partially impaired. CD28 represents the cognate receptor for the co-stimulatory molecules (CD80 and CD86) expressed on antigen presenting cells [98]. The age-associated decline of CD28+ T cells demonstrates that a greater portion of the T cell repertoire is already committed to
the memory phenotype (CD45RO+ in mice) leaving fewer naïve CD8+ T cells to respond to new infections [61, 99, 100]. These age-induced CD8+ T cell impairments suggest another means through which an older individual is at a greater risk of developing severe infections and responding poorly to vaccination [101].

*B Cells and the Aging Immune System*

Thymic involution occurs as an individual ages and explains the gradual decline in the circulating numbers of naïve T cell found with aging. B cell populations are affected in much the same way as the bone marrow compartment, and also decrease with age. The hematopoietic compartment composed of supportive cells (i.e. stromal cells, fibroblasts) that promote the survival of hematopoietic stem cells are lost with advancing age, and are replaced with adipocytes. This dysregulated microenvironment has obvious implications in aging immunology and the development of B cell progenitors. It also appears likely that plasma B cells returning to the bone marrow compartment after terminal differentiation may also be affected by this altered microenvironment and may account for the reduction in long term antibody titers in older individuals [85]. Much like the reduced number of naïve T cells and increased memory T cells that are found in an aged host, the B cell population seems to undergo a similar age related alteration. For instance, a greater portion of circulating B cells do not express CD27, the naïve B cell marker, indicating that again a greater percentage of the B cell population is of an already committed memory phenotype [102]. Furthermore, work by Chong et al. (2005) showed that these memory B cells are resistant to apoptosis, suggesting that they can continue to proliferate and produce antibody, yet the specificity of antibody may be of little value to the aged host [103]. Another aged related impairment found in the B cell response is an apparent
development of low affinity antibody. This finding is partially explained by a predominant shift away from the development of IgG and instead towards the maintenance of the IgM subtype in B cells with aging. The cause of this shift, or resistance to antibody switching, could be related intrinsic dysfunctions in the B cell itself, or may also be explained by the extrinsic influences of other cells [102, 104]. Reduced T helper cell interactions with the B cells during development, and low cytokine production by T cells and other innate/adaptive cells (plasmacytoid dendritic cells or antigen presenting dendritic cells) could contribute to this impairment in the antibody specific response to infection and vaccination. Therefore, both age-related changes of B cell intrinsic function as well as alterations in functions of other cell populations may ultimately affect B cell response. [105-107].

*Dendritic Cells and Their Alterations within the Aging Immune System: Conventional Dendritic Cells, Plasmacytoid Dendritic Cells, Follicular Dendritic Cells, and Langherhan’s Cells*

Dendritic cells represent a family of immune cells that exhibit various phenotypes. This portion of the review on the impact of aging on dendritic cell function will focus on the conventional dendritic cell. However, a few alternative subsets of DCs will briefly be discussed as they also have been shown to have immune impairments as a result of the aging process. The three alternative subsets to be discussed include, Langhehan’s cells (LC), plasmacytoid dendritic cells (pDC) and follicular dendritic cells (FDC). Langerhan’s cells are a skin-localized type of dendritic cell that are important for the development of an effective immune response to vaccination. These cells acquire antigen deposited by vaccination and deliver it to the draining lymph node to interact with its cognate T cell or B cell [108, 109]. In the skin of elderly humans, these cells have been found to be reduced in number [110], with the same results confirmed in
Plasmacytoid dendritic cells are another unique subset of dendritic cells that function minimally in the process of antigen presentation. Instead, pDC’s are well-known for their potent production of type-I interferons (mostly IFNα, but also IFNβ) in response to contact with viral RNA and CpG oligodeoxynucleotide through their respective receptors TLR-7 (ssRNA) and TLR-9 (DNA) [114, 115]. Interferon-α in particular, serves to activate a complex antiviral response that may limit viral replication during infection if sufficient levels of IFNα are produced. In aged hosts, reductions in circulating numbers of pDC have been identified along with reductions in the amount of IFNα produced by pDC [116]. The reduction in IFNα is thought to be due to a reduced expression of TLRs on pDC, leading to reduced stimulation of TLR by inactivated virus, or by Guardiquimod (TLR-7 agonist) and CpG Oligodeoxynucleotide (TLR-9 agonist) [117-120]. Finally, age-associated alterations have been identified in the follicular dendritic cells residing in the draining lymph nodes. These cells primarily provide cytokine stimulation and structural components that aid in the development of germinal centers within secondary lymphoid tissue. Several of the FDC dysfunctions identified with reduced function in aging include, antigen trapping, iccosome (Immune complexes of antigen and antibody) on the cell surface, as well as reduced expression of complement receptors (CR1 and CR2) leading to reduced presentation due to lack of antigen availability [63, 121]. These alterations in FDC function may result in reductions in cell-mediated and humoral responses that have been documented with aging. Another interesting finding, based on an early study involving germinal center formation in aged mice, was that FDC tended to get trapped in the subcapsulary sinuses, resulting in decreased migration of these cells into the proper areas of the lymph node for germinal center formation after antigenic challenge [122, 123]. Although these three subsets of dendritic cells represent relatively smaller numbers of dendritic cells, their
dysfunctions are very likely to contribute significantly to the age-associated declines of other immune cells.

Conventional dendritic cells (cDC), also known as professional antigen presenting cells, or APCs, represent a unique cell that functions both in the innate and adaptive immune responses. Immature dendritic cells are constantly surveying peripheral environments around the body, and upon encounter with a pathogen, phagocytosis of the pathogen occurs, resulting in a migratory response towards a draining lymph node. Along with the migratory process, the differentiation of this antigen presenting cell into a mature phenotype occurs [124]. The mature phenotype can be identified by increased expression of the B7 molecules (CD80 and CD86) and CD40, as well as processing and presentation of antigen in the context of an extracellular MHC (I or II) molecule [125-128]. These migratory/maturation events and the arrival of these cells into the draining lymph node, allow for APC interaction with T and B cells specific for the cognate antigen presented in the MHC-molecule [129, 130]. Not surprisingly, conventional dendritic cells isolated from aged individuals have been shown to have various impairments (reduced co-stimulatory molecule expression, reduced MHC molecule expression) in some studies [131-133], while other studies have not shown the same outcome [134]. Various alterations in experimental design could possibly explain the difference in results between studies. Variations such as the type of host (animal or human for example), culturing conditions for in vitro studies, and even the tissue from which the DC was isolated can contribute to varying results [63, 135]. Regardless of the differences in results obtained from aging studies these functional aspects of the dendritic cells are indispensable to the host defense against viral and bacterial pathogens [98].
Up-regulation of co-stimulatory (CD80 and CD86) molecules on APCs represents an integral part of acquired immunity. CD80 and CD86 interact with secondary receptors (CD28) to the T cell and B cell receptor complexes, thereby delivering a signal that enhances the development of potent immune responses to infection, and may aid in the development of functional antibody after vaccination. Work by Sambhara, et al. (2001) and others have implicated reduced expression of CD80 and CD86 as a potential explanation for the impaired immune response to influenza infection [136, 137]. Similar studies further examined CD80 and CD86 expression in aged rodents and showed that there was a delayed period of time until the expression of these co-stimulatory molecules in aged mice reached the levels found in young mice [138]. It has also been shown that a reduced level of co-stimulatory expression on monocytic cells isolated from PBMCs obtained from aged donors was associated with reduced vaccine response [139]. While it seems the majority of evidence in the aging immunology literature suggests reduced total expression or delays in expression of costimulatory molecules, some studies have shown no change in co-stimulatory molecule expression on dendritic cells in aged rodents[134], or that alterations in the expression of co-stimulatory molecules could be corrected with the addition of cytokine stimulation [63]. Again, it is possible that the experimental design affected the results of these rodent studies. For example, some studies have expanded dendritic cells from bone marrow derived stem cells by treatment with GM-CSF and IL-4, and in other studies splenic dendritic cells were isolated and underwent expansion [137]. These types of treatments with cytokines may prevent identification of true age-associated impairments by overcoming the impairment with ex vivo stimulation of a particular cell with cytokine. In summary, the method for attaining DCs in aging studies can alter the outcome leaving one to conclude that there are no changes on a particular cells with aging [1]. However,
that is not to say that these studies do not have their place in immunological research as they allow the intrinsic alterations in cell function to be examined [140].

Expression of the major histocompatibility complex (MHC), on dendritic cells has also been examined in studies of aging immunology. The majority of studies reported reductions of MHC expression on various antigen presenting populations (including macrophages) examined in aged humans and rodents. A few studies reported no change in the expression of these molecules with aging [141, 142]. In spite of the inconsistent findings, it is important to consider the implications of reduced MHC-II expression on DC after stimulation with respect to the development of an effective immune response [137]. After antigen is acquired by phagocytosis, antigen is processed in an endocytic vesicle and presented in the context of an MHC-class II molecule [143]. Cross presentation involving MHC-class I expression can also occur in various dendritic cells populations, but primarily by lymphoid tissue derived CD11c+CD8α+ cells [144, 145]. Only one study examined the lymphoid resident CD11c+CD8α+ DC for impairments with aging, and found a reduced percentages of these cells in the spleen aged mice in comparison to young [146]. This study did not look any further into the intrinsic functions of these cells, so it is unknown at this point whether these cells are functionally altered by aging or whether there are only changes in cell number. In addition to cross-presentation, the eventual migration and extracellular presentation of antigen in the MHC-II molecule must occur in order for the cognate T-cell or B-cell to find the appropriate antigen. Therefore, these molecules are very important in the development of a pathogen specific immune response in aged hosts, and their reduction may contribute to impaired vaccine and immune responses.
Innate aspects of DC function are also important in immune function. Dendritic cells survey the peripheral environments around the body, and as their name suggests, these cells possess finger-like protrusions (dendrites) across their surface [147]. These dendrites increase the surface area of the cell thereby increasing the chances for antigen exposure in the peripheral environment. These dendrites can also fit through tight junctions between epithelial cells increasing access to pathogens on the luminal side that have yet to cross the epithelial cell layer, and this is one way antigen in the gut and alveoli of the mucosal environments is acquired by these cells. Phagocytosis occurs once an antigen of interest is encountered, and then migration will begin towards secondary lymphoid tissue. The impact of age on these DC functions has not been well studied. In one study by Donnini, et al. (2002), age did not alter phagocytic function, but did enhance DC migration [134]. Results from a study by Paula, et al (2009), showed no difference in phagocytic function in bone marrow derived DCs, but suggested decreased processing in the endocytic vesicle as an explanation for an age-induced impairment found in DCs isolated from aged rodents [137]. Because there are so few studies examining these specific functions of DCs with aging, it is difficult to draw firm conclusions about these innate DC functions at this time.

Alterations in the Innate Immune Response with Aging

The innate cell response also experiences alterations with aging that contribute to the increased susceptibility of the elderly to infectious diseases as well as reduced antibody and cell-mediated function in response to infection and vaccination. Franceschi et al.(2000), has suggested that the levels of baseline systemic cytokines (TNFα and IL-6), or increased acute phase proteins important in the development of any inflammatory immune response, remain
elevated in the aged compared to young. It has also been suggested that the evolutionarily conserved innate immune response may remain intact with aging, while the more intricate advanced adaptive response undergoes the greatest changes with immune senescence [148]. This theory further implies an age-related increase in the numbers of circulating macrophages as an example of an intact innate response. Contrary to the Franceschi theory, many aspects of innate immunity have been examined over the past decade, and multiple age-induced impairments in innate cell interactions, including reduced cytokine production, phagocytosis, and migration have been identified. These findings don’t completely negate Franceschi’s theory as other immunologists studying immune senescence have suggested greater levels of alterations in the adaptive response than the innate response as well [74, 78, 149]. Most likely both the arms of the immune response will undergo alterations as a host ages.

Neutrophils, Macrophages and Natural Killer Cells Functioning into Aged Peripheral Environments

The effects of age on specific innate immune cell populations (neutrophils, macrophages and NK cells) have also been studied [150-153]. Neutrophils are bone-marrow derived granulocytic cells whose numbers seem to be preserved in the aged host [154]. These cells tend to be the first responders to infection as they migrate out of circulation into the peripheral environment following a chemokine gradient produced at the site of infection or injury. Migration does not appear to be greatly affected by the aging environment [155], however some studies have shown that neutrophil arrival at the site of infection may be delayed or a reduction in phagocytosis may occur [156]. Work by Wenish, et al (2000) and others showed reductions in phagocytic capacity of neutrophils isolated from aged donors with respect to their ability to
phagocytose opsonized *Escherichia coli* and *Staphylococcus aureus* [157, 158]. This observation may result from a reduction in Fc receptors, given that unopsonized bacterial products (LPS - lipopolysaccharide) have been phagocytosed at equal levels in young and aged donors [155, 156]. In addition to decreased phagocytosis, microbial killing has also been examined in neutrophils isolated from aged healthy donors and shown to be decreased 10-50% in comparison to young healthy donors [159, 160]. These reductions in killing capacity by the neutrophil are thought to be linked to a reduced production of reactive oxygen species, myeloperoxidase activity, as well as a reduction in intracellular Ca²⁺ (which would imply impairments exist in signaling cascades) [151, 160-163]. Although neutrophils are short lived cells, an age-related resistance to apoptosis has been observed in some studies that ultimately may lead to an excessive accumulation of these cells at the site of infection. This accumulation could block new neutrophils from effectively killing pathogens and delay wound healing (also shown to be impaired in the elderly) [164] as new cells would be unable to readily access the site of infection. It is likely as well that the increased susceptibility of the aged population to infection is also related to the age-associated impairments in neutrophil function [165].

Natural killer cells represent another group of innate cells that respond very early to infection and aid particularly in the establishment of an inflammatory environment necessary for the clearance of intracellular pathogens [166]. Since aged individuals are at a heightened risk of infections by viruses, it is important to determine whether an impairment of NK function contributes to the increased risk of viral infection in aged populations. With respect to viral infection, there are two primary functions that NK cells will carry out. First, NK cells produce perforin and granzymes that are important for cytotoxic killing of infected cells. A second function is the production of type-II interferon (IFN-γ) which serves to activate phagocytic cells,
such as macrophages, in order for them to perform more effectively [166-168]. Findings regarding differences in the number of circulating NK cells between aged and young hosts (humans and mice) have been mixed, especially when infectious experimental models are used [169, 170]. However, when SENIUR guidelines (guidelines that only ensure the healthiest elderly are included in a study) are strictly adhered to in human studies, there is an increase in NK cell numbers among the aged [171, 172]. Despite this age-related increase in cell number that has been identified, NK cytotoxic killing and interferon-γ production have both been shown to be decreased on a per cell basis [63, 173-176]. Additionally, work by Plett, et al (2000), showed higher IFNα/β receptor expression on these cells isolated from aged mice in comparison to young. However, even with receptor expression enhanced by aging, this study found no differences in the ability of NK cells to respond to type-I interferon stimulation. Also, an increased percentage of NK cells undergo apoptosis after stimulation with IFNα/β. This suggests a possible defect in signaling via the type-I interferon pathways in aged mice, a finding that was also reported in later studies by this group with respect to CD8+ cells and their responsiveness to interferons [177]. Finally, a few studies have reported NK cell production of chemokines (MIP-1α, RANTES, and IL-8) and cytokine (IL-2) responsiveness is reduced in aged hosts [178, 179]. These findings taken together might explain in part why elderly are more susceptible to viral infections.

Macrophage dysfunctions have been examined extensively in aging immunology research. Macrophages have been shown to have reduced phagocytic capability, delayed migration, formation of reactive oxygen species, formation of nitrogen intermediates important for antimicrobial activity, and chemokine/cytokine production and responsiveness in aged host [134, 135, 180, 181]. Macrophages are constantly surveying the peripheral environments and
are, therefore, considered to be one of the first responders to pathogens and antigens, particularly in the lung microenvironment where the alveolar macrophage resides [182]. Knowing the plasticity of resident macrophages that exist in specific tissues, a theory presented by Stout et al (2005) and others, suggested that dysregulation in the tissue environment, possibly due to chronic inflammation associated with aged hosts, might further impact the phenotype and function of the macrophage in that specific environment [183, 184]. Wound healing impairments noted in aged animals might be the best evidence for this theory. Aged tissue cells have been shown to have reduced production of growth factors (vascular endothelial growth factor and epithelial growth factor, tissue growth factor-β) during inflammatory responses, and in experiments involving small incisional wound repair [185, 186]. The growth factors and the receptors (expressed on innate cells, i.e. macrophages) must interact to function appropriately. Results from several studies have identified deficiencies in angiogenesis, collagen deposition, and re-epithelialization identified in aged hosts [164, 185, 186]. Thus, it seems that the environmental changes induced with aging might affect the function of these innate cells, perhaps through reduced communication leading to dysfunctions in wound healing. Furthermore, another study conducted in mice in 2003 by Haynes et al. further delineated that it is the aged microenvironment in which the immune response develops that contributes to reduced functional memory T cells. Using young and aged transgenic mice, memory cells from young mice were transferred into aged mice and functioned at the same level as the young in response to antigenic challenge in a young host, meanwhile CD4+ memory T cells developed in an aged mouse in response to antigenic stimulus functioned poorly when transferred in young mice [187].
In addition to environment and tissue related effects on macrophage function with advancing age, several intrinsic changes in macrophage function have been observed. Toll-like receptor (TLR) expression has been examined on various cell subsets, including macrophage, and in general, the findings in both human and murine studies show a decline in expression and function with advancing age [181]. Toll-like receptors were first identified in *Drosophila melanogaster* and were shown to be evolutionarily conserved among many species. These types of receptors recognize innate components of pathogens, collectively called pathogen associated molecular patterns (PAMPs). There are approximately ten TLRs that recognize PAMPs ranging from bacterial cell wall components (e.g., LPS via TLR-4) to genetic material (viral RNA via TLR-7 in mice [139]). TLR interaction with PAMPs causes activation of the multiple immune response leading to the activation of pro-inflammatory cytokine production (IL-6, TNFα and Type-I Interferons), chemotaxis of other innate and adaptive immune cells (via KC, RANTES, MCP-1 production) and potentially an enhanced engulfment or phagocytosis of pathogens (that may serve to limit spread of the infection to other areas of the body) [188, 189]. Expression of TLR on macrophages has been shown to be reduced on splenic and thioglycollate-elicited macrophages in aged hosts [190]. Furthermore, downstream inflammatory responses to TLR agonists (LPS), led to reduced IL-6 and TNF-α production by macrophages from aged mice [190-192]. Furthermore, mRNA transcripts in macrophages were examined by microarray to determine if downstream signaling was altered in the aged macrophage [193]. This study observed a reduced production of IL-6 and IL-1, but showed IL-10 was increased. Furthermore, TLR-associated signaling leading to NF-κB activation was also shown to be reduced with increasing age whereas IRAK-M (negative TLR pathway regulator) was increased. These findings, along with those by Mahbub, et al (2012), suggested that macrophage activation
induced by LPS is dysregulated with advancing age by resulting in an “anti-inflammatory” effect in the aged in contrast to the proinflammatory effect observed in the young [194]. In summary, given the important role of TLRs in promoting a proinflammatory environment necessary for an effective immune response, the age-related reduction TLR expression may explain reductions in the early innate immune response leaving elderly hosts more susceptible to infections.

*Introduction to the Obesity Epidemic: Obesity and Public Health*

The obese state is now recognized for its involvement in the development of many diseases, including atherosclerosis, hypertension, cardiovascular diseases, and type-2 diabetes [195-198]. Obesity is also recently recognized for its role in susceptibility of the host to many infectious disease states, including but not limited to influenza, *Mycobacterium tuberculosis*, *Helicobacter pylori*, encephalomyocarditis virus and coxsackievirus [4, 199-201]. Body Mass Index (BMI) is used widely in human research to classify an individual as underweight (BMI < 18.5 kg/m²), normal weight (BMI < 25 kg/m²), overweight (BMI > 25 kg/m²), obese (BMI > 30 kg/m²) and morbidly obese (BMI > 40 kg/m²) [6]. In general, there seems to be a positive correlation between the susceptibility/severity of various diseases/disorders and increasing body weight. A few of these findings are highlighted in the studies referenced above, but most importantly, as the unhealthy obese population has continued to increase it has caused increased economic pressure on the health care system and on individuals who cover the costs for treatments related to obesity-associated illnesses and co-morbidities [202]. This makes understanding possible treatments for obesity-related impairments important for overall public health.
Obesity: Caused by Imbalance between Energy Intake and Energy Expenditure

Nutrition is recognized for having a positive or negative impact on the human body as a whole. Not surprisingly, a balanced nutritional status, or a balanced amount of energy intake with the proper amount of energy expenditure, seems to yield better quality of life in humans than an imbalanced state [4]. A review written by Pi-Sunyer, XF (2002) provides reasonable evidence for the rise of the obesity epidemic in the United States [200]. This paper along with others, have evaluated the change in obesity over the past 50 years and shown that the obesity epidemic has increased dramatically since the 1990s. From the 1960 to 1980, the rates of obesity had gradually increased in States across the United States. In the 1980s, only 4 States had an obese populous of 15% or higher; however, from 1991-1998, this number had grown to 37 States with a population composed of greater than 15% obese individuals. By end of the 1990s, in all 50 States the obesity epidemic had taken hold, affecting men and women and every age group, including children. Pi-Sunyer et al. (2002) have implicated technological advances across developed countries for their direct involvement in the obesity epidemic. For example, these technological advances have led to automated jobs that require less energy expenditure throughout the work day. Additionally, this daily work environment coupled with easy access to high-calorie, processed, yet palatable foods, has left a greater percentage of the human population in the state of energy imbalance mentioned above.

Obesity Impacts Immune Responses in the Lung

The effects that obesity may have in the lung environment are only beginning to be examined. However, there is accumulating research suggesting an association between lung function and obesity. Chronic respiratory conditions including asthma and COPD have been
linked to overweight children and adults [203]. Obesity also seems to be involved in decreased vaccine responses to influenza and hepatitis B vaccines [204-206]. Additionally, a decreased antibody response to tetanus toxoid in overweight children has been reported [207]. During the 2009 pH1N1 outbreak, an increased number of obese individuals were admitted for hospitalization due to increased severity of the infection in these patients. These findings suggest that obesity is somewhat of an immunosuppressive condition. Fat deposits are thought to be the source of the systemic impairments associated with obesity [208, 209], not only has a resident macrophage population been identified in this adipose tissue [210, 211], but this tissue is thought to be the source of a chronic baseline level of inflammation (TNFα, IL-6, CRP) identified in obese humans and animals [212]. ‘Adipokines’, or fat-specific cytokines, such as leptin and adiponectin [213, 214], are also produced by adipocytes in this environment, and each of these proteins has been implicated in lung impairments associated with obesity.

Much like insulin resistance, which develops in obese humans and diet-induced obese rodents, leptin resistance occurs as well. Leptin is produced by many cells in the body in addition to adipocytes. It is elevated in the normal weight host to regulate appetite by inducing a state of satiety. In the obese host, when calorie intake high, leptin levels often times are also high. Researchers have been able to show that excessive high fat calorie intake leads to impaired leptin signaling much the same way insulin resistance develops leading to diabetes. However, aside from appetite regulation, leptin does influence immune cell function through the leptin receptor (Ob-R) and has been shown to activate the JAK/STAT pathway (via STAT3), MAPK and PI3K pathways inducing a protective inflammatory response during infection [212]. Leptin has also been shown to alter macrophage activation and phagocytosis [215], and to induce the activation, proliferation and maturation of many immune cells, such as NK cells, T lymphocytes,
dendritic cells, and neutrophils [216]. Additionally, one of the most interesting findings for leptin involvement in immunity came from studies of the ob/ob knockout mouse. This knockout mouse completely lacks leptin production, and it was of interest to note that these mice present with thymic involution [217]. This suggests that T cells are directly affected by the loss of leptin, suggesting one potential means by which adaptive immunity is altered by the obese state. If obesity is generating a state of leptin resistance leading to significant metabolic effects, it is possible that this same resistance might be occurring in immune cells as well [218].

A study by Smith et al. (2007) measured serum leptin levels in obese mice infected with influenza, and found that infection caused reductions in serum leptin from day 0 to 6 in the obese mice whereas increased serum leptin was found in the lean mice from day 0 to 3 followed by significant reductions by day 6 [219]. This group suggested that the altered response to leptin in the obese mice might explain the delayed/reduced levels of innate cell infiltration (monocytes, macrophages, etc) into the lungs of these mice between day 0 and 6. However, it is possible that leptin expression in the lung (by testing for protein levels in BAL or lung RNA) might directly influence immune response to influenza. Interestingly, Sood et al. (2010), mentions the lack of information regarding respiratory leptin response as a significant drawback to the determination of the role of leptin in asthmatic humans. This review highlights that systemic levels of leptin may not reflect the true levels of leptin in the lung environment, and whether some aspect of the asthmatic response is altered by leptin has not been determined in humans [220]. Obviously, systemic levels of leptin are easily measureable in human sera samples as blood can be collected by venapuncture which is only mildly invasive. However, in order to determine lung levels of leptin, bronchoalveolar lavage collection is an option, but this procedure is far more invasive and requires trained personal to be carried out.
The diet-induced obese (DIO) mouse has become an excellent model for further research into the immune impairments associated with obesity. Immune alterations in DIO mice infected with influenza A virus (IAV) led to a poorer disease outcome in comparison to lean mice [219, 221-223]. Two studies by Karlsson, et al. (2010), were able to show reduced T cell memory and reduced maintenance of those memory cells in DIO mice, leading to 25% mortality in DIO mice compared to no mortality in lean mice after challenge with a heterologous strain of the virus [222, 223]. These results fit with the results seen in another study showing reduced cell-mediated and humoral response to trivalent influenza vaccination in obese humans in comparison to normal weight controls [206]. Furthermore, a study by Smith et al. (2006), showed immune impairments in the primary response to influenza infection in DIO mice leading to increased morbidity and mortality in these mice. In particular, immune cell infiltration and cytokine/chemokine production (IFNα/β, MCP-1 and RANTES) was delayed in the lungs of DIO mice, and the CD8+ T cell response was delayed and reduced in comparison to lean mice. Another study further implicated dendritic cell impairments in the loss of a robust immune response leading to the same early immune impairments found in the 2006 study. Not only were the numbers of plasmacytoid dendritic cells reduced in the lungs after influenza infection, but conventional dendritic cells had a reduced capacity to stimulate influenza primed CD8+ T cells. Additionally, IL-12 mRNA was reduced in lymph node cells, further implicating dendritic cells in the diet-induced obese impairments associated with influenza infection.

Exercise Immunology

The field of exercise immunology began to take full shape in the 1980s. However, studies involving physical activity and immunity had been documented as long as 100 years ago
Early findings of an increased incidence of upper respiratory tract irritations or illnesses in elite athletes or individuals training for endurance event provided a considerable driving force to the development of the field. It appeared during this early time in the field of exercise immunology that excessive exercise could negatively impact immune function, and the possibility that moderate exercise might have beneficial effects on immune responsiveness was not an area of focus. More recently, findings from a few studies suggest that exercise does provide some benefit with respect to immune function.

Many aspects of the innate immune response are altered with exercise. Neutrophilia has been reported shortly after the onset of exercise, and a second round of neutrophil influx into the peripheral blood will occur after exercise has subsided. The magnitude of this second wave of neutrophils entering circulation is usually dose dependent and based on exercise intensity as well as duration. Even though increased numbers of neutrophils begin to circulate after acute exercise, studies have shown decreased degranulation and oxidative burst when these cells are stimulated with bacteria [225-227]. Furthermore, regular exercise training seems to impact the neutrophil to an even greater degree, as attenuated respiratory burst has been shown to last for several days post-exercise [228]. Animal studies have shown an effect of exercise on tissue specific macrophages. For example, moderate exercise and highly intense exercise stimulated the phagocytic function of these cells and chemotaxis was also altered in a corticosterone dependent manner [229, 230]. Other studies of macrophage alterations have shown an exercise induced reduction in MHC-II expression along with decreased antigen presentation in studies of exhaustive exercise [231-233]. Natural killer cells have also been a topic of interest to exercise immunologist as natural killer cell cytotoxicity has been shown to increase in an exercise dose (duration and intensity) dependent fashion. This is thought to be related to increased numbers of
circulating NK cells in the blood after exercise, because on a per cell basis this enhanced activity changes very little with exercise [234]. Finally, the last cell that has been examined in the innate immune response is the dendritic cell. Only two studies have been carried out with respect to the effects of exercise on dendritic cell function. One study observed increased numbers of DC cells in response to exercise training [235]. The other study observed increased expression of MHC-class II along with increased production of IL-12 in exercise-trained rats using a mixed leukocyte reaction involving bone marrow derived and expanded dendritic cells [236]. Finally, the findings for innate immune cell alterations seem to be a little mixed, but this confusion is most likely due to the differences in dose of exercise used in the determination of some alteration on host cell immunity. Studies in animal models highlight this point very well as exhaustive exercise practiced prior to HSV-1 infection led to increased mortality [237], while studies of regular moderate exercise prior to influenza virus infection caused improvements in illness scores in treadmill exercised mice [238]. This indicates that before conclusions can be drawn about some of the cell-specific alterations associated with exercise, the dose, intensity, duration, and frequency of exercise must be considered.

Adaptive immunity is also known to be altered with exercise. As with innate immune cells that enter circulation shortly after the onset of exercise, lymphocytes undergo the same increase in circulation. However, after exercise has ceased lymphocyte numbers will fall below baseline levels detected prior the exercise. Furthermore, the decrease in circulating numbers of lymphocytes (T and B cells) seems to be related to the duration and intensity of the exercise session and the amount of recovery time between sessions [239-241]. This makes sense as the T cell among others immune cells are thought to be influenced by epinephrine (adrenaline) and other hormones which would be produced by the host in response to the exercise. Further studies
have confirmed that the decline in adaptive immune cell function is associated with increased stress hormones, such as cortisol, which are elevated with exercise [242, 243]. While lymphocyte numbers have been shown to be reduced with exercise, T cell activation in response to mitogenic or antigenic stimulation in two separate studies was shown to be increased using expression of activation markers on T cells as a measure of T cell function. [244, 245]. These findings indicate that adaptive immunity is altered in response to exercise, and further suggest that even with the changes in circulating numbers these cells might be functioning at a higher level based upon the activation results.

Finally, the amount of research that has accumulated over the past years since the discipline of exercise immunology was established has grown exponentially. Now studies exist that have evaluated the effect of exercise on many disease states and have been able to show immune cells alterations leading to better disease outcomes in several animal models and even in humans. The studies in this dissertation will hopefully further contribute to the field of exercise immunology and to the broad concept that a healthier lifestyle can be achieved through regular physical activity.
Figure 1: Distribution of influenza-associated illness among various age groups within the human population. Very young (less than 5 years of age) and elderly (65 years of age and older) tend to be at a higher susceptibility to seasonal influenza virus (red line) in comparison to young (18 to 30 years of age) and middle-aged (approximately 30 to 55 years of age) adults. In years when pandemic strains (black line) of influenza virus arise in circulation the young to middle-aged are at an increased risk of susceptibility to pandemic strains as they have yet to acquire immunity to the new pandemic strains. Very young and elderly remain susceptible to pandemic influenza outbreaks.
Table 2. Toll-like receptors identified as of 2012 in mice (human). Toll like receptors form dimers (column one) in order to recognize ligands (column two) and pathogens that express those ligands are represented in column three.

<table>
<thead>
<tr>
<th>TLR dimer:</th>
<th>Ligand:</th>
<th>Pathogen:</th>
</tr>
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<tbody>
<tr>
<td>TLR 1/2</td>
<td>Triacylated lipopeptides</td>
<td>Bacteria/Mycobacteria</td>
</tr>
<tr>
<td>TLR 2/6</td>
<td>Diacylated lipopeptides/zymosan</td>
<td>Bacteria/Fungus</td>
</tr>
<tr>
<td>TLR 3/3</td>
<td>dsRNA</td>
<td>Viruses</td>
</tr>
<tr>
<td>TLR 4/4</td>
<td>Lipopolysaccharide</td>
<td>Gram Negative Bacteria</td>
</tr>
<tr>
<td>TLR 5/5</td>
<td>Flagellin</td>
<td>Bacteria</td>
</tr>
<tr>
<td>TLR 7/7 (or TLR 8/8)</td>
<td>ssRNA</td>
<td>Viruses</td>
</tr>
<tr>
<td>TLR 9/9</td>
<td>CpG DNA</td>
<td>Bacteria/Viruses</td>
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CHAPTER II

ANTIBODY AND RECALL RESPONSE TO INFLUENZA A/PR/8/34 INFECTION IS REDUCED IN TREADMILL EXERCISED MICE YET STILL PROTECTIVE.

A manuscript to be submitted to The Journal of Infectious Disease as a Brief Report

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Abstract:

Previous findings have demonstrated that exercise training prior to influenza infection minimized symptom severity, reduced lung viral load, and decreased serum anti-influenza antibody. The possibility that an exercise-associated reduction in antibody during primary infection might compromise resistance to a secondary infectious challenge exists, thus the mechanisms that may explain decreased antibody were investigated. BALB/c mice were exercise-trained for eight weeks and then infected with Influenza virus through an intranasal route. The results showed that exercise reduced morbidity/mortality and decreased serum anti-influenza IgG and IgG2a from day 8-180 post-primary infection. In response to secondary infectious challenge, exercised mice were equally protected from severe morbidity/mortality, but demonstrated reduced serum anti-influenza IgG, IgG2a, and decreased influenza-NP peptide-specific CD8+ Granzyme B+ recruited and lung resident cells. When influenza virus was
administered by the intraperitonial route, exercise did not reduce serum antibody IgG or IgG2a. These findings suggest that the exercise-associated declines in antibody titer were most likely due to an early reduction in lung viral load, prior to the initiation of antibody response.

**Background:**

Regular, moderate exercise provides benefits to various host systems, including cardiovascular, musculoskeletal, and immune systems [174, 246, 247]. The beneficial effects of exercise have been observed for multiple disease conditions including cancer, asthma, respiratory infection, cystic fibrosis, and HIV [248-252]. These findings are not insignificant as exercise represents an inexpensive means through which individuals can improve their quality of life without further burdening the healthcare system in any way. One infectious disease for which regular exercise may improve disease outcome is influenza infection [253, 254]. Influenza viruses infect approximately 100,000 individuals in the U.S. annually causing symptoms (malaise, fever) ranging from mild to very severe. In severe cases of infection, which tend to occur in the more susceptible populations (very young, elderly and obese), hospitalization and death may occur [1-3].

Work by our research group has examined the effect of exercise in a murine model of influenza infection. We have shown that 10-12 weeks of moderate exercise training in either young or aged BALB/c mice minimized symptom severity and reduced viral load over the course of infection in comparison to non-exercised controls. Also, exercise-associated reductions in serum anti-influenza antibody titers (IgG, IgG1 and IgG2a) at day 7 and day 10 post-primary infection (PPI) were observed. Based on this previous work, it was unclear whether the decreased anti-influenza antibody was 1) a result of reduced antigen load (reduced lung viral
titers), 2) an exercise-associated impairment in antibody production, or 3) predominance towards a Th1 cell response developing in the exercised mice during primary infection. Furthermore, the reduction in anti-influenza antibody might result in an insufficient level of antibody needed for a protective response during secondary challenge with influenza virus. In this study, we evaluated the length of time for which the exercise-associated reduction in serum antibody persisted and whether secondary challenge with a lethal dose of influenza would result in compromised resistance or diminished memory response. In order to determine whether the reduction in antibody was due to 1) an exercise-associated impairment of antibody function, or 2) an exercise associate enhancement of early viral clearance in the lung thus decreasing antigen load, virus was administered through an i.p. route, bypassing the respiratory tract.

**Materials and Methods:**

Eight week old BALB/c mice (n = 45) were obtained from Jackson Laboratories and randomly assigned to either an exercise treatment group or a control group. After a one week acclimation period, a 5 day/week exercise training program began in which the exercise sessions gradually increased in speed and duration, from 12 meters/min for 10 minutes to 45 min per day at a maximum speed of 18 meters/min for 2.5 months. Non-exercised control mice (n = 15) were housed in the same room and subjected to daily handling stress and treadmill noise as a control. All studies were performed according to Institutional Animal Care and User Committee guidelines at Iowa State University and within the guidelines set by the NIH for the care and use of laboratory animals. Approximately 24 hours after the last exercise session, all mice were anesthetized with aerosolized isoflurane and infected via an intranasal route with a sublethal dose of A/PR/8/34, approximately 0.545 HAU/mouse [stock virus - HAU=8192/0.5mL or $10^{10.45}$]
EID$_{50}$/mL - diluted 1:15,000 in sterile saline]. Body weight was monitored until day 28 post-primary infection (PPI), and blood was collected at day 14, 21, 28 and month six PPI by saphenous vein bleeds. Two days after the six-month PPI blood collection, all mice including original uninfected mice (n = 4) were anesthetized using aerosolized isoflurane, and challenged with a lethal dose of A/PR/8/34 (approximately 4.36 HAU/mouse) by an intranasal route. A subset of uninfected mice (n = 3) were included in this secondary challenge experiment as controls. Body weight was monitored through re-challenge until day eight post-secondary challenge (d8 PSC). Blood and whole lungs were collected at day 8 PSC.

A second experiment was carried out in which exercised mice were trained in the same manner reported above at ~24 hours after the last exercise session all mice (including non-exercised controls) were infected by intraperitoneal (i.p.) injection with a total volume of 100 µL containing live A/PR/8/34 virus, approximately 1.09 HAU/mouse (n = 6 per group). Serum anti-influenza virus IgG, IgG1, and IgG2a levels were determined by indirect ELISA (method reported below). Body weight was monitored daily for 14 days. Blood was collected at day 14 (saphenous vein bleed) and day 28 (CO$_2$ euthanasia) post-challenge for serum anti-influenza antibody levels.

Blood was collected by saphenous vein bleeds or heart puncture and centrifuged at 3500 rpm for 15 min. Sera were stored at -80 °C until tested by indirect ELISA for anti-influenza IgG, IgG1 and IgG2a. For the indirect ELISA protocol, ELISA plates (Immulon, Alexandria, VA) were coated overnight at 4 °C with influenza virus A/PR/8/34 diluted in carbonate coating buffer (pH 9.6) at a concentration of 200 HAU/ml for anti-influenza-IgG, IgG1, and IgG2a. The wells were blocked with 0.1% BSA solution at 37°C for one hour. Plates were washed three times with
PBS/0.05% Tween 20 between each step. Serum was added to the plate in duplicate and incubated at 37°C for 3 hour. Alkaline phosphatase-conjugated goat anti-mouse IgG (H&L), IgG1, and IgG2a (Southern Biotechnology Associates) were added and then incubated overnight at 4°C. Finally, phosphatase substrate (Sigma) was added and incubated at room temperature for 20 minutes. Optical density (OD) of each well was measured using a microtitration plate reader at 405 nm wavelength.

Lungs were removed from mice euthanized at day 8 PSC, minced and pressed through a wire screen. Single cell suspensions were cultured at 5 x 10^5 cells/well in the presence of 1 μg/ml brefeldin A and 400 ng/mL recombinant mouse IL-2. Lung cultures were treated with 1 μM of influenza peptide NP_{147-155} (TYQRTRALV), PMA/Ionomycin or media control. After 6 hours, cells were harvested, stained with rat anti-mouse antibodies (CD8β FITC, CD3ε PE-Cy7 and CD11a PerCP-Cy5.5), fixed, permeabilized and stained with rat anti-mouse IFNγ APC and Granzyme B PE. NP-specific lung resident (CD3⁺CD8⁺CD11a⁺) and lung recruited (CD3⁺CD8⁺CD11a⁻) Granzyme B⁺ and IFNγ⁺ cells were determined. All antibodies were obtained from BD Biosciences, and flow cytometry was performed using a FACSCanto flow cytometer. Data were analyzed with FlowJo (Tree Star).

Changes in body weight or food intake over time were assessed with a mixed ANOVA (time as repeated measure * treatment). A one-way ANOVA was used to compare serum anti-influenza immunoglobulins (IgG, IgG1, and IgG2a) and lung cell populations at each time point p.i.. All data are reported as mean ± standard error of the mean (SEM). Values of p< 0.05 were considered statistically significant.
Results:

The results showed that the non-exercised (No-Ex) mice in comparison to exercised (TM-Ex) had increased body weight loss over the course of infection and recovery (determined by mixed ANOVA, t*e signifies a significant time by exercise interaction; p < 0.05) (Fig.1A). Mortality measures included mice that lost greater than 20% body weight and were euthanized. A greater percentage of No-Ex mice were euthanized due to significant body weight loss in comparison to the TM-Ex mice (53% mortality No-Ex versus 27% mortality TM-Ex) (Fig.1B). By day 28 PPI, most mice had returned to their initial weights with only few exceptions, and no mice were showing signs of influenza-associated illness at this time. All mice rested for an additional 5 months. Exercised mice did not resume exercise training during the rest period or before lethal dose re-challenge. After re-challenge, previously infected mice did not lose body weight and did not exhibit clinical signs of illness. In contrast, mice that had not been previously exposed to influenza virus infection succumbed to the lethal dose within five days p.i..

Levels of serum anti-influenza antibody were determined at day 14, 21, 28 and month six PPI (Fig. 1C and 1D) and at day 8 PSC (Fig. 2A). Significant exercise-induced reductions (e* signifies a significant exercise effect by one-way ANOVA, p < 0.05; e+ p < 0.1) in serum IgG and IgG2a were found at all time points examined, including six months PPI and at day 8 PSC. IgG1 was also reduced at day 14, 21, and 28 PPI, not different at month six PPI, yet significantly elevated in the TM-Ex mice 8 days after lethal dose re-challenge in comparison to the No-Ex mice. TM-Ex and No-Ex mice that received an i.p. injection of live influenza virus (instead of intranasal infection) had no differences in the amount of IgG and subtypes detected in serum at day 14 and 28 post-challenge (Supplemental Figures - S1 and S2).
Finally, the NP-specific CD8+ recall response was also evaluated in whole lung tissue for both exercised and non-exercised mice 8 days after re-challenge (Fig. 2B). NP-specific CD8+IFN-γ+ cells tended to be reduced in the TM-Ex mice compared to No-Ex in response to re-challenge. Meanwhile, NP-specific CD8+Granzyme B+ recruited and lung-resident cells were both reduced post-secondary challenge in the TM-Ex compared in No-Ex. Findings for the reductions in resident and recruited granzyme B+ populations were both statistically significant ($p < 0.1$ for CD11a- and $p < 0.05$ for CD11a+ population).

**Summary and conclusions:**

In this brief report we show that regular moderate exercise enhances the immune system in such a way that leads to improvements in illness severity associated with influenza infection as well as a reduced time to recovery. Previous findings showed that multiple pro-inflammatory mediators in the bronchoalevolar lavage fluid were reduced as a result of exercise treatment [238], along with a decline in serum anti-Influenza IgG (recently submitted). These previous findings led to questions regarding the potential mechanisms by which exercise might impair the development of the antibody response, and/or compromise the response to a subsequent lethal dose re-challenge with homologous virus. In view of our finding that exercise did not result in decreased antibody levels when virus was administered through an i.p. route, it is likely that exercise does not impair antibody response. Instead, the reduction in serum antibody when virus is administered through an intranasal route most likely reflects the exercise-induced decrease in viral load at very early time points post-infection. Results from this study show that the exercise-induced reductions in anti-influenza IgG and IgG2a were still present at 6 months p.i. and up to 8 days after re-challenge. The exercise-associated reductions in anti-influenza virus
IgG and IgG2a continued through re-challenge even though exercise mice did not return to regular treadmill training. It is possible that fewer memory B cells exist in exercise-trained mice due to the reduced antigen load at the time of primary infection. Anti-influenza IgG1 levels were also reduced to day 28 PPI in exercise-trained mice, but in contrast to IgG and IgG2a, IgG1 was not different at six months PPI, and surprisingly higher at 8 days post-secondary challenge in the exercised trained in comparison to non-exercised mice. One possibility to explain the differential effect on IgG1 and IgG2a antibody is an exercise-associated change in lung cytokine environment, i.e., less Th1, greater Th2 consistent with findings from another research group [255].

Finally, NP-specific CD8+ Granzyme B+ cells were also reduced after lethal dose re-challenge. These findings are consistent with the possibility that an early reduction in viral load in exercised mice led to a less of a need for a potent CD8+ response to the infection. It is also possible that suggest that the memory T cell response in the exercised mice developed in a Th2>Th1 lung cytokine milieu during primary infection, a finding which has also been shown in the lung epithelium of murine models of asthma [248]. Finally it is important to note that even though exercise reduces the antibody and NP-specific CD8+ response, those reductions are not indicative of an impaired immune response to influenza.

As a brief summary, and possible explanation for the results, we believe the shortened time to recovery and decreased antigen led to the alterations seen in the developing memory populations. Past results in our model have shown that IFNγ is reduced over the course of infection, IL-5, which is a characteristic cytokine of a Th2 response, is also reduced. This suggests other aspects (i.e. co-stimulation) might also be reduced over time. If the exposure to
antigen and subsequent exposure to co-stimulation/cytokines is reduced, perhaps this is decreasing the overall magnitude of the memory CD8+ response, and reducing the time of exposure of the IgG1-specific B cell population to IFNγ leading to reduced class switching (to IgG2a). This explanation is purely speculative and further work into the development of the specific memory cell populations in the exercised host would need to be carried out to make this conclusion. More importantly, in this study we’ve shown that the exercised mice developed a protective memory CD8+ and antibody responses, in spite of the reductions in NP-specific CD8+ cells and antibody, as these mice were still protected from lethal dose re-challenge.
Figures Chapter II:

Figure 1A.
Figure 1B.

% Mortality

8/15

3/11

No-Ex

TM-Ex
Figure 1C.
Figure 1A-1D: Body weight loss, percent mortality and serum anti-influenza antibody levels after primary infection. 1A) Grams of body weight loss for treadmill exercised (TM-Ex) mice (n = 11) compared to non-exercised (No-Ex) (n = 15). A significant time by exercise interaction (t*e; p < 0.05) was found by statistical analysis using a mixed ANOVA. Non-infected mice (n = 4) were included in the experiment and did not lose weight (data not shown) 1B) Percent mortality was decreased in the treadmill exercised mice in comparison to the non-exercised. 1C) Serum anti-influenza IgG and subtypes measured by indirect ELISA at day 14, 21, and 28 PPI, 1D)and six months PPI (non-infected data not shown). Bars represent mean optical density (405nm) ± SEM; e* significant effect of exercise, p < 0.05 and e+, p < 0.1 by one-way ANOVA.
Figure 2A.

Serum anti-influenza Ab day 8 post secondary challenge

[Diagram showing optical density (405nm) for Total IgG, IgG1, and IgG2 comparing No-Ex and TM-Ex groups.]
Figure 2B. Recruited (left) and Resident (right) NP-specific CD8+ cells

![Bar chart showing cell counts per 5X10^5 lung cells for recruited and resident NP-specific CD8+ cells with e* indicating a significant effect of exercise; p < 0.05, and e+ for a trend for an effect exercise; p < 0.1.]

Figure 2A and B: Percentage of total lung NP-specific CD8+ cells expressing granzyme B and serum anti-influenza antibody post-secondary challenge. 2A) Serum anti-influenza IgG and subtypes (IgG1 and IgG2a) measured by indirect ELISA and expressed as mean optical density at 405nm ± SEM. 2B) Lung resident (CD3ε+ CD11a+) and recruited (CD3ε+ CD11a-) NP-specific CD8β+ Granzyme B+ cells measured in whole lung homogenates by flow cytometry expressed as counts per 5X10^5 lung cells. For both TM-Ex and No-Ex n = 7, non-infected n = 3 with e* indicating a significant effect of exercise; p < 0.05, and e+ for a trend for an effect exercise; p < 0.1. Statistical significance was determined by one-way ANOVA.
Supplemental Figure 1 (S1).

Day 14 Influenza i.p. injection experiment
Supplemental Figure 2 (S2).

Supplemental Figure S1 and S2: Anti-influenza IgG and subtypes 14 and 28 days after challenge with an i.p. injection of live A/PR/8/34. IgG and subtypes detected in the serum at S1) day 14 and S2) day 28 post-primary challenge by indirect ELISA. One-way ANOVA was used to determine statistical significance however the findings were not different across groups. Sample size (n) equals 6 mice per group.
CHAPTER III

DIFFERENTIAL EFFECT OF EXERCISE ON THE ANTIGEN PRESENTING CELL FUNCTION IN YOUNG AND AGED, TREADMILL EXERCISED MICE INFECTED WITH INFLUENZA A/PR/8/34.

A manuscript to be submitted to *Mechaisms of Ageing and Development*

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Abstract:

Influenza A virus (IAV) is a common respiratory pathogen that infects the respiratory epithelium causing morbidity and even mortality in many cases. Aged hosts are known to have increased susceptibility to seasonal IAV infection. Young adults, in addition to being susceptible to seasonal IAV infections, also appear to be at heightened risk for more severe disease during pandemic IAV outbreaks. The severity of respiratory illness can vary among individuals, and the practice of moderate exercise may serve as a host factor that can influence illness severity. This study was carried out to examine the effect of exercise on dendritic cell (DC) function within the host immune response that might be enhanced with regular exercise. Lymph nodes were collected from young and aged, exercised and non-exercised mice at day 10 p.i. (p.i.) and CD11c⁺, CD19⁺ and CD8⁺ cell populations were evaluated. In addition, antigen uptake, processing, and cell migration capacity of respiratory dendritic cells between the lungs and
lymph nodes were also assessed. Finally, we evaluated the extent to which exercise may alter the antigen presenting cells’ ability to activate influenza virus-specific CD8^+ IFNγ^+ and CD4^+ IL-2^+ cells. Results indicate that exercise caused decreased total numbers of dendritic cells isolated from aged and young exercised hosts at day 10 p.i. infected with IAV. However, these results are not the result of a loss of DC function, based upon the exercise induced enhancements in phagocytosis, migration, antigen processing and presentation by the dendritic cells isolated from young and aged exercised mice compared to non-exercised. The results from this study highlight alterations in dendritic cell function that might contribute significantly to the enhanced immunity seen in exercised aged and young hosts infected with IAV.

**Introduction:**

Influenza A virus (IAV) causes a severe infection of the human respiratory tract resulting in approximately 250,000-500,000 annual deaths worldwide [206]. A great amount of research has been devoted to developing better anti-viral therapies, more effective annual vaccinations and even holistic means (i.e. diet, nutrition, exercise) to enhance immunity to IAV. Increased susceptibility to seasonal IAV infection, along with increased morbidity and mortality, have been well-documented in aged populations (65+ years of age) when compared to younger adults [256, 257]. In addition, there is an ever-present threat of the emergence of new strains of pandemic IAV that leave even the younger adult population at risk of severe infection. One example of this scenario occurred in 2009, when the pH1N1 strain of influenza arose and quickly spread worldwide. During the 2009 pH1N1 outbreak, the younger adult population exhibited an increased number of hospitalizations and increased risk of mortality in comparison to seasonal IAV outbreaks [258]. These findings highlight the importance of examining both young and
aged populations as the immune response is not always appropriate in the young adult population, in comparison to the elderly. Therefore, it is important to examine the effects of exercise on the immune response in both young and aged mice challenged with IAV.

Exercise is associated with improvements in overall human health and wellness. Work by Lee et al. (2010), identified regular physical activity as an indicator of a lengthened lifespan and reduced incidence of chronic disease state in adults [246, 259]. Physical activity can be thought of as a relatively inexpensive means of maintaining a healthier lifestyle, thus possibly reducing the overall burden on healthcare systems due to individuals simply remaining healthier over a lengthened lifespan [246, 260]. Of importance to the phenomenon of improved health with exercise is its relationship to the decreased incidence of upper respiratory infection when exercise is practiced at a moderate level. Implementing moderate exercise as a means of possibly reducing the young and aged susceptibility to these types of infections may therefore be of value to the general public health.

Early studies by our group and others have shown that aged and young, treadmill-exercised mice have improvements in symptom severity and reduced illness scores to IAV when they are exercised prior to infection [238, 261]. While an exact mechanism of action has yet to be determined, studies such as this are undertaken to determine if intrinsic functions are enhanced on specific cells of the immune system. The respiratory dendritic cell (rDC) is one subset of antigen-presenting cell paramount in the host defense against IAV and should not be overlooked as a potential target for the effect of exercise at the cellular level. Studies have shown increased expression of MHC-class II on dendritic cells [235], along with increased production of IL-12 in exercise-trained rats [236]. Given these findings and the fact that rDCs
serve as the mediator between the innate and adaptive immune response to influenza virus, it is important to determine the effect of exercise on these cells. During influenza virus infections, uptake of viral antigen in the lung by an immature rDC triggers a migratory response toward the lung draining lymph node (LDLN) and a differentiation of this rDC into a mature phenotype. The mature DC phenotype can be identified by the increased expression of the co-stimulatory B7 molecules (CD80 and CD86) and CD40, processing and presentation of influenza peptides in the context of extracellular MHC (class I and II) molecules, and cytokine production. These migratory/maturation events and the arrival of these cells into the lung draining lymph node (LDLN) where they can interact with naïve T and B cells, are indispensable to the host defense against viral pathogens. However, several dendritic cell functions have been found to be dysregulated with aging. A study by Sambhara et al. (2001) identified reduced expression of CD80 and CD86 on dendritic cells, while Toapanta et al. (2009) reported an age-associated reduction in the number of respiratory dendritic cells accumulating in the lungs during an IAV infection. Contrary to these results, Donnini et al. (2002) found no difference in the expression of the co-stimulatory molecules but still observed reduced numbers of CD8+ T cells producing IFNγ in response to culture with aged antigen-presenting cells compared to young.

In this investigation, we sought to determine the extent to which exercise may alter rDC function in both young and aged populations. The objective of the first experiments was to determine whether numbers of lymph node dendritic cells were altered by exercise in IAV-infected young and aged hosts. A second study was carried out to identify the migration of lung dendritic cells to LDLN to better understand how this trafficking pattern may be altered with exercise and therein contribute to a possible enhancement of the adaptive immune response to
IAV. Finally, we determined the effect of exercise on the antigen presentation function of dendritic cells to determine if exercise would cause these cells to intrinsically present antigen to T cells and activate them in a more efficient way. To our knowledge, these are the first experiments to extensively examine the exercise-effect on dendritic cell function in aged and young mice.

Materials and Methods

Mice, Exercise Training, Virus, Infection and Tissue Collection

Young (eight week) and aged (16-18 months) male BALB/c mice were obtained from Jackson Laboratories and allowed to acclimate for two weeks prior to beginning the exercise protocol. Speed and duration of each exercise session was gradually increased (from 12 m/minute for ten minutes) over the two to three month training period and by the beginning of week three of the training period, mice were running 45 min/day, five days per week at a speed between 16-18 m/min on a treadmill. Young and aged, non-exercised controls were housed in the same room as the treadmill exercised mice and exposed to daily handling stress and treadmill noise. Approximately 24 hours after the last exercise session, young and aged, exercised and non-exercised mice were anesthetized with isoflurane and infected via an intranasal route with influenza virus H1N1 (A/PR/8/34) at approximately 0.545 HAU per 50 uL [stock virus - HAU=8192/0.5mL or $10^{10.45}$ EID$_{50}$/mL - diluted 1:15,000 in sterile saline]. Young and aged uninfected controls were included in every experiment. Body weight and food intake were monitored as a means of determining symptom severity and effect of illness over the course of infection. Mice were euthanized by cervical dislocation at various time points p.i., out to day 10.
Bronchoalveolar lavage (BAL), lungs, lymph nodes and spleens were collected from each mouse.

**Flow Cytometry and Lymph Node Dendritic Cell Characterization**

Lymph nodes from aged mice were collected at day 4, 5 and 10 post-influenza virus infection (p.i.), at day 5 and 10 p.i. from young mice, and held in cold cRPMI (Gibco Invitrogen) until they could be homogenized by pressing them through a 20μm cell strainer in a petri dish using a plunger from a 3mL syringe. The strainer and petri dish were thoroughly washed with cRPMI and cells were collected and filtered by centrifugation at 1500rpm in the bottom of a 5ml polypropylene tube. Lymph node cells were resuspended in 0.1% BSA Stain Buffer (BD Biosciences) and counted using a Coulter Particle Counter. Cells were treated with Fc block (BD Biosciences) and stained using the following antibodies: APC rat anti-mouse CD11c, PerCP-Cy 5.5 rat anti-mouse CD8α, FITC rat anti-mouse CD40, PE-Cy 7 rat anti-mouse CD86, and PE rat anti-mouse CD80. Samples were fixed using BD Stabilizing Fixative (BD Biosciences) and analyzed by flow cytometry. Cell type determination and gating was carried out using FlowJo 7.6 software.

**APC Activation Assay**

Spleens from uninfected young and aged exercised and non-exercised mice were collected and sorted for CD11c⁺ cells to be included in an APC activation assay outlined by Smith, et al with some modifications [221]. Spleens from two to three mice were combined and homogenized into single cells suspension. Cells were treated with RBC lysis buffer and washed three times with Hank’s Balanced Salt Solution (Sigma Aldrich). After the final wash, cells were resuspended in AUTOMACS Rinsing Buffer (with 1% BSA), incubated for 10 minutes
with Fc block, and then incubated for 15 minutes with microbeads specific for CD11c. After one more wash to remove excess CD11c-specific microbeads, cells were sorted using a FACS AUTOMACS cell sorter. Cells were maintained on ice after sorting until subjecting them to a two hour incubation with UV inactivated influenza virus (H1N1 – A/PR/8/34). After this incubation period CD11c+ were washed three times with cRPMI (Gibco Invitrogen) media and plated with influenza virus-primed T cells.

In order to obtain our influenza virus primed T cells, young non-exercised mice were infected with a sublethal dose of influenza and necropsied (at day seven post-influenza infection) for splenocyte collection on the same day as the APC activation assay. Spleens were collected, homogenized, treated with RBC lysis buffer, resuspended in cRPMI and placed in a polystyrene petri dish for one to two hours. Non-adherent cells were collected and found to be 78-82% CD3+ T cells. These cells were washed once with HBSS and resuspended at a concentration of 1 x 10^6 cells/mL. Sorted and virus-treated CD11c+ cells were plated with non-adherent primed T cells at two ratios 1:1 and 1:2 (DC:T) in a 96-well polystyrene plate (Costar #3096). After a two hour incubation, BD Golgi Stop (BD Biosciences) was added to each well of the 96 well plate and a four hour incubation at 37°C, 5% CO2 followed. Cells were pelleted and resuspended in 0.1% BSA-Stain Buffer (BD Biosciences) and stained with rat anti-mouse FITC CD8β and PE rat anti-mouse CD4 for 30 minutes at 4°C. Cells were fixed and permeabilized then stained with APC rat anti-mouse IFNγ, PE-Cy7 rat anti-mouse IL-2 and PerCP-C7 5.5 rat anti-mouse TNFα. Samples were analyzed by flow cytometry and gated using FlowJo 7.6 Software.

*FITC-OVA Uptake and Trafficking Experiments*
Mice were anesthetized using an isoflurane chamber approximately 24 hours after the last treadmill exercise session. Once mice were sedated, they were treated via an intranasal route with 50 μL of FITC-labeled ovalbumin (FITC-OVA) at a concentration of 6.6 μg/μL diluted in sterile saline. Mice were returned to their cages and allowed to recover from anesthesia. At 3, 12, and 24 hours post FITC-OVA instillation, subsets of mice were euthanized so that lungs and lung draining lymph nodes could be collected. Each of these tissues was homogenized and treated with RBC lysis buffer for two to three minutes on ice, then filtered three times with HBSS (Sigma Aldrich) and centrifugation. Cells were next suspended in BSA wash buffer (0.01% BSA-PBS) and counted using a Coulter Particle Counter. Each sample was adjusted for cell concentration of 2.0 x 10⁶ lungs cells and 1x 10⁶ lymph nodes cells were combined and stained using rat APC anti-mouse CD11c, Alexa Flour 700 anti-mouse MHC II, PE anti-mouse CD103, and Alexa Flour 647 anti-mouse CD11b (BD Pharmigen). Mice from young and old, non-exercised and exercised groups were included in every experiment, and unlabeled-OVA included as a background control. Each sample was analyzed on the FACS Canto flow cytometer and gating strategies were applied to each of the samples using FlowJO 7.6 software.

Statistical Analysis

SPSS software was used for all statistical analysis. Two-way ANOVAs were utilized to determine age by exercise interactions for all the above experiments. One-way ANOVA was used to determine effects of exercise and age separately for previously described experiments. Statistical significance was assigned for a $p < 0.05$ and statistical trends $p < 0.1$. 
Results:

*Lymph Node DC populations in Aged vs. Young, Exercised Mice Infected with IAV*

Aged treadmill trained mice were infected with influenza then euthanized at day 4, 5 and 10 p.i. (p.i.). Young treadmill trained mice were included in the day 5 and day 10 experiments. Lung draining lymph nodes were collected from the aged and young mice at the various time points and the total number of cells per lymph node was determined (Fig.1A). Exercise significantly reduced the total cells attained per lymph node in the aged exercised mice at day 4, 5, and 10 p.i., while there were significant reductions in lymph node cellularity in the young mice exercised relative to non-exercised at day 10 p.i. only (e, exercise effect; \( p < 0.05 \), e+, \( p < 0.1 \)). At day 10 p.i. percentages of CD11c\(^+\), CD19\(^+\) and CD8\(^+\) cells were determined per lymph node for young and aged, exercised and non-exercised mice (Fig.1B). In the young exercised and non-exercised mice, the percentages of CD11c, CD19, and CD8\(^+\) cells were all increased in comparison to aged exercised and non-exercised, respectively (a; age effect; \( p < 0.05 \)). When percentages of each population were applied to total cell counts per lymph node, exercise led to reductions in all three cell types (Table 1) in the both young and aged exercised compared to young and aged non-exercised at day 10 p.i. (e, exercise effect; \( p < 0.05 \), e+; \( p < 0.05 \)). These results suggest that the exercise-associated reduction in viral load (reported in published data, data not shown for these experiments) during the IAV infection leads to detectable reductions in the lymph node cell numbers.

*Exercise Alters Antigen Uptake in the Lungs of Young and Aged Treadmill Trained Mice*

Total numbers of FITC-OVA\(^+\) lung cells (Table 2A) and median fluorescence intensity for FITC-OVA in each of these common lung phagocytic populations (Table 2B) were
determined by flow cytometry. In the lungs of aged and young exercised and non-exercised mice FITC-labeled OVA was detected in SSC+ cells (most likely neutrophils – see supplemental 2A-2E) and FSC+ cells further identified as alveolar macrophages (aMΦ), interstitial macrophages (iMΦ), airway dendritic cells (arDC), alveolar dendritic cells (alvDC), and interstitial dendritic cells (iDC) (See supplemental figures flow cytometric gating 2F-2J). In the aged mice, numbers of total aMΦ, alvDC and iDC positive for FITC-OVA were reduced compared to young, however at 3 hours post-instillation, a smaller subset of arDC was increased in the aged mice compared to young (a, main effect of age, p<0.05, a+ p<0.01). By 12 hours post-instillation, more total FSC+ cells, SSC+ cells, iMΦ, and arDC were positive for FITC-OVA in aged compared to the young, while fewer aMΦ were FITC-OVA+. Similarly, at 24 hr, FITC-OVA+ SSC+ cells were increased, along with FITC-OVA+ arDC and alvDC in the aged mice compared to young, while again FITC-OVA+ aMΦ were decreased in the aged versus young. Interestingly, exercise altered the numbers of FITC-OVA+ aMΦ and iMΦ cells. At 3 and 12 hours post-instillation, exercise decreased the number of FITC-OVA alveolar macrophage in young and aged mice. At 12 and 24 hours exercise treatment increased the number of FITC-OVA+ interstitial macrophages in the young and aged mice (e, main effect of exercise p < 0.05 and e+, p < 0.1). Some cell populations were differentially affected by exercise treatment in the young mice compared to old mice. At 3 hours, FITC-OVA+ iDC were decreased with exercise in the aged by ~ two-fold, while not different with exercise treatment in the young. At 12 hr, total FITC-OVA+ FSC+ cells were increased with exercise in the young and decreased with exercise in the aged (a*e, a significant age by exercise interaction p < 0.05). Finally, at 24 hr the numbers of iMΦ were approximately two-fold increased in the aged
exercised compared to aged non-exercised, meanwhile, young groups did not significantly differ from each other (a*e, p < 0.05).

FITC-OVA MFI levels for total FITC-OVA+ lung cells and the various lung cells were examined at all three of the previous time points (3, 12 and 24 hours post-instillation) to determine if exercise or age affects phagocytic capacity in a cell-specific way (Table 2B). At 3 hours post-instillation, only effects of age were noted for all lung cell subsets examined, excepting SSC+ granulocytes. The FITC-OVA MFI for FITC-OVA+ aMΦ, iMΦ, arDC, alvDC and iDC was higher in both the aged groups compared to both of the young groups. At 12 hours post-instillation, both effects of age (greater MFI in iMΦ, arDC, alvDC, and iDC in aged mice) and exercise were found. At this 12 hour time point, exercise was associated with increased MFI in SSC+ cells, iMΦ, and iDC. Similar to the results at 12 hours, at 24 hours multiple cell populations in the aged mice had increased MFI compared to young (aMΦ, iMΦ, arDC, alvDC and iDC). However, at 24hr post-instillation, exercise decreased the MFI of SSC+ cells and iMΦ to levels lower than what was identified at 12 hours post. This suggests that FITC-labeled OVA had begun to be cleared from the lungs by various phagocytic cells in the exercised mice (young and aged) by 24 hours post-instillation, especially since these cell types represented two of three populations found in highest numbers per total lung cells. Finally, a significant age by exercise interaction was associated with SSC+ cells at 12hr post-instillation, such that FITC-OVA MFI was slightly higher in the aged exercised in comparison to the aged non-exercised, yet greatly increased (approximately 1.5-fold) in the young exercised in comparison to the young non-exercised (a*e, p < 0.05). In conjunction with the reduced FITC-OVA MFI from 12 hr to 24 hr post-instillation in SSC+ cells and the reduced numbers of these cells, suggest again that
exercise is altering the kinetics leading to better clearance by these cells, and possibly others, in aged and young mice.

*Antigen Traffic to Lymph Nodes is Altered with Exercise in the Aged and Young*

Antigen trafficking was assessed by collecting lymph nodes from mice that had been treated with FITC-labeled OVA at 12 and 24 hours post-instillation. Total FITC-OVA+ cell numbers of the following populations per lymph node were determined: Total FSC+ (Total FITC+ cells), lymph node CD11c^{hi} (conventional dendritic cells - cDC) and CD11c^{int} (dendritic cells - DC) and CD11b^{+}11c^{lo} (macrophage - MΦ) (Table 2A). Age-associated alterations were identified for total FITC-OVA+ cells and DC such that each of these cell subsets were increased in the aged mice compared to young at 12 hr post-instillation. Exercise increased the numbers of FITC-OVA+ cDC, and DC in both young and old mice at 12 hours post-instillation. Exercise also appeared to enhance the numbers of FITC-OVA+ MΦ in the young however old mice had no difference in FITC-OVA+ MΦ at this same time point. The magnitude of the exercise-associated increase in cDC and DC was greater in aged mice than young at 12 hours. At 24 hours, a time effect was found such that the total numbers of FITC+ cells, most dendritic cell numbers (excepting DC in the young non-exercised group), and MΦ were reduced, suggesting that FITC-OVA was starting to be cleared from the lymph node as well in most groups. At 24 hours, different effects of exercise were found in young and old mice. FITC-OVA+ cells were generally decreased by exercise in young mice, but increased by exercise in aged mice. Mean fluorescence intensity for FITC-OVA was also examined in migrating cells (Table 2B). FITC-OVA MFI was greater in the cDC and DC populations in comparison to MΦ, however there
were no differences in MFI at 12 or 24 hours post-instillation between age groups and exercise treatments.

**Antigen Processing is Enhanced by Exercise in Aged Treadmill Trained Mice**

The ability of CD11c+ dendritic cells to process antigen was detected using DQ-OVA. DQ-OVA can only be detected in the 515 nm channel by flow cytometry after it has been processed in the endocytic vesicles. CD11c+ cells were isolated from the spleens of uninfected young and old, exercised and non-exercised mice then cultured with DQ-OVA or FITC-labeled OVA (Figure 4). FITC-labeled OVA was included as a control to ensure that these cells did not phagocytose antigen at different rates after 24 hr of culture. We were able to confirm that the rate of phagocytosis was consistent as equal numbers of FITC-OVA+CD11c+ cells were identified after a 24 hr culture period across groups. However, DQ-OVA+CD11c+ cell counts were different (a*e; p < 0.05) between all of the groups, indicating a difference in the antigen processing function in the CD11c+ cells isolated from the various hosts. Higher DQ-OVA+ cells were found per CD11c+ cells isolated from the aged treadmill exercised mice compared to the aged non-exercised, but conversely DQ-OVA+ CD11c+ cells were lower in the young treadmill trained mice in the comparison young non-exercised.

**Activation of CD8+IFNγ+ and CD4+IL-2+ Primed T Cells is altered with Exercise in the Young and Aged**

CD11c+ cells were sorted from spleens obtained from uninfected young and old, exercised or non-exercised mice. The CD11c+ cells were then combined with splenocytes derived from influenza-primed young hosts in order to evaluate the activation capabilities of CD11c+ cells from each treatment group (Fig. 5A and 5B). The percentage of CD8+ IFNγ+ per
CD3+ cells activated relative to the maximum activation (determined by representing activation in media or NP treatment as a percentage of PMA/ionomycin) is reported in the results. The percentage of maximum activation was significantly increased in the young exercised compared to non-exercised, but not altered in the in the aged with exercise (e+; trend for a main effect of exercise, p < 0.05) (Fig. 5A). The percentage of CD4+ IL-2+ cells per CD3+ cells were elevated in the young exercised compared to the young non-exercised and decreased in the aged exercised compared to aged non-exercised. Mean fluorescence intensity (MFI) for IFNγ and IL-2 were examined in their respective cell types (CD8+ IFNγ+ and CD4+ IL-2+ cells) (Fig. 5B), there were no differences in IFNγ MFI in CD8+ IFNγ+ cells between groups and there was a significant age by exercise interaction for IL-2 MFI in CD4+ IL-2+ cells (a*e, p < 0.05). IL-2 MFI was reduced in CD4+ IL-2+ cells isolated from the young exercised compared to the young non-exercised and the IL-2 MFI was increased in the aged exercised compared to aged non-exercised. These results suggest an alteration in the antigen presentation function with exercise more so in the young CD11c+ cells than in the aged with exercise.

**Discussion:**

Early results from our lab, and others, have identified regular moderate exercise for its role in reducing illness severity and viral load over the course of an influenza infection. The results reported in this paper assess intrinsic functions of professional antigen presenting cells and their capacity to enhance the immune response to influenza in ways that benefit the treadmill-trained host. Knowing that the innate and adaptive immune responses to influenza infection are mediated by respiratory dendritic cells, we hypothesized that we would find a more rapid kinetics of migration in the lung draining lymph nodes of aged and young exercised mice.
during influenza infection. Contrary to our expectations, our experiments identify significant reductions in total lymph node cells collected from aged exercised, along with reduction in total numbers of CD11c+, CD19+, and CD8+ cells in the aged exercised compared to aged non-exercised mice at days 4, 5 and 10 p.i. Similar reductions in total lymph node cells were seen in the young exercised compared to non-exercised at day 10 p.i. along with reduced CD11c+, CD19+ and CD8+ cells. Although we did identify reductions in numbers of total lymph node cells and more specific populations in the young and aged exercised mice compared to non-exercised, the past studies should not be overlooked as exercised (aged and young) mice had reduced symptom severity, time to recovery (recently submitted), and reduced lung viral titer (data not shown for this experiment) [238]. Taken together, these results suggest that exercise-induced reduction in viral load is the cause of the reduced lymph node cell numbers and not an indication of an exercise-induced impairment in the lymph node environment. Further experimentation was carried out in various non-infectious models to assess specific dendritic cell functions, in this way the results would not be confounded by the reduced antigen induced by exercise in our murine model of influenza.

We next examined the ability of various lung cell populations to phagocyteFITC-labeled OVA then transport that antigen to the lung draining lymph nodes in aged and young exercised and non-exercised uninfected mice (Table 1A and 1B, Table 2A and 2B). Previous work in other labs has identified respiratory dendritic cell populations involved in uptake and trafficking of antigen from the lung to the lung draining lymph nodes during influenza infection [262-264]. Work has also been done to characterize specific locations within the lung in which these dendritic/phagocytic cells reside [129, 265]. We used this information to assess the ability of specific respiratory DC subsets, along with granulocytes (SSC+ cells) and two types of
macrophages, to uptake FITC-labeled OVA and determine if this phagocytic function was altered with exercise. Age was the greatest cause of differences among populations at 3hr after FITC-OVA instillation; numbers of certain cell subsets (aMΦ, arDC and iDC) were reduced in the aged compared to young. This was initially interpreted as an age-associated impairment in these cells until FITC-OVA MFI was taken into consideration as it showed that these cells isolated from aged mice were taking up more FITC-OVA on a per cell basis than the cells isolated from the young mice. Exercise reduced the numbers of aMΦ taking up antigen in both the young and aged mice at 3 and 12 hr post-instillation, and exercise was associated with reduction in iDC numbers in the aged exercised, yet increased iDC numbers in the young at 3hr. This possibly suggests that exercise is altering the kinetics and cell populations composing the lung environment even after 3hr of antigenic stimulation. By 12 hr, total FSC+ cells that had taken up FITC-OVA were increased in the young with exercise, yet decreased in the aged with exercise. Furthermore, at 12 hr, while reduced numbers of aMΦ were identified in the young and aged exercised, iMΦ were increased in these same groups. Taken together with the results at 3hr, this indicates the populations of macrophages were altered with exercise, especially taking into account that FITC-OVA MFI was increased in the iMΦ population at 12 hr in the exercised young and aged mice. By 24 hr the exercise associated decreases in cells numbers or MFI are most likely the product of increased uptake and trafficking of FITC-OVA out of the lungs with exercise. While iMΦ numbers remained increased at 24hr in both young and aged exercisers, FITC-OVA MFI per iMΦ was decreased by this time point in both exercised groups. The iMΦ might be a resident cell that does not migrate out of the lung as readily as dendritic cells. If iMΦs are residing in the tissue and continuously taking up FITC-OVA, as our data implies, this could be of benefit to the young and aged exercised mice for containing and clearing viral pathogens.
such as influenza as they might be phagocytosing cells that have died after becoming infected [266-268]. Macrophage and SSC+ cell populations represented greater total numbers than the dendritic cells subsets in the lung and have been shown to be involved in cleanup and wound repair in the lungs, whereas the smaller DC subsets are thought to be more important for trafficking antigen to the lymph nodes [269, 270]. The only exercise-associated changes seen in dendritic cell populations were the increased iDC numbers in the young exercised at 3 hr p.i. compared to young non-exercised, and the increased FITC-OVA MFI in iDC at 12 hours in both young and aged exercised mice in comparison to young and aged non-exercised.

Next we examined the migration of dendritic cells to the lung draining lymph nodes at 12 and 24 hours post FITC-OVA instillation. We were able to show with these experiments that exercise was associated with increased trafficking of conventional dendritic cells and general dendritic cells to the lung draining lymph nodes at 12 hr and 24 hr post-instillation in both the young and aged exercised mice (Table 3A). FITC-OVA MFI within each cell type was not different in any of the treatments (Table 3B), but much higher in the two dendritic cell populations in comparison to general macrophages. While there is a slight potential for lung macrophages to have migrated from the lung, most likely this population is composed of a lymph node resident macrophage. The lymph node resident macrophage may be a subcapsular macrophage that functions to take up FITC-OVA in this environment given the possibility that FITC-OVA might enter the lymph node from circulation. This aside, the data for this experiment indicates that since the MFI is much higher in each of the dendritic cell populations (at least in comparison to the general macrophage) these cells are actively taking up FITC-labeled OVA in the lungs and trafficking it to the lymph nodes to a greater degree in both the young and aged exercised mice in comparison to the non-exercised control mice.
Taken all together the results suggest an increase in migration of the two dendritic cell subsets. Given that FITC-OVA MFI was higher in iDC at 12 hours in the aged and young exercised mice compared to non-exercised, but not different between the non-exercised and exercised at 24 hr; this finding may indicate an earlier peak of antigen uptake in this lung interstitial cell population. In addition, since we identified greater numbers of both cDC and DC in the lymph nodes at 12 hr in the exercised mice this could be indicative of increased migration. This increased migration is beneficial to the exercised host, as the earlier antigen is trafficked to the lung draining lymph node to find its cognate T-cell and B-cell population, the sooner clonal expansion can occur in both of these adaptive cell subsets leading to earlier clearance of the influenza infection from the lungs. Furthermore, if there is a shift in early innate cell kinetics leading to peak antigen uptake and earlier clearance, the shift in kinetics could limit the virus at a very early time point (< 24 hr after infection) this could reduce total lung viral load over the course of the infection as viral replication generally peaks between 2-3 days after infection begins. The results from these experiments might provide a partial explanation for the early results from our lab which have identified reduced viral antigen through infection. These results also show that apart from the dendritic cell migration, the capability of the other respiratory phagocytic subsets (increased FITC-OVA MFI in granulocytes as well as increased numbers and MFI of interstitial macrophages) is enhanced with exercise.

We also examined antigen processing in CD11c+ cells isolated from aged and young exercised and non-exercised mice. Results were mixed for the antigen processing experiment which showed increased antigen processing (more DQ-OVA+ cells) in the aged exercised, but not young, than non-exercised. The results from the experiment are promising for the aged mice, but suggest an impairment induced by exercise in the young exercised mice compared to non-
exercised. One possible explanation is that CD11c is a widely used marker for various dendritic cell subsets including conventional dendritic cells, lymphoid resident (CD8α+) dendritic cells and plasmacytoid dendritic cells. Conventional dendritic cells and lymphoid resident DC could process and present antigen, though the conventional dendritic cells would do so at a faster rate most likely, while the plasmacytoid dendritic cell is not known for its antigen processing capability. Since the CD11c+ cells were isolated from young and aged mice which have been shown to have variations in the numbers of the various types of dendritic cells making up the total lymph node population, perhaps the reduced antigen processing is not the product of a dysfunction in the young mice induced by exercise but simply the product of an alternative DC population that is less likely to present antigen making up a greater portion of the total CD11c+ cells isolated from the spleens of the various hosts. Therefore results seem promising for the aged mice that exercised however, unless the various populations of dendritic cells isolated from the spleen of the varied hosts (young/old, exercised/non-exercised) can be better characterized conclusions about the antigen processing results should be made with caution.

Knowing that CD4+ and CD8+ T cells are necessary for resolving an influenza infection, the capability of CD11c+ cells derived from exercised and non-exercised hosts to activate influenza-specific T cells was examined. In the young mice, exercised increased the percentages of CD8+IFNγ+ cells as well as the MFI for IFNγ in CD8+ cells relative to the maximum amount of activation achieve by leukocyte activation cocktail. Additionally CD4+IL-2+ percentages were also increase by exercise treatment in the young mice, yet IL-2 MFI was not different. In contrast, in the aged mice exercise reduced the percentage of CD4+IL-2+ cells but enhanced the IL-2 MFI. The results suggest that the exercise-induced effects on CD11c+ cells and their ability to stimulate the adaptive response (via CD4 and CD8) cells were enhanced to a
greater extent in the young mice. However, given that a reduction in IL-2 is a hallmark of aging, the exercise-associated increase in CD4+ IL-2 MFI may be an important effect of exercise.

To our knowledge, we were the first group to compare the differential effects of exercise on uptake and trafficking by dendritic cells of the lung and to assess the activation potential of dendritic cells isolated from both young and aged exercised hosts. Many of the immune improvements that we have identified in aged and young exercised mice could be explained in part by the results from this study. Furthermore physical activity remains as a relatively inexpensive means of improving quality of life in the elderly and young and should not be overlooked for its potential role in improving overall health in the human population as a whole.
Figures Chapter III:

Figure 1A.
Figure 1A and B: Lymph node immune cell populations in aged mice at day 4, 5 and 10 p.i. and young mice at day 5 and 10 p.i. 1A) Aged exercised and non-exercised mice infected with influenza were euthanized at days 4, 5 and 10 p.i. (p.i.). Young exercised mice were included at day 10 p.i. for determination of total numbers of lymph node cells per lymph node. 1B) Percentages of CD11c+ (CD19- CD8α-), CD19+ (CD11c- CD8α-) and CD8α+ (CD11c- CD19-) cells at day 10 p.i.. Data are mean values ± the SEM and representative of three separate experiments; day 4 n = 10-12, day 5 n = 10-11 and day 10 n= 6-8 for infected groups, n = 6-7 for uninfected mice.
Table 1. Day 10 p.i. lymph node cell populations: CD11c+, CD19+CD11c-CD8α-, CD8α+CD19-CD11c- cells (see S1A and S1B graphs for gating strategy).

<table>
<thead>
<tr>
<th>Percentage/Count</th>
<th>Y-No-Ex</th>
<th>Y-TM-Ex</th>
<th>A-No-Ex</th>
<th>A-Tm-Ex</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11c%</td>
<td>4.18±0.66</td>
<td>8.24±4.24</td>
<td>7.23±0.43</td>
<td>7.87±0.36</td>
</tr>
<tr>
<td>#s per LN</td>
<td>10320±1629</td>
<td>7550±13444</td>
<td>42523±2471</td>
<td>25763±1178</td>
</tr>
<tr>
<td>CD19%</td>
<td>49.09±1.47</td>
<td>47.37±0.95</td>
<td>35.84±2.88</td>
<td>36.38±1.48</td>
</tr>
<tr>
<td></td>
<td>120981±3629</td>
<td>109176±3000</td>
<td>205928±16944</td>
<td>119095±4845</td>
</tr>
<tr>
<td>CD8+%</td>
<td>10.21±0.63</td>
<td>10.68±0.51</td>
<td>8.5±1.65</td>
<td>8.13±1.38</td>
</tr>
<tr>
<td></td>
<td>25208±1553</td>
<td>13643±1609</td>
<td>50008±9884</td>
<td>26614±4517</td>
</tr>
</tbody>
</table>
Table 2A. FITC-OVA positive cells per total lung cells collected.

<table>
<thead>
<tr>
<th>Cell Populations</th>
<th>Y-No-Ex ±SE</th>
<th>Y-TM-Ex ±SE</th>
<th>A-No-Ex ±SE</th>
<th>A-TM-Ex ±SE</th>
<th>Age/Ex effects &amp; Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lungs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>3hr</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total FSC+</td>
<td>506000±51268</td>
<td>485777±51559</td>
<td>558400±50616</td>
<td>543500±32598</td>
<td></td>
</tr>
<tr>
<td>Granulocytes (SSC+)</td>
<td>72422±11107</td>
<td>74592±15826</td>
<td>84563±15884</td>
<td>85615±21215</td>
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</tr>
<tr>
<td>Alveolar MΦ</td>
<td>175555±14988</td>
<td>132666±17838</td>
<td>130170±9162</td>
<td>81678±15749</td>
<td>a, e</td>
</tr>
<tr>
<td>Interstitial MΦ</td>
<td>56011±7566</td>
<td>50166±7350</td>
<td>57010±4851</td>
<td>52112±8680</td>
<td></td>
</tr>
<tr>
<td>Airway DC</td>
<td>2931±494</td>
<td>2348±503</td>
<td>4479±774</td>
<td>5100±1297</td>
<td>a</td>
</tr>
<tr>
<td>Alveolar DC</td>
<td>8501±1091</td>
<td>12637±2555</td>
<td>7288±1529</td>
<td>6176±2167</td>
<td>a+</td>
</tr>
<tr>
<td>Interstitial DC</td>
<td>11431±1282</td>
<td>13523±2612</td>
<td>10153±1414</td>
<td>5467±2081</td>
<td>a+ e, a</td>
</tr>
<tr>
<td><strong>12hr</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Total FSC+</td>
<td>489400±40449</td>
<td>581428±7498</td>
<td>600375±12953</td>
<td>571600±24896</td>
<td>a*e, a</td>
</tr>
<tr>
<td>Granulocytes (SSC+)</td>
<td>48460±8081</td>
<td>6382±2491</td>
<td>72137±7400</td>
<td>68600±4102</td>
<td>a</td>
</tr>
<tr>
<td>Alveolar MΦ</td>
<td>97420±7599</td>
<td>70628±2917</td>
<td>73162±7350</td>
<td>47640±3599</td>
<td>a, e</td>
</tr>
<tr>
<td>Interstitial MΦ</td>
<td>38100±2120</td>
<td>50000±2661</td>
<td>50062±3209</td>
<td>66020±5987</td>
<td>a, e</td>
</tr>
<tr>
<td>Airway DC</td>
<td>3726±726</td>
<td>3834±595</td>
<td>6670±1034</td>
<td>6742±1304</td>
<td>a</td>
</tr>
<tr>
<td>Alveolar DC</td>
<td>16600±2436</td>
<td>15127±2017</td>
<td>17725±2360</td>
<td>20570±5609</td>
<td>a</td>
</tr>
<tr>
<td>Interstitial DC</td>
<td>17160±3106</td>
<td>15414±1276</td>
<td>13565±1421</td>
<td>12814±2486</td>
<td>a+ e, a</td>
</tr>
<tr>
<td><strong>24hr</strong></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Total FSC+</td>
<td>464333±18923</td>
<td>496666±21501</td>
<td>493500±15597</td>
<td>492428±29409</td>
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</tr>
<tr>
<td>Granulocytes (SSC+)</td>
<td>513556±6755</td>
<td>53450±7711</td>
<td>89650±6072</td>
<td>84857±18110</td>
<td>a</td>
</tr>
<tr>
<td>Alveolar MΦ</td>
<td>77111±9325</td>
<td>98333±8583</td>
<td>57175±8477</td>
<td>54957±6369</td>
<td>a</td>
</tr>
<tr>
<td>Interstitial MΦ</td>
<td>36655±3551</td>
<td>33550±3090</td>
<td>28750±3031</td>
<td>48928±4890</td>
<td>a*e, e</td>
</tr>
<tr>
<td>Airway DC</td>
<td>3610±414</td>
<td>3191±473</td>
<td>5233±577</td>
<td>4641±347</td>
<td>a+</td>
</tr>
<tr>
<td>Alveolar DC</td>
<td>7475±1660</td>
<td>8660±2122</td>
<td>4920±1230</td>
<td>5600±1443</td>
<td>a</td>
</tr>
<tr>
<td>Interstitial DC</td>
<td>8964±2681</td>
<td>9056±2169</td>
<td>3425±948</td>
<td>7907±1988</td>
<td></td>
</tr>
</tbody>
</table>

'e' significant exercise effect (p<0.05), 'e+' p<0.1, 'a' a significant age effect (p<0.05), 'a+' p<0.1, 'a*e' significant age by exercise interaction where p<0.05 (Two-Way ANOVA), 'a+e' p<0.1

Table 2A. Antigen uptake with exercise in the lungs of aged and young treadmill trained mice. Intranasal instillation of FITC-labeled OVA was carried out to assess antigen uptake by lung phagocytic cells with and without exercise in aged and young mice. Subsets of mice that had been treated with FITC-OVA were euthanized at 3hr (n = 8-10), 12hr (n = 6-8) and 24hr (n = 8-9) post-instillation. Total FITC-OVA+ FSC+ and SSC+ cells (general granulocytes) and FITC-OVA+ subsets of lung cells (alveolar macrophages, interstitial macrophages, airway dendritic cells, alveolar dendritic cells and interstitial dendritic cells – See Supplemental graphs 2A-2K for flow cytometric gating strategy). Total FITC-OVA+ counts per cell type were related
back to the total lung cells collected per mouse. Data are represented by mean counts for each cell population ± SEM. ‘e’ significant exercise effect ($p < 0.05$), ‘e+’ ($p < 0.1$), ‘a’ a significant age effect ($p < 0.05$), ‘a+’ ($p < 0.1$) ‘a*e’ significant age by exercise interaction where $p < 0.05$, ‘a+e’; $p < 0.1$. Statistical significance was determined by two-way ANOVA. Data are representative of two experiments, 3hr n = 8-10, 12hr n = 10-12 and 24hr n = 6-8.
Table 2B. Mean Fluorescence Intensity of FITC-OVA in FITC-OVA+ lung cell subsets.

<table>
<thead>
<tr>
<th></th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lungs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3hr</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Granulocytes (SSC+)</td>
<td>19761 ± 2928</td>
<td>16251 ± 2521</td>
<td>20525 ± 1923</td>
<td>23605 ± 3003</td>
<td></td>
</tr>
<tr>
<td>Alveolar MΦ</td>
<td>1220 ± 196</td>
<td>1032 ± 270</td>
<td>1504 ± 258</td>
<td>1800 ± 394</td>
<td>a+</td>
</tr>
<tr>
<td>Interstitial MΦ</td>
<td>1587 ± 352</td>
<td>1746 ± 541</td>
<td>2531 ± 659</td>
<td>4226 ± 1218</td>
<td>a</td>
</tr>
<tr>
<td>Airway DC</td>
<td>1459 ± 208</td>
<td>1124 ± 228</td>
<td>1788 ± 250</td>
<td>2312 ± 237</td>
<td>a</td>
</tr>
<tr>
<td>Alveolar DC</td>
<td>1070 ± 192</td>
<td>930 ± 283</td>
<td>1884 ± 416</td>
<td>3616 ± 1151</td>
<td>a</td>
</tr>
<tr>
<td>Interstitial DC</td>
<td>1570 ± 316</td>
<td>1579 ± 469</td>
<td>2434 ± 491</td>
<td>3563 ± 698</td>
<td>a</td>
</tr>
<tr>
<td>12hr</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Granulocytes (SSC+)</td>
<td>23000 ± 2207</td>
<td>36835 ± 2269</td>
<td>30575 ± 1104</td>
<td>33960 ± 2742</td>
<td>a*e, e</td>
</tr>
<tr>
<td>Alveolar Macs</td>
<td>1381 ± 332</td>
<td>1621 ± 53</td>
<td>1708 ± 166</td>
<td>1893 ± 230</td>
<td>a</td>
</tr>
<tr>
<td>Interstitial MΦ</td>
<td>1994 ± 488</td>
<td>3264 ± 186</td>
<td>2889 ± 338</td>
<td>3964 ± 535</td>
<td>a, e</td>
</tr>
<tr>
<td>Airway DC</td>
<td>1259 ± 156</td>
<td>1525 ± 96</td>
<td>1727 ± 171</td>
<td>1889 ± 239</td>
<td>a</td>
</tr>
<tr>
<td>Alveolar DC</td>
<td>1070 ± 254</td>
<td>1355 ± 71</td>
<td>1812 ± 225</td>
<td>1979 ± 407</td>
<td>a</td>
</tr>
<tr>
<td>Interstitial DC</td>
<td>1537 ± 402</td>
<td>2188 ± 93</td>
<td>2403 ± 263</td>
<td>2873 ± 441</td>
<td>a, e+</td>
</tr>
<tr>
<td>24hr</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Granulocytes (SSC+)</td>
<td>38922 ± 4379</td>
<td>3050 ± 3706</td>
<td>42752 ± 4473</td>
<td>33585 ± 4801</td>
<td>e+</td>
</tr>
<tr>
<td>Alveolar Macs</td>
<td>2065 ± 317</td>
<td>1790 ± 304</td>
<td>2598 ± 140</td>
<td>2256 ± 314</td>
<td>a+</td>
</tr>
<tr>
<td>Interstitial MΦ</td>
<td>4481 ± 663</td>
<td>3786 ± 707</td>
<td>7726 ± 722</td>
<td>5708 ± 1018</td>
<td>a, e+</td>
</tr>
<tr>
<td>Airway DC</td>
<td>2065 ± 275</td>
<td>1843 ± 357</td>
<td>3235 ± 259</td>
<td>2721 ± 402</td>
<td>a</td>
</tr>
<tr>
<td>Alveolar DC</td>
<td>2254 ± 404</td>
<td>1970 ± 497</td>
<td>4679 ± 726</td>
<td>3690 ± 748</td>
<td>a</td>
</tr>
<tr>
<td>Interstitial DC</td>
<td>2835 ± 484</td>
<td>2329 ± 500</td>
<td>4141 ± 289</td>
<td>3501 ± 524</td>
<td>a</td>
</tr>
</tbody>
</table>

\*e\* significant exercise effect (p<0.05), \*e\*; p<0.1, \*a\* a significant age effect (p<0.05), \*a\*; p<0.1, \*a\*e\* significant age by exercise interaction where p<0.05 (Two-Way ANOVA), \*a\*e\*; p<0.1

Table 2B. Median fluorescence intensity of FITC-OVA of lung phagocytic cells. For each lung cell subset represented in Table 1A, FITC-OVA median fluorescence intensity was determined (excepting total FSC+ cells). Mean MFI is represented for each cell population ± SEM. Utilizing a two-way ANOVA for statistical analysis ‘e’ significant exercise effect (p < 0.05), ‘e’ (p < 0.1), ‘a’ a significant age effect (p < 0.05), ‘a+’ (p < 0.1) ‘a*e’ significant age by exercise interaction where p < 0.05, ‘a+e’; p < 0.1.
Table 3A. FITC-OVA+ cells (counts per total cells from single lymph node) trafficking to lung-draining lymph nodes.

<table>
<thead>
<tr>
<th>Cell Populations</th>
<th>Y-No-Ex</th>
<th>Y-TM-Ex</th>
<th>A-No-Ex</th>
<th>A-TM-Ex</th>
<th>Age/Ex effects &amp; Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean±SE</td>
<td>Mean±SE</td>
<td>Mean±SE</td>
<td>Mean±SE</td>
<td></td>
</tr>
<tr>
<td><strong>12hr</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total FITC+</td>
<td>20846±8338</td>
<td>61011±16554</td>
<td>83384±33461</td>
<td>117661±72082</td>
<td>a</td>
</tr>
<tr>
<td>CD11c hi (cDC)</td>
<td>1061±227</td>
<td>1161±167</td>
<td>824±174</td>
<td>2845±1010</td>
<td>a*e, e</td>
</tr>
<tr>
<td>CD11c int (gDC)</td>
<td>2290±1107</td>
<td>4089±785</td>
<td>3228±670</td>
<td>14378±2750</td>
<td>a*e, a, e</td>
</tr>
<tr>
<td>CD11b+11c lo(MΦ)</td>
<td>10074±3426</td>
<td>24795±3144</td>
<td>40320±17756</td>
<td>36498±6188</td>
<td>e</td>
</tr>
<tr>
<td><strong>24hr</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Total FITC+</td>
<td>33090±19927</td>
<td>16974±3619</td>
<td>16521±4226</td>
<td>46568±21701</td>
<td>a</td>
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<tr>
<td>CD11c hi (cDC)</td>
<td>748±226</td>
<td>604±172</td>
<td>533±152</td>
<td>913±221</td>
<td>a+e</td>
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<td>CD11c int (gDC)</td>
<td>3237±1521</td>
<td>1739±838</td>
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<td>5251±2311</td>
<td>a+e</td>
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<tr>
<td>CD11b+11c lo(MΦ)</td>
<td>15592±3561</td>
<td>8424±881</td>
<td>6523±1108</td>
<td>15271±3093</td>
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</table>

‘e’ significant exercise effect (p<0.05), ‘e+’; p<0.1, ‘a’ a significant age effect (p<0.05), ‘a+’; p<0.1, ‘a*e’ significant age by exercise interaction where p<0.05 (Two-Way ANOVA), ‘a+e’; p<0.1

Table 3A. Antigen trafficking from lungs to lung draining lymph nodes. FITC-OVA+ total FSC+ cells, general macrophages (CD11b+ and CD11clo-int, and high autoflourescence), and dendritic cells (conventional dendritic cells (CD11c^hi) and dendritic cells (CD11c^int)) were characterized in the lymph nodes to determine trafficking of these cells to the lung draining lymph node at 12 and 24 hours post intranasal instillation of FITC-labeled OVA. Cell counts are relative to total cells collected per lymph node in each mouse. Counts are represented as total cell count per lymph node ± SEM. Statistical analysis was carried out using a two-way ANOVA, ‘e’ significant exercise effect (p < 0.05), ‘e+’(p < 0.1), ‘a’ a significant age effect (p < 0.05), ‘a+’(p < 0.1) ‘a*e’ significant age by exercise interaction where p < 0.05, ‘a+e’; p < 0.1. Data is representative of one experiment; 12 hr n = 10-12 and 24hr n = 6-8.
Table 3B. Median fluorescence intensity for FITC-OVA in lymph node cells positive for FITC-OVA.

<table>
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<td><strong>12 hr</strong></td>
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<td></td>
</tr>
<tr>
<td>CD11c hi (cDC)</td>
<td>2992±752</td>
<td>2470±227</td>
<td>4057±1927</td>
<td>3295±830</td>
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<tr>
<td>CD11c int (gDC)</td>
<td>1478±277</td>
<td>1151±153</td>
<td>1746±732</td>
<td>1268±239</td>
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<tr>
<td>CD11b+11c lo(MΦ)</td>
<td>335±70</td>
<td>280±37</td>
<td>205±23</td>
<td>264±29</td>
<td></td>
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<tr>
<td><strong>24 hr</strong></td>
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</tr>
<tr>
<td>CD11c hi (cDC)</td>
<td>3761±557</td>
<td>3218±617</td>
<td>2070±25</td>
<td>2449±547</td>
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<tr>
<td>CD11c int (gDC)</td>
<td>1301±211</td>
<td>1218±57</td>
<td>1060±179</td>
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<tr>
<td>CD11b+11c lo(MΦ)</td>
<td>212±27</td>
<td>283±14</td>
<td>253±43</td>
<td>441±207</td>
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</table>

‘e’ significant exercise effect (p < 0.05), ‘e+’ (p < 0.1), ‘a’ a main effect of age (p < 0.05), ‘a+’ (p < 0.1), ‘a*e’ significant age by exercise interaction where p<0.05 (two-way ANOVA), ‘a+e’ (p < 0.1).

Table 3B. Median fluorescence intensity of FITC-OVA for lymph node dendritic cell. For cell populations represented in Table 2B MFI for FITC-OVA was determined per cell. Data is represented as MFI ± SEM. Statistical analysis by two-way ANOVA, ‘e’ significant exercise effect (p < 0.05), ‘e+’ (p < 0.1), ‘a’ a significant age effect (p < 0.05), ‘a+’ (p < 0.1) ‘a*e’ significant age by exercise interaction where p < 0.05, ‘a+e’ (p < 0.1). Data is representative of one experiment; 12 hr n = 10-12 and 24 hr n = 6-8.
Figure 4. Endocytic processing by dendritic cells. Spleen-derived CD11c+ cells were sorted from exercised and non-exercised mice and culture with DQ-OVA or FITC-OVA to determine uptake and to assess endocytic processing in CD11c+ cells. Spleens from two to three mice were combined prior to cell sorting to obtain enough CD11c+ cells to carry out the experiment (n = 4). 1.0 x 10^6 CD11c+ cells were cultured per well with DQ-OVA and FITC-OVA for 24 hours. Unlabeled OVA was included as background control but not detectable by flow cytometry (data not shown). Cell counts of DQ-OVA+ cells (right) and FITC-OVA+ cells (left) were determined by flow cytometry. Two-way ANOVA was used to determine the statistical significance of the findings and whether there was a significant interaction between age and exercise (a*e; p < 0.05).
Figure 5A.

Percentage of Maximum (LAC) Activation

- CD8+IFNg+
- CD4+IL-2+

Percentage of CD3+ cells

- Y-No-Ex
- Y-TM-Ex
- A-No-Ex
- A-TM-Ex

Percentage of Maximum (LAC) Activation

- e+
- a+e

Percentage of CD3+ cells

CD8+IFNg+
CD4+IL-2+
Figure 5A and B. Antigen presentation and activation capacity of spleen derived CD11c+ cells isolated from exercised hosts. Data is represented as a percentage (% of CD3 cells and MFI for IL-2 and IFNγ) of the maximum activation induced by leukocyte activation cocktail in each treatment group. CD11c+ cells isolated from uninfected mice were treated with UV-inactivated influenza post sorting then cultured with influenza primed splenocytes from day 7 influenza infected young mice. Antigen presentation and activation capability of these cells was determined by representing the 5A) percentage of CD8+ IFNγ+ or CD4+ IL-2+ cells per CD3+ cells to the maximum percentage of each of these same subsets activated by leukocyte activation.
cocktail. 5B) Percentage of maximum IFNγ MFI per CD8+ IFNγ+ and IL-2 MFI per CD4+ IL-2+ produced in response to leukocyte activation cocktail. Two-way ANOVA was used to determine main effects of age (a; \( p < 0.05 \) and a+; \( p < 0.1 \)) and exercise (e; \( p < 0.05 \) and e+; \( p < 0.1 \)) and interactions between age and exercise (a*e; \( p < 0.05 \) and a+e; \( p < 0.1 \)). For each group, \( n = 5-6 \).
Supplemental Figures Chapter III:

Supplemental 1A and 1B
Supplemental Figures 1A and 1B: Flow cytometry gating strategy for the determination of lymph node cell subsets of IAV infected mice. S1A) Gate 1 – $\text{CD11c}^{\text{neg-lo}}$ cells and Gate 2 – $\text{CD11c}^{\text{int-hi}}$ cells and S1B) Gate 1 – $\text{CD11c}^{\text{neg-lo}}$ $\text{CD19}^{+}\text{CD8}^\alpha^{-}$ cells versus Gate 2 – $\text{CD8}^\alpha^{+}\text{CD19}^{-}$ cells.
Supplemental Figures S2A-S2E.

Supplemental Figure 2A-2E: Respiratory gating for SSC+ cells using FITC-labelled OVA and unlabelled OVA. S2A) SSC+ cells positive for FITC-labeled OVA, and S2B) unlabelled OVA. S2C and S2D) SSC+ cells that are positive for FITC-labeled OVA, and S2D)
undetectable SSC+ cells unlabeled treatment. S2E) Majority of OVA+ cells are also positive for CD11b+ and CD11c\textsuperscript{lo} (neutrophil phenotype).
Supplemental Figure S2F-S2J: Gating strategy for the detection of FSC+ lung phagocytic cell subsets. 

S2F) FSC+ cells selected. 

S2G) Interstitial macrophages detected, along with CD11c+CD11b+ subset and CD11c+CD11b- subset. 

S2H) Represents the interstitial dendritic
cell subset gate. S2I) CD11c+ alveolar macrophages, and S2J) airway dendritic cells CD103+ and alveolar dendritic cells.
Supplemental Figures 3A-3D.

3A. CD11c+MHC-II+ dendritic cells - Day 5 and 10 p.i.

3B. CD11c+CD8α+ resident LN dendritic cells of the lymph node

3C. Surface Expression Activation Markers: Day 5

3D. Surface Expression Activation Markers: Day 10
Supplemental Figures 3A-3D. Dendritic cell numbers and surface expression of CD86, CD80, MHC II, and CD40 on CD11c+ MHC II+ cells. 3A) CD11c+ MHC II+ cells at day 5 and 10 p.i. per 5.0X10^5 lymph node cells. 3B) CD11c+ CD8α+ cells at day 5 and 10 p.i. per 5.0X10^5 lymph node cells. Mean fluorescence intensity, or surface expression, of CD86, CD80, MHC II and CD40 on CD11c+ MHC II+ conventional dendritic cells at 3C) day 5 and 3D) day 10 p.i. One-way ANOVA was used for statistical analysis; * significant effect of exercise in the aged mice; p < 0.5, + trend for an exercise effect in the aged mice; p < 0.1. ** significant effect of exercise in the young mice; p < 0.5, ++ trend for an exercise effect in the young mice; p < 0.1. *** significant effect of age; p < 0.5, +++ trend for an age effect; p < 0.1.
Supplemental Figures 4A-4I.

Supplemental Figure 4A.

![Graph showing FITC-OVA+ FSC+ cells per Mouse over time for different conditions.](image-url)
Supplemental Figures 4B-4E.
Supplemental Figures 4F-4I. Phagocytic lung cell populations involved in FITC-OVA uptake at 3, 12 and 24 hours post FITC-labeled OVA instillation. 4A) Total FITC-OVA+ FSC+ cells isolated from the lungs collected from young and aged, exercised and non-exercised mice comparitively. 4B-4E) FITC-OVA+ granulocytes, alveolar macrophages, and interstitial macrophages isolated from the lungs of all groups at 3, 12 and 24 hours post FITC-OVA instillation. 4F-4I) FITC-OVA+ airway, alveolar and interstitial dendritic cells at 3, 12 and 24 hours post-instillation. All results are relative to total lung cells collected per mouse.
Supplemental Figures 5A-5F.

Supplemental Figure 5A.

Count per Lymph Node

Hour Post-Instillation

12hr 24hr

Y-No-Ex 
Y-TM-Ex 
A-No-Ex 
A-TM-Ex
Supplemental Figure 5B.

Lymph Node Macrophage

Count per Lymph Node

Groups vs Y-No-Ex
Groups vs Y-TM-Ex
Groups vs A-No-Ex
Groups vs A-TM-Ex
Supplemental Figure 5A-5F. Lymph node cell populations involved in FITC-OVA trafficking isolated 12 and 24 hours post FITC-labeled OVA instillation.  5A) Total FITC-OVA+ FSC+ cells isolated from lymph nodes collected from young and aged, exercised and non-exercised mice.  5B) FITC-OVA+ lymph node macrophage population isolated from all groups at 12 and 24 hours post FITC-OVA instillation.  5C-5F) FITC-OVA+ conventional dendritic cells (CD11c<sup>hi</sup>) and other CD11c<sup>int</sup> dendritic cells isolated from the lymph nodes at 12 and 24 hours.
post-instillation. Results represent the mean ± SEM for total cells collected per lymph node per mouse.
Supplemental Figures 6A-6I.

6A. Percentage of Activated CD4+IL-2+ cells per Total CD3+ cells

6B. Percentage of CD3+ cells CD4+IFN+ cells

6C. CD4+TNF+ cells per Total CD3+ cells

6D. APC Activation Assay - CD4+IL-2+ cell MFI

6E. APC Activation Assay - CD4+IFN+ MFI

6F. CD4+TNF+ cell TNF MFI

6G. IL-2 Protein Levels

6H. Protein Levels IFN in Supernatants

6I. TNF Protein Levels
**Supplemental Figure 6J.**

96hr Dendritic Cell Supernatants

![Chart showing cytokine levels (IL-6, IP-10, KC, MIG) for different groups (Young, No-Ex, Young, TM-Ex, Old, No-Ex, Old, TM-Ex).](chart.png)
Supplemental Figure 6K.

96 hr Dendritic Cells Supernatants

- IL-10
- RANTES

Young, No-Ex
Young, TM-Ex
Old, No-Ex
Old, TM-Ex
Supplemental Figure 6L.

Supplemental Figures 6A-6L. Antigen presentation and activation utilizing CD11c+ cell isolated from each treatment group. 6A-6C) CD3+CD4+ T cells activation based on 6A) IL-2 6B) IFNγ and 6C) TNFα expression. Mean fluorescence intensity for 6D) IL-2, 6E) IFNγ and 6F) TNFα in each of the cooresponding populations (CD3+ CD4+ cells – positive for each of the relative cytokines). Cytokine production measured in supernatants at 24, 48 and 96 hours post culture, 6G) IL-2, 6H) IFNγ and 6I) TNFα. 6J) IL-6 and other inflammatory mediators measured at 96 hours post-culture. 6K) IL-10 and RANTES detected at 96 hours post culture. 6L) Chemotaxis factors, MCP-1, MIP-1α, MIP-1β and MIP-2 in cell culture supernatants at 96 hours post-culture. One-way ANOVA was used for statistical analysis; * significant effect of exercise in the aged mice; $p < 0.5$, + trend for an exercise effect in the aged mice; $p < 0.1$. ** significant effect of exercise in the young mice; $p < 0.5$, ++ trend for an exercise effect in the young mice; $p < 0.1$. *** significant effect of age; $p < 0.5$, +++ trend for an age effect; $p < 0.1$. 
Supplemental Figure 7A and 7B.

Figure 7A.

CD8b+ cells from BAL at Day 10p.i.
Supplemental Figures 7A and 7B. BAL cells populations detected at day 5 and 10 p.i. in young and aged, exercised and non-exercised mice. 7A) CD8+ T cells detected at day 10 post influenza infection and 7B) inflammatory monocytes detected at day 5 and 10 p.i..
**Supplemental Table 8.** Lung mRNA levels in day 5 infected mice.

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Supplemental Table 8. mRNA levels from the lungs of day 5 infected mice. Young and aged uninfected mice included, along with aged non-exercised compared to aged exercised mice.

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CHAPTER IV

LACK OF INTERFERON α IN YOUNG, BUT NOT AGED MICE, LEADS TO AN
EXACERBATED DISEASE STATE IN RESPONSE TO INFLUENZA INFECTION.

manuscript to be submitted to *The Journal of Immunology*

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Abstract:

Interferon-α produced early during Influenza A virus (IAV) infection has antiviral and immunomodulatory roles. Although older populations experience greater severity of IAV infection, the extent to which the IFNα response may be compromised by aging is largely unknown. In this study we examined the effect of age on IFNα response to IAV by blocking IFNα via anti-IFNα antibody treatment during day -1 to day 4 post-infection (p.i.). The results showed that IFNα antibody treatment increased morbidity among young mice, but reduced morbidity in aged mice. At day 4 p.i., anti-IFNα treatment reduced the bronchoalveolar lavage (BAL) concentration of most chemokines including eotaxin, IP-10, MCP-1, MIP1α, MIP1β, MIP-2, RANTES, as well as cytokines IL-1α, IL-1β, IL-6, IL-12p70 and TNFα. Also, anti-IFNα treatment resulted in greater numbers of inflammatory monocytes, neutrophils, and CD8+ cells, but reduced IL-15 in the lungs at day 8 p.i., and increased serum anti-influenza IgG and IgG1. A
lack of IFNα during primary infection resulted in a skewed IgG1/IgG2a response to secondary challenge along with a reduced number of influenza nucleopeptide (NP) specific lung resident CD8+ cells. Differential age effects of anti-IFNα treatment to primary infection included increased lung viral load in young mice at day 10 p.i., but reduced viral load in aged mice, and greater concentrations of IFNγ, TNFα, IL-12p70, IL-10 in BAL of aged mice but reduced IFNγ and IL-10 in young mice. Overall the findings suggest that IFNα may delay recovery from IAV in aged mice, but appears important for optimal recovery in young mice.

**Introduction:**

Influenza A virus is a highly contagious upper respiratory infection that causes widespread morbidity and mortality each year [17, 271]. Within hours of infection virus can be detected in the upper and lower respiratory tract of which is followed by an influx of immune cells (i.e. neutrophils, pDCs) and production of chemokines and cytokines, including type-I IFNs (IFNα/β) within the first 24 hours of infection[37, 138]. One segment of the population that is at increased risk for infection and complications from influenza are the elderly, due to the decreased efficacy of the annual vaccination among other immune alterations linked to the dysregulation of immunity that comes with aging [1, 272, 273]. With the aging of the U.S. population, there is a growing need to identifying differences in immune function between the aged and young in an effort to begin to develop effective treatment strategies.

Interferon alpha (IFNα) is a potent immunomodulatory protein that may be induced by signaling through different pathways (via TLRs, RIG-I) in leukocytes in response to viral infection [143, 274, 275]. Recently, type-I interferons were shown to play a role in linking innate and adaptive immune responses to influenza infection, primarily by enhancing dendritic
cell function (increased trafficking of these cells to the draining lymph nodes and up-regulation
of CD80 and CD86) and ‘fine tuning’ the adaptive response by inducing isotype switch in B-
cells [276-281]. Although specific roles for IFNα/β have been identified within the immune
response to influenza, little work has been done to distinguish the separate functions of IFNα and
IFNβ. Both IFNα and IFNβ serve an antiviral function by inducing signaling pathways within
various immune cells leading to the production several antiviral proteins known collectively as
interferon stimulated genes (ISG) [37, 282]. As examples, mxyovirus (influenza) resistance 1
(Mx1) and 2’5’oligoadenylate synthetase (OAS2) are both known to interfere with viral
replication and are up regulated in immune cells stimulated with virus or type-I interferons [277,
283, 284].

Studies have shown that interferons are essential for increasing the primary antibody
response to influenza vaccination [285] and promoting antibody isotype switch [276, 286].
Within the mouse model of influenza, it has been shown that in STAT1/-/- mice, the normal
expression of type I interferons is attenuated, leading to the induction of higher anti-influenza
antibody titer as compared to relative induction of influenza-specific cytotoxic T cells (CTL)
[280]. Influenza virus is commonly recognized as inducing a Th-1 response based upon the
requirement for a lung CTL response in order to achieve viral clearance. However, work by
Doherty et al. (2006), among others, highlights the necessity for both responses (CTLs and
antibody) in order to eventually clear virus from the lungs [17]. Since type-I interferons have
been shown to have a modulatory role in the development of both these responses, they remain
under investigation in terms of using IFNα/β as vaccine adjuvant [281, 287, 288]. A vaccine
trial conducted in older adults tested sublingual administered IFNα just prior to influenza
vaccination. However, this clinical trial failed to elicit any enhancement of primary antibody
response to vaccination [289]. In a mouse model of influenza infection, Beilharz et al (2007) showed increased protection in mice when an oral dose of type-I interferon was administered prior to lethal A/PR/8/34 infection [290]. These studies are somewhat contradictory, as one indicates no change in immune response of elderly individuals treated with oral IFNα whereas the murine study with live virus suggests an IFNα-induced improvement in the host response to a lethal dose of influenza. However, these models are very different, and therefore it is difficult to draw any conclusions regarding the role of IFNα in aged compared to young individuals. Since an aged host is consistently at a higher risk of infection with the seasonal strains of influenza it is reasonable to consider this area of research as it remains important to public health [119, 177, 291, 292].

The purpose of this study was to determine the role of IFNα in response to influenza infection in young and aged mice by administering daily anti-IFNα monoclonal antibody during the first four days of infection (a time point consistent with the innate immune response shaping of adaptive immunity). Sickness severity, antibody response, antibody class switching, expression of interferon-inducible genes, among other immune measures, were examined after primary infection in both treatment and age-matched control mice. The primary role of IFNα in the development of T-cell memory and antibody subtypes after lethal secondary challenge was also examined.
Materials and Methods

Animals, Injection, and Influenza Virus

Young (8 weeks) and aged (18 months) BALB/c mice were purchased from Jackson Laboratory (Bar Harbor, Maine) and housed in a pathogen-free environment. Mice were injected daily with 20 µg/mouse of rat IgG1 anti-mouse interferon-α, clone RMMA-1 (PBL Interferon Source, Piscataway, NJ) diluted in 0.01% BSA saline. Antibody administration began the day before (day -1) infection, and continued until day 4 p.i. (p.i.). Both young and aged control mice were injected with 20 µg/mouse of an irrelevant antibody, rat IgG1, at the same dose and time of day as anti-IFNα treated mice. Mice were anesthetized using aerosolized isoflurane and infected via an intranasal route with influenza virus strain A/PR/8/34 (H1N1) at a dose of 0.546 HA units per mouse [stock virus - HAU=8192/0.5mL or $10^{10.45}$ EID$_{50}$/mL - diluted 1:15,000 in sterile saline]. Over the course of the experiment mice were housed separately for body and food weight to be recorded daily in each of the experiments. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC).

Euthanization and Tissue Collection

On day 4 p.i. a small subset of mice from each group was euthanized. Lung tissue was collected and lung RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, California). Lung RNA was further purified using the RNeasy Micro Kit (QIAGEN, Valencia, California) then frozen at -80°C until further testing was carried out by PCR. Bronchoalveolar lavage (BAL) fluid was collected by inserting a catheter attached to a syringe through an incision in the trachea, and airways were flushed three times with 1 mL of PBS. BAL was then centrifuged and frozen at -80°C until further testing could be carried out by ELISA or Multiplex. On day 8 p.i.
and day 10 p.i. mice were also euthanized. Bronchoalveolar lavage (BAL), lung tissue, whole blood, and lymph nodes were collected at these time points. Whole blood was collected by cardiac puncture and centrifuged at 10000xg for 10 minutes for serum separation and stored at -80°C for further testing by ELISA. BAL and lung RNA were collected as described above and also tested by ELISA, Multiplex and PCR. Finally lymph nodes were homogenized through 20 micron filters into cRPMI complete media, treated with red blood cell lysis buffer, and remained in PBS until subsequent analysis of cell populations by flow cytometry.

**Long Term Antibody Response, Secondary Infection, and Lung Memory Response**

Subgroups of mice were set aside to survive primary infection to be re-challenged with a secondary lethal dose infection (4.36 HAU per mouse [stock virus - HAU=8192/0.5mL or $10^{10.45}$ EID$_{50}$/mL - diluted 1:1875 in sterile saline]) of influenza to assess the recall response to influenza after anti-IFNα treatment. Mice were housed separately through the course of primary infection, over a 6 month rest period and during re-challenge so that body weight and food weight could be monitored over the course of each infection (primary and secondary). Blood was collected by saphenous vein bleeds on these mice at Day 14, 21 and 28 for long term serum anti-Influenza antibody determination. It should be noted that none of the aged mice survived to secondary re-challenge. Aged mice were a minimum of 18 months of age at the time of the primary infection, and during the 6 month rest period most of the aged mice died of natural causes. Primary infection was carried out following the same procedure described above, but re-challenge mice were anesthetized with aerosolized isoflurane and infected via the intranasal route with a lethal dose of influenza virus strain (A/PR/8/34 H1N1) at a dose of 3 HAU per mouse. No anti-IFNα monoclonal antibody was injected after Day 4 post-primary infection. At
day 11 post-secondary infection (p.s.i.) mice were euthanized using the same protocol described so that whole blood and lungs could be collected. Serum was collected from whole blood by centrifugation and stored at -80°C until later analysis of serum IgGs and subtypes by ELISA. Whole lungs were also collected and homogenized by pressing through a 20 micron filter. These cells were treated with RBC lysis buffer, filtered three times using cRPMI and centrifugation (1500 rpms). Memory CD8+ T cells were determined by stimulation with NP peptide and subsequent flow cytometry.

*Identification Lung Cell Populations by Flow Cytometry*

Neutrophils (SSC+ CD45+ CD11b+ Gr-1+), inflammatory monocytes (FSC+ CD45+ CD11b+ CD11c- Gr-1+), plasmacytoid dendritic cells (CD11c<sup>int</sup> mPDCA-1+), and CD8+ T cells (CD45+ CD8β+) were identified in the BAL fluid using the following antibodies, hamster anti-mouse CD45 PE-Cy7, rat anti-mouse CD11c APC, rat anti-mouse CD11b PerCP-Cy5.5, rat anti-mouse Gr-1 PE, rat anti-mouse CD8β Alexa Flour 488, and rat anti-mouse mPDCA-1 PE (BD Pharmingen). Lung cells were plated at a rate of 1X10<sup>6</sup> cells per well and duplicate wells were treated with one of the following: 1uM of NP<sub>147-155</sub> (TYQRTRALV) peptide, brefeldin (BD Pharmingen) as a positive control, or no stimulation (media control) as a negative control. After an 8 hour treatment period with the peptide, cells were cultured with Golgi Plug for 2 hours then stained with rat anti-mouse CD8β Alexa Flour 488 and rat anti-mouse CD11a PE-Cy7. Cells were then fixed, permeabilized and stained using rat anti-mouse Granzyme B-PE and rat anti-mouse IFNγ-APC. After staining each sample was analyzed by flow cytometry for resident granzyme B producing CD8+ cells (CD8β+ CD11a+ GranB+), resident gamma-IFN producing (CD8β+ CD11a+ IFNγ+) CD8+ T cells and recruited CD8+ T cells (CD8β+ CD11a-). Each of
these cell populations were influenza-specific based upon in vitro stimulation with NP-peptide. Flow cytometry was performed using a FACSCanto flow cytometer. FlowJo 7.6 software was used for gating and analysis.

*IgG, IgG1, and IgG2a ELISA*

Mouse serum was tested for influenza specific total IgG, IgG1 and IgG2a antibody by indirect ELISA. ELISA plates (Immulon, Alexandria, VA) were coated with 100uL of Influenza A/PR/8/34 (200HAU/mL) diluted in carbonate coating buffer (pH 9.6) and incubated overnight at 4°C. Plates were blocked with PBS-0.1% BSA-blocking buffer at 37°C for one hour. Plates were washed three times with PBS/0.05% Tween 20 between each step. Serum was diluted and added to the plates in duplicate then incubated for four hours at room temperature. AP-conjugated goat anti-mouse IgG, IgG1, IgG2a diluted 1:100 were added to the plate and incubated for 8 hours at 4°C. Plates were developed using phosphatase substrate (Sigma Aldrich, St. Louis) for 15 minutes at room temperature. Optical density (OD) of each well was measured using a microtitration plate reader (FluoStar Galaxy) at 405 nm.

*PCR Microarray*

1,500 ng of purified lung mRNA was analyzed for interferon inducible gene expression using the 96-well Mouse Interferon α, β Response RT² Profiler™ PCR Array (SABiosciences, Frederick, Maryland) and protocol provided with the array. Samples were analyzed using the BioRad iCycler and corresponding MyIQ Optical System Software Version 1.0.
**Statistical Analysis**

All statistical analysis was performed using SPSS Statistics 17.0. A two-way ANOVA (age X antibody treatment) was used to establish main effects of age and antibody on immune measures, including antibody titers, BAL cytokines and chemokines, and dendritic cell populations. A mixed ANOVA (age X antibody treatment X time) with time as repeated measures (within subjects) variable was used to analyze change in body weight or food intake over time. Statistical trends were denoted by $p<0.1$ and significance levels for all tests were set at $p<0.05$.

**Results:**

*Illness Severity and Viral Load*

Daily body weight and food consumption were monitored over the course of three separate experiments as a means of assessing illness severity to influenza infection with and without IFNα involvement. The pattern of weight loss was the same over three replicate experiments. Weight loss over time was more severe in young mice treated with anti-IFNα antibody, and the opposite pattern was observed in the aged mice such that anti-IFNα treatment resulted in reduced weight loss (Fig. 1A). The analysis of body weight data from day 8 and 10 showed a significant age by antibody by time interaction (Mixed ANOVA; $p < 0.05$), and follow up analyses within each age group showed an antibody treatment by time interaction (greater weight loss in young anti-IFNα treated mice and reduced weight loss in aged anti-IFNα treated mice). As a further means to assess illness severity, food consumption was monitored (Fig.1B). Again, a significant age by antibody treatment interaction ($p < 0.05$) was found suggesting that young mice and old mice did not respond in a similar manner at both day 8 and day 10 post-
infection (p.i.). Results showed that young anti-IFNα treated mice ate significantly ($p < 0.05$) less food than the young control mice, indicating an exacerbated disease state in the young when IFNα was blocked at day 8 (similar trend at day 10). Conversely, the aged anti-IFNα treated mice consumed more ($p < 0.1$) food than the old control mice over the course of infection (both day 8 and day 10 p.i experiments). Bronchoalveolar lavage fluid (BALF) was also tested for influenza virus at day 4, 8 and 10 p.i. to assess the effect of anti-IFNα treatment on lung viral load (Fig. 1C). At Day 4 p.i., there were no significant differences between the treatment groups. At day 8 p.i., aged mice had slightly greater viral loads in the BAL fluid compared to young mice ($p < 0.1$), whereas at day 10 p.i., aged mice had lower viral loads than young mice ($p < 0.05$). At day 8 p.i., there was not a significant effect of anti-IFNα treatment on viral load, but by day 10 p.i., a significant interaction was observed such that anti-IFNα treatment in young mice was associated with greater viral load whereas in old mice, anti-IFNα treatment resulted in reduced virus.

_Serum anti-Influenza IgG are Altered in Response to anti-IFNα Treatment in Both Young and Aged mice_

Serum antibody titers for total anti-influenza IgG and subtypes (IgG1 and IgG2a) were evaluated as early as day 8 p.i., and until Day 28 p.i. Blocking IFNα resulted in significant increases in total anti-influenza IgG and IgG1 in the serum of both young and aged mice at Day 8 (Fig. 2A) and 28 p.i. (Fig. 2B), whereas IgG2a did not seem to be as greatly affected by anti-IFNα treatment. A significant age by antibody interaction was only noted in the levels of total IgG at Day 8 p.i. such that old and young mice did not response to anti-IFNα treatment to the
same extent. As a result of anti-IFNα treatment, the fold change in IgG in young was a 1.6 fold increase, whereas the increase in aged mice was 6-fold. Also a main effect of age was found such that old mice had reduced antibody levels compared to young (IgG, IgG2a, IgG1) at day 8 and 28 p.i. The ratio of IgG2a/IgG1 was also calculated for each group at each time point (Table 1). The typical BALB/c antibody response to influenza infection is a gradual increase over time in the IgG2a/IgG1 ratio. However, the anti-IFNα treatment skews this ratio in both young and old mice such that IgG1 levels remain much higher than IgG2a at both 8 days and 28 days p.i..

**Effects of anti-IFNα Treatment during Primary Infection Persist into Secondary Infection**

Treatment with anti-IFNα during the early days of primary infection influenced antibody response to secondary challenge (even though neutralizing antibody treatment was not administered during secondary challenge). Mice that had been injected with monoclonal antibody against interferon α during the first 4 days of the primary infection retained a more robust IgG serum antibody and IgG1 subtype at day 11 post-secondary challenge (Fig. 3A). There was no difference in symptom severity at the time of secondary challenge (both anti-IFNα and control mice showed no weight loss or reduced food intake). Unfortunately, aged mice had succumbed to natural mortality before re-challenge so we were unable to carry out these experiments in the aged mice. With respect to lung cell memory response, a comparison was made between young mice that had received neutralizing antibody treatment during the initial infection with young mice that did not receive antibody treatment. Lung cells from the young mice were isolated and stimulated with NP-peptide to determine the numbers of CD8β+ CD11a+ GranB+ and CD8β+ CD11a+ IFNγ+ cells, both populations represent a lung resident NP-specific CD8CD8+ cells, involved in killing (GranB+ cells) or available to activate other aspects of the
immune response (IFNγ+ cells) important during re-challenge. Mice treated with anti-IFNα during the first 4 days of primary infection had significantly fewer ($p < 0.05$) total CD8β+ CD11a+ IFNγ+ cells and reduced ($p < 0.1$) CD8β+ CD11a+ GranB+ cells than control mice 11 days after re-challenge (Fig. 3B).

**Expression of Interferon Inducible Genes is altered with Age and anti-IFNα Treatment**

Lung mRNA expression of interferon-stimulated genes (ISG) was measured by PCR microarray at day 4, 8 and 10 p.i. (Fig. 4A-4C). At Day 4 p.i. the mice received their last anti-IFNα injection, and the pattern of gene expression at this time point suggested a downregulation in several IFNα-inducible genes including Ifi204, IRF7, ISG15, Mx1, and OAS2 (Fig. 4A). IFNβ appeared to be affected differentially in the young and aged mice. In the aged mice, IFNβ was reduced with anti-IFNα treatment, whereas in the young, anti-IFNα appeared to increase the levels of IFNβ transcripts at day 4. At day 8 p.i., IFNβ tended to increase in both young and aged mice that were previously treated with anti-IFNα antibody, which may be a compensatory response for the temporary lack of IFNα. An age-related effect was also found at day 8 p.i., such that ISG15 gene expression was increased in aged mice. By day 10 p.i., a similar effect of aging and antibody treatment was observed across all multiple genes. In young mice, anti-IFNα antibody treatment resulted in increased expression, whereas in aged mice treated with antibody, gene expression was reduced to the level observed in non-infected mice.

**Effects of anti-IFNα Treatment on BAL Cytokines and Cell Populations**

Bronchoalveolar lavage (BAL) was tested for protein levels of proinflammatory chemokines and cytokines commonly elevated in the respiratory tract during an influenza infection (Table 2). Anti-IFNα treatment significantly reduced cytokine and chemokine
production at Day 4 p.i. in both young and old mice. The anti-IFNα treatment reduced the production of G-CSF, GM-CSF, IL-1α, IL-1β, IL-6, IL-12p70, IL-15, MCP-1, IP-10, MIP-1β, MIP-2, RANTES and TNFα in the aged and young mice (b, p < 0.05). There were large changes in BAL cytokine and chemokine concentrations, yet there were no significant difference in the percentage or counts of neutrophils or plasmacytoid dendritic cells (pDC) in young or old mice as a result of anti-IFNα treatment, although aging was associated with fewer pDC (Table 3). However, the anti-IFNα treatment resulted in a greater percentage of inflammatory monocytes at day 4 which was more pronounced in aged mice. Also at day 4 p.i. significant effects of aging were noted in the levels of IL-6, IL-12p70 and IL-10. In contrast, MIP-1β and RANTES were decreased in the aged mice compared to young.

At day 8 p.i., anti-IFNα-treated mice generally had greater numbers cells present in the BAL including neutrophils, CD8+ cells and inflammatory monocytes, although this effect was most pronounced in aged mice with ~ 3 fold increase in the number of inflammatory monocytes. Also, blocking IFNα resulted in decreased IL-15 in the BAL. Other cytokine differences between young and aged mice were observed in response to anti-IFNα treatment. Aged anti-IFNα mice showed ~ 2 fold increase in IFNγ, IL-10, IL-12p7- and TNFα, whereas young treated mice showed no change or a decrease in these cytokines.

At day 10 p.i., again the effects of antibody treatment differed by age, with the exception of IFNγ, which was decreased in the BAL of young and old anti-IFNα-treated mice. In aged mice, anti-IFNα treatment resulted in decreased BAL concentration of the following cytokines or chemokines, IL-6, IL-10, IFNγ, G-CSF, TNFα, IL-12p70, KC, MCP-1, IP-10, MIP-1β, and mig. In young mice, an opposite effect was found such that G-CSF, IL-6, IP-10, MCP-1, TNFα, and
IL-10 BAL concentrations were increased. The changes in cell populations mirror cytokine and chemokine changes in that anti-IFNα treatment which varied by age (significant age by antibody interaction). Inflammatory monocytes and CD8+ cells were decreased in the old anti-IFNα-treated mice compared to the old control mice. In contrast, these same cell populations were significantly increased in the young mice treated with antibody (Table 3).

**Discussion:**

The results from this investigation demonstrated that the effects of IFNα during influenza infection clearly differed in young and aged mice. Body weight and food consumption were monitored over the course of each experiment, and interestingly, young mice treated with anti-IFNα experienced greater symptom severity whereas aged mice lacking IFNα appeared to fare better with respect to illness severity. These young mice lost much more weight and consumed less food when treated with neutralizing antibody against IFNα than their age matched untreated controls. Conversely, aged anti-IFNα treated mice lost less weight and ate more food than their age-matched controls. These same results were observed in three separate experiments.

A partial explanation for these differential effects of anti-IFNα treatment in the young and aged comes from work by Jiang et al (2003), who demonstrated insensitivity to IFNα in the aged host in comparison to young. Early after influenza infection naïve CD8+ T cells were shown to be depleted by apoptosis from the spleens of young mice, later work showed that this IFNα-dependent process did not occur in aged mice, suggesting that the cells from the aged mice were resistant to this phenomenon[292-294]. Further investigation led to the discovery that naïve CD8+ T cells did not undergo depletion by apoptosis due to lower production of IFNα/β in the aged mice. In aged mice there was also a decreased sensitivity to type-I interferons in CD8+
T cells, despite the finding that aged mice had enhanced numbers of the IFNaR1 on the surface of CD8+ T cells. While this paper does highlight an age-associated dysregulation in type-I interferon signaling, it also shows that type-I interferons are important in clearing the splenic T cell compartment to make ‘space’ for influenza specific CD8+ T cells during infection. The results from our study show an increase in CD8+ T cells in the BAL at day 8p.i. in the young anti-IFNα treated mice (Table 3.), however, we are also able to show reduced numbers of lung resident and recruited NP-specific CD8+ T cells after recall challenge in the young anti-IFNα treated mice suggesting a possible defect in the development of the influenza-specific CD8+ T cell response due to loss of IFNα during the first 4 days of infection (Fig. 3B). Additionally IL-15 expression was significantly reduced in the BAL of young mice treated with anti-IFNα at both Day 4 and 8 p.i. (Table 2). Work by Verbist et al, (2011) has shown that loss of IL-15, a pro-survival cytokine, during the primary response to influenza leads to severe reductions in the influenza-specific CD8+ memory response, and work by Nakamura, et al (2009) showed reductions in NP-specific CD8+ T cells in IL-15 KO during primary immune response [295-297]. Perhaps these studies explain the delayed clearance from the lungs and exacerbated disease state in the young anti-IFNα treated mice due to a lack of a sufficient cytokines leading to the development of influenza-specific CD8+ T cell response. Unfortunately, aged anti-IFNα treated mice did not survive to re-challenge so we do not know of the levels of resident NP-specific CD8+ T cells that remained after primary infection or whether they were comparable to that of the young anti-IFNα mice.

In our model, results for the aged and young anti-IFNα treated mice are similar at day 4 p.i., and suggest that IFNα has an important role in activating multiple cytokines and chemokines. The purpose of these experiments was to investigate the effect of IFNα in the early
phase of infection (first four days). After day 4p.i, anti-IFNα antibody treatment ceased, allowing the examination of the role IFNα had in shaping the subsequent adaptive immune response. Young and aged anti-IFNα treated mice had a similar alteration in lung cytokine and chemokine responses at Day 4p.i. indicated by significant reduction in protein levels of several cytokines (Table 2) in the BALF. However, lung populations examined in BALF at day 4p.i. included pDCs and neutrophils, which were not altered by anti-IFNα treatment. Alternatively, percentage of inflammatory monocytes were higher in both young and aged mice treated with anti-IFNα, however the increase in these cells was much more dramatic in the young IFNα treated group of mice. This might suggest an alternative means by which the immune response can be up regulated in the young and aged mice apart from IFNα. It is possible that the levels IFNβ may have increased in the young to compensate for the loss of IFNα, and the pattern of IFNβ expression tracks well with the percentage of inflammatory monocytes collected in the lavage from the young and aged mice. Furthermore, this might explain why there were significantly less inflammatory monocytes in the aged compared to the young at day4p.i., considering the levels of IFNβ mRNA were higher in both the young groups in comparison to the aged [298].

By day 8 p.i. young and aged anti-IFNα treated mice had elevated neutrophil, CD8+ cell, and inflammatory monocyte infiltration in comparison to the control controls. Additionally, the levels of neutrophils and inflammatory monocytes were increased in the aged anti-IFNα treated mice in comparison to the young anti-IFNα treated mice. Since neutrophils are known to be involved in wound healing perhaps the elevated levels in the aged mice might be a means through which illness and immunopathology might be reduced with anti-IFNα treatment (due perhaps to an enhanced wound repair mechanism). In the BALF of aged mice, anti-IFNα
treatment resulted in significantly greater monocyte infiltration and CD8+ T cells, along with increased IFNγ, IL-12p70 and IL-10, all of which were not increased in the young anti-IFNα treated mice. In comparison to aged anti-IFNα mice, the levels of IFNγ, IL-12p70, IL-10, KC, and IL-15 were all reduced in the young anti-IFNα treated mice at day 8 p.i. Even though cell populations might be increased in these young anti-IFNα mice, the reduced cytokine production suggests a reduced cell function in the young anti-IFNα mice in comparison to the aged anti-IFNα treated mice. If young anti-IFNα treated mice don’t recover their influenza-specific cytokine response, this would lead to delayed viral clearance, which is suggested by the day 10 viral titer data. Young anti-IFNα treated mice had the highest levels of virus remaining in their lungs at this late time point in comparison aged anti-IFNα treated mice, which had almost no virus remaining at day 10 p.i. (Fig. 1C). Additionally, the presence of IL-10 might be of importance to these findings. Both the aged anti-IFNα treated and young-control mice had higher levels of IL-10 in the BALF at day 8p.i. in comparison to the aged control and young anti-IFNα treated mice. Given that IL-10 can serve to limit inflammation, there may be a degree of regulation (along with wound healing via increased neutrophils in the aged mice) occurring in these two groups, leading to an improved resistance to influenza virus in the aged anti-IFNα treated mice and young control in comparison to their age counterparts [42].

The antibody response in both of these groups of mice treated with neutralizing antibody against IFNα was similar in young and aged mice, and confirms the role of IFNα in shaping the specific antibody response to influenza. The results from our study and others show that loss of innate receptors, such as TLRs, which are involved in the induction of type-I interferons, or transcription factors involved in type I interferon secretion (STAT1 -/-), and even the loss of type I interferon (IFNaR1/2) receptors by genetic knockouts leads to a decline in the Th1 (IgG2a
antibody) response and an increase in the Th2 (IgG1 antibody) response. Therefore, higher serum antibody titers develop in these mice in response to immune stimuli. IgG1 is produced early on in the humoral response to influenza, but because of isotype class switching typically driven by IFNγ production, IgG2a will become the dominant influenza specific subtype later in the immune response to influenza. Results from our study show that young and aged mice treated with anti-IFNα antibody even for only the first four days of influenza infection produced far more IgG1 than the untreated young and aged mice (Fig 2A, 2B and Table 1). Additionally, the increase in the IgG1 response with anti-IFNα treatment was still detectable after secondary infection in the young mice, suggesting the antibody response to influenza has been permanently altered by lack of IFNα early in the immune response to influenza (Fig 3A). Work by Doherty et al. (2006) shows that while the virus-specific CD8+ response is important to clearing influenza virus from the lungs, the humoral response is indispensable as well to host resistance to primary infection. Even though the young mice have a robust antibody response that is protective during secondary infection, it is not completely clear whether their virus specific CD8+ response was impaired leading to delayed viral clearance. The reduced numbers of memory CD8+ T cells suggests that the memory population developed from a smaller population of clonally expanded influenza-specific CD8+ T cells during the primary infection (Fig. 3B). Therefore, if influenza specific CD8+ T cell population were reduced in the young anti-IFNα mice, even if they were producing higher levels of IgG1 in comparison to the young untreated control mice, this might suggest an imbalance between CD8+ antiviral response and humoral response to influenza infection in the young anit-IFNα treated mice which agrees with the work by Doherty et al. (2006). This imbalance between adaptive and humoral immunity might explain the exacerbated disease state in the young mice treated with anti-IFNα and increased time to clear the virus.
Depending on the strain of virus and susceptibility of the host to infection an appropriate degree of inflammation is needed for clearance of the infection [49]. Immune dysregulations have been identified in the young (‘cytokine storm’) and aged (‘inflamm-aging’) infected with influenza both leading to excessive inflammation and subsequent immunopathology in the lung. The ‘cytokine storm’ has been suggested as the cause of a higher degree of inflammation that contributes to the virulence of avian H5N1 in the young and thought to be the cause of increased morbidity of the younger population to the 2009 pH1N1 outbreak in comparison seasonal influenza to which young are not generally as susceptible [46, 49, 299]. A/PR/8/34 does not induce the same amount of immunopathology as the highly virulent strains in the young, and young tend to elicit an appropriate amount of inflammation in response to A/PR/8/34 to clear the infection. The results in our study suggest that the loss of IFNα early in the infection led to a reduced amount of inflammation leading to delayed viral clearance, reduced levels of cytokines important to an influenza-specific immune response and increased illness severity in the young. Meanwhile, in the aged population, the concept of ‘inflamm-aging’ has been a topic of investigation among researchers as an aged host has an underlying systemic level of inflammation (i.e. IL-6, IL-1, TNFα), leading to a chronic state that possibly impacts the aged hosts response to viral infection [300, 301]. In our study, while we did identify multiple cytokines altered with age it was not surprising to find IL-6 increased at day 4 and 8 in the aged mice compared to young. When we examined the IL-6 levels between the aged anti-IFNα treated mice and control mice, IL-6 was dramatically reduced at day 4 in the aged anti-IFNα treated mice. This finding with respect to IL-6, along with reduced TNFα, IL-1α and IL-1β might be a contributing factor to reduced illness severity in the aged, notwithstanding that these cytokines are also involved in inflammaging and the associated complications during infections.
Perhaps the early (day 4 p.i.) reductions in cytokines led to maintenance of appetite in the aged anti-IFNα leading to improvements in illness severity, while increased cell infiltration and cytokine production (day 8 p.i.) led to the eventual clearance of the virus by day 10 in most of the aged anti-IFNα mice [60].

In conclusion, the results from this study provide a unique perspective for how IFNα, apart from IFNβ, is differentially utilized by young and aged hosts in influenza infection. Different disease outcomes were established for each age-group when they were treated with neutralizing antibody against IFNα, indicating that this type-I interferon modulates the immune response in the young and aged by separate pathways.
Figures Chapter IV:

Figure 1A.
Figure 1B.

![Graph showing food consumption over days 7 and 10 for different groups labeled Y, Y-anti-IFN, O, and O-anti-IFN. The graph indicates a significant difference marked by "a*b" for both days with error bars indicating variability.]
Figure 1A-1C: Effect of anti-IFNα monoclonal antibody treatment on illness severity to influenza and lung viral titer in young adult and aged mice. 1A) Body weight loss over time was assessed for a time by age by antibody interaction (t*a*b) and 1B) total food consumption was also examined at Day 4 and Day 7 in each of the experiments. 1C) Viral titer was detected in bronchoalveolar lavage fluid by quantitative PCR for influenza NP protein. Results are a product of three separate experiments where mice were euthanized at day 4 (n=6-8), day 8 (n=5-8) and Day 10 (n=5-7) p.i. a*b indicates a significant (p < 0.05) age by antibody interaction by two-way ANOVA, a+b (p < 0.1), a indicates a significant (p<0.05)effect of age, a+ (p < 0.1), b indicates a significant (p < 0.05) effect of antibody, b+ (p < 0.1).
Figure 2A.

Day 8 Serum anti-Influenza Antibody
(serum diluted 1:5)

Optical Density (405nm)

- a*b, a, b
- b, a+

Y
Y-anti-IFN
O
O-anti-IFN

Total IgG
IgG2a
IgG1
Figure 2B.

Day 28 Serum anti-Influenza Antibody
(serum diluted 1:50)

Figure 2A and 2B: Effect of anti-IFNα monoclonal antibody treatment on serum anti-influenza IgG, IgG1, and IgG2a in young and aged mice at Day 8 and Day 28 post influenza infection. Serum anti-influenza antibody levels determined by ELISA are represented as optical density (OD) levels at 405nm at 2A) Day 8 and 2B) Day 28 p.i.. Results are the product of two separate experiments where n=5-8 for Day 8 experiment and n=6-7 for Day 28 experiment.
Table 1. Ratio of IgG2a to IgG1 developed during influenza infection

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Day 8</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y-</td>
<td>0.45</td>
<td>1.25</td>
</tr>
<tr>
<td>Y-anti-IFN</td>
<td>0.21</td>
<td>0.51</td>
</tr>
<tr>
<td>O-</td>
<td>0.63</td>
<td>1.77</td>
</tr>
<tr>
<td>O-anti-IFN</td>
<td>0.11</td>
<td>0.33</td>
</tr>
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</table>
Figure 3A.
Figure 3B. Effect of anti-IFNα monoclonal antibody treatment on anti-influenza IgG, IgG1, IgG2a, as well as resident lung NP-specific CD8+ cells in young mice at Day 11 post-secondary influenza challenge. A subset of young anti-IFNα treated mice (n=7) and young control mice (n=6) were kept alive through infection to be re-challenged with a lethal dose (HAU ~1.6 used for re-challenge, compared to 0.2 HAU for primary challenge) of A/PR/8/34 6 months post-primary infection. 3A) Total serum anti-influenza IgG and IgG1 were measured by indirect ELISA, 3B) while lung resident influenza NP-peptide specific CD8+ T cells (IFNγ+ and granzyme B+) were measure in the lungs by flow cytometry per 1X10^6 lung cells. Results represent one experiment with statistical significance assigned by one-way ANOVA; b signifies a significant ($p < 0.5$) effect of anti-IFNα treatment, while b+ indicates a trend ($p < 0.1$) for an effect of anti-IFNα treatment.
Figure 4A.

Day 4

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change relative to uninfected</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFI204</td>
<td>Y</td>
</tr>
<tr>
<td>IFN-b</td>
<td>Y-anti-IFN</td>
</tr>
<tr>
<td>IRF7</td>
<td>O</td>
</tr>
<tr>
<td>ISG15</td>
<td>O-anti-IFN</td>
</tr>
<tr>
<td>Mx1</td>
<td></td>
</tr>
<tr>
<td>OAS2</td>
<td></td>
</tr>
</tbody>
</table>

In the graph, the fold change for different genes (IFI204, IFN-b, IRF7, ISG15, Mx1, OAS2) is shown for different conditions (Y, Y-anti-IFN, O, O-anti-IFN) on Day 4.
Figure 4B.

[Bar chart showing fold change relative to uninfected for various genes on Day 8. The chart includes bars for Ifi204, IFN-b, IRF7, ISG15, Mx1, and OAS2, with Y, Y-anti-IFN, O, and O-anti-IFN conditions.]

Legend:
- Y
- Y-anti-IFN
- O
- O-anti-IFN

Note: a
Figure 4C.

Figure 4A-C: Effect of anti-IFNα monoclonal antibody treatment on whole lung mRNA expression of innate interferon-stimulated genes at 4A) day 4, 4B) day 8 and 4C) day 10 post-influenza infection. Results are represented as fold change relative to young. Sample size (n) is 4-5 for day 4, 4-6 for day 8 and 4-5 for day 10. One-way ANOVA was used to determine the effects of age between either anti-IFNα treated mice or mock injected mice (a, p < 0.05 and a+, p < 0.1) and antibody effects separately in the aged mice or young mice. Two-way ANOVA was used to assess age by antibody interaction between young and aged mice treated with anti-IFNα antibody.
Table 2. Protein Levels of BAL cytokine and chemokines at Day 4, Day 8 and Day 10 p.i.

<table>
<thead>
<tr>
<th>Protein (Day p.i.)</th>
<th>Y- ±SE (p-value)</th>
<th>Y-anti-IFN ±SE (p-value)</th>
<th>0- ±SE (p-value)</th>
<th>0-anti-IFN ±SE (p-value)</th>
<th>Age by Antibody Interaction</th>
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<td>5522 ± 1933</td>
<td>1166 ± 563</td>
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<td>10p.i.</td>
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<td>3 ± 1</td>
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<td></td>
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<td>1 ± 1</td>
<td>1 ± 1</td>
<td>1 ± .33</td>
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<td>7979 ± 2907</td>
<td>1200 ± 619</td>
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<td>7710 ± 1598</td>
<td>6852 ± 1085</td>
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<td>8 ± 3</td>
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<td>77 ± 16</td>
<td>65 ± 33</td>
<td>5 ± 1</td>
<td>a*b</td>
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<td>a+b</td>
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<td>91 ± 16</td>
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<td>11 ± 5</td>
<td>9 ± 5</td>
<td>a+</td>
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<td>5482 ± 2705</td>
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<tr>
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<td>36 ± 10</td>
<td>46 ± 10</td>
<td>72 ± 14</td>
<td>39 ± 5</td>
<td>a+b</td>
</tr>
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<td>410 ± 266</td>
<td>7 ± 4 (a)</td>
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<td>76 ± 17</td>
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<td>35 ± 6</td>
<td>53 ± 4</td>
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<td>22 ± 4</td>
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Table 2. Bronchoalveolar lavage fluid (BALF) was tested for cytokines and chemokines at 4, 8, and 10 days p.i. Two-way ANOVA was used to determine main effects of age (a, $p < 0.05$ and a+, $p < 0.1$) and antibody (b, $p < 0.05$ and b+, $p < 0.1$) and interactions between age and antibody (a*b, $p < 0.05$ and a+b, $p < 0.1$) Results are representative of 3 separate experiments. Day 4 n = 6-8, Day 8 n = 5-8 and Day 10 n = 5-7.
Table 3. Percentage and number total of BAL represented by cell populations Day 4, 8 and Day 10 p.i.. Cell percentage indicated first, followed by cell number below.

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<th>Cell population</th>
<th>% Total/Counts</th>
<th>Y-</th>
<th>Y-anti-IFN</th>
<th>O-</th>
<th>O-anti-IFN</th>
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<td>36870±1350</td>
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<td>12.16±0.43</td>
<td>6.79±1.67(a)</td>
<td>10.47±1.06</td>
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<td>54960±4800</td>
<td>58660±4600</td>
<td>23569±7560</td>
<td>31433±9325</td>
<td>a</td>
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<td>Plasmacytoid DC</td>
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<td>2.40±0.47</td>
<td>1.52±0.36</td>
<td>1.80±0.13</td>
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<td>10682 ± 752</td>
<td>11263±2450</td>
<td>6180±450</td>
<td>5655±12450</td>
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<td>2.03±0.23(b)</td>
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<td>12372±6400</td>
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<td>Inflammatory Monocytes</td>
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<td>18.90±5.6</td>
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<td>37387±17307</td>
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<td>6.59±0.89</td>
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<td>21239±6315</td>
<td>9520±6031</td>
<td>21523±7213</td>
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<td>4.02±0.75</td>
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<td>1407±265</td>
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<td>Inflammatory Monocytes</td>
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<td>20.1±1.47</td>
<td>7.97±4.31</td>
<td>0.50±0.23</td>
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<td>2860±1200</td>
<td>8975±1832</td>
<td>126±72</td>
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<td>CD8+ T cells</td>
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<td>9.95±1.71</td>
<td>4.48±1.15</td>
<td>1.09±0.46</td>
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<td>3067±785</td>
<td>4321±909</td>
<td>1518±275</td>
<td>286±95</td>
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Table Legend: ‘a’ signifies a main effect of age where p<0.05, ’a+’; p<0.1. ‘b’ signifies a main effect of antibody treatment where p<0.05; ‘b+’; p<0.1, ’a*b’ for a significant age by antibody interaction by Two-Way ANOVA where p<0.05, ‘a+b’; p<0.1

Table 3. Lung cell populations at day 4, 8, and 10 p.i. in bronchoalveolar lavage fluid determined by flow cytometry. Neutrophils (SSC+ CD45+ CD11b+ Gr-1+), inflammatory monocytes (FSC+ CD45+ CD11b+ CD11c- Gr-1+) plasmacytoid dendritic cells (CD11c<sup>int</sup> mPDCA-1+) and CD8+ T cells (CD45+ CD8β+) were determined by flow cytometry. Results are representative of 3 separate experiments. Two-way ANOVA was used to determine main effects of age (a, p < 0.05 and a+, p < 0.1) and antibody (b, p < 0.05 and b+, p < 0.1) and interactions between age and antibody (a*b, p < 0.05 and a+b, p < 0.1) Results are representative of 3 separate experiments. Day 4 n = 6-8, day 8 n = 5-8 and day 10 n = 4-6.
Supplemental Figures Chapter III:

Supplemental Figure 1A.

Supplemental Figure 1A. Food consumption over time to day 10 post-influenza infection for young and aged anti-IFNα treated mice.
Supplemental Figure 2A and 2B.

Supplemental Figure 2A.

Day 8 dendritic lymph node cell populations

![Bar chart showing cell counts for CD11c+MHC-II+ and CD11c+CD8a+ populations at Day 8. The chart includes comparisons between mock and anti-IFN conditions.](chart.png)
Supplemental Figure 2B.  

Total Expression per Lymph node dendritic cell

Supplemental Figure 2A and 2B. Activation markers on dendritic cells subsets of the lymph nodes: Cell numbers and MFI for CD80, CD86, MHC-II and CD40 at day 8 post-influenza infection. 2A) Lymph node dendritic cells subsets, CD11c\textsuperscript{hi} MHC II+ conventional dendritic cells and CD11c\textsuperscript{int} CD8α+ resident lymph node dendritic cells, detected in the lymph nodes at day 8 post-influenza infection. 2B) Mean fluorescence intensity for activation and costimulatory markers (CD86, CD80, MHC II, and CD40) on conventional dendritic cells isolated from lung draining lymph nodes at day 8 post-influenza infection. Two-way ANOVA was used to determine main effects of age (a, $p < 0.05$ and a+, $p < 0.1$) and antibody (b, $p < 0.05$ and b+, $p < 0.1$) and interactions between age and antibody (a*b, $p < 0.05$ and a+b, $p < 0.1$)
CHAPTER V

EXERCISE IMPROVES HOST RESPONSE TO INFLUENZA VIRUS IN OBESE AND NON-OBESE MICE.

A manuscript to be submitted to the *Journal of Infectious Diseases*

Kristi Warren¹, Molly Slattery¹, Todd Wyatt², K.J. Yoon¹, Marian Kohut¹

¹Iowa State University, Ames, IA 50011, ²University of Nebraska Medical Center, Omaha, NE

Abstract:

Obesity may impair host response to influenza A virus (IAV) infection. During the 2009 pH1N1 outbreak obese (BMI > 30) individuals were found to have a higher rate of hospitalization than normal weight (BMI < 25) individuals indicating that disease severity was increased in these individuals. Findings such as these highlight the importance of understanding the role obesity plays in the exacerbation of various disease states. Exercise may improve resistance to infection in obese hosts. In order to test this hypothesis, C57/BL6 mice were fed either a normal or high fat diet and assigned to either exercise or non-exercise treatment for 8 weeks. Twenty-four hours after the last exercise session, mice were infected with influenza A/PR/8/34. Exercise reduced BAL cytokines/chemokines in non-obese and reduced lung viral titer in non-obese and obese mice. Exercise augmented type-I interferon lung mRNA levels in the obese exercised and improved immune cell infiltration 3 days after infection, an impairment that has been identified in other studies of diet-induced obesity and influenza virus infection. These results suggest exercise might improved host resistance to IAV infection in diet-induced obese mice.
Introduction:

Obesity is a growing epidemic worldwide and increases the risk of developing a variety of metabolic, cardiovascular and respiratory disorders [203]. Along with non-infectious disorders obesity is now linked with an increased risk of viral and bacterial infections. Epidemiological findings and recent research suggests immune impairments exist that contribute to the development of these disorders induced by the obesogenic state. Influenza-associated illness is known to affect 3-5 million people worldwide annually [302, 303], and notwithstanding that in 2008 approximately 1.5 billion people around the world were thought to be overweight (BMI > 25), it was not entirely surprising for epidemiological studies to identify the great impact the 2009 pH1N1 virus had on the obese population [4, 304]. Along with increased hospitalizations and illness severity in obese individuals caused by 2009 pH1N1 outbreak [304], further research showed reduced pH1N1 vaccination response in obese individuals [206]. Earlier work identified reduced antibody responses in obese individuals to hepatitis vaccination and tetanus toxoid as well [204, 205, 207]. Taken together these findings suggest that immunosuppression might be correlated with obesity.

The diet-induced obese (DIO) mouse has become an excellent model for further research into the immune impairments associated with obesity. Immune alterations in DIO mice infected with influenza A virus (IAV) led to a poorer disease outcome in comparison to lean mice [219, 221-223]. Two studies by Karlsson, et al (2010), were able to show reduced T cell memory and reduced maintenance of memory T cells in DIO mice, these reductions led to 25% mortality in DIO mice compared to no mortality in lean mice after challenge with a heterologous strain of the virus [222, 223]. These results are consistent with the findings from another study showing
reduced cell-mediated and humoral response to trivalent influenza vaccination in obese humans in comparison to normal weight controls [206]. Furthermore, a study by Smith et al (2006), showed immune impairments in the primary response to influenza infection in DIO mice leading to increased morbidity and mortality. In particular, immune cell infiltration and cytokine/chemokine production (IFNα/β, MCP-1 and RANTES) was delayed in the lungs of DIO mice. In addition, the CD8+ T cell response was delayed and reduced in comparison to lean mice. Another study further implicated dendritic cell impairments in the loss of a robust immune response leading to the same early immune impairments in cell infiltration and CD8+ T cell function found in the 2006 study. Not only were the numbers of plasmacytoid dendritic cells reduced in the lungs after influenza infection, but conventional dendritic cells had a reduced capacity to stimulate influenza primed CD8+ T cells. Additionally, IL-12 mRNA was reduced in lymph node cells, further implicating a role for dendritic cells in the diet-induced obese impairments associated with influenza infection.

It is also known that fat depots around the body contribute systemically to various dysregulated host systems (metabolic, endocrine, immune etc.) [211, 305]. Examples of this dysregulation include insulin and leptin resistance or the low grade inflammation detected in the serum of obese individuals [306]. Diet intervention alone has been shown to be effective at reducing BMI and fat percentage. However, the best results for reducing BMI, total body fat percentage and possibly improving immunity, come from implementing proper diet and regular physical activity together. Epidemiological studies have shown that individuals that exercise regularly, but with a BMI classified as overweight (BMI = 25-30), had a similar lifespan in relation to normal weight individuals (BMI < 25) that did not exercise [307]. The mechanisms are not yet fully understood but this finding suggests that regular moderate exercise provides a
benefit to the host, even in the overweight state. Therefore, the purpose of this study was to test the extent to which exercise might improve the immune impairments identified in DIO mice infected with IAV.

In this study the diet-induced obese (DIO) state was established in C57/BL6 mice, and regular moderate exercise was administered for 8 weeks prior to IAV infection. Mice were euthanized at two time points p.i., at the time of peak viral load (day 3 p.i.) and at a time in which optimal CD8+ T cell recruitment and viral clearance are expected to occur (day 8 p.i.). Overall, the results show that illness was improved in the lean and DIO mice with exercise as body weight loss was reduced and calorie intake was increased over the course of infection in comparison to non-exercised mice. In addition, exercise reduced lung viral titer at both day 3 p.i. and day 8 p.i. in lean and DIO mice, and increased cell infiltration, type-I interferons and cytokines in obese exercised mice in comparison to non-exercised at day 3 p.i.. This finding suggests that exercise led to early improvements in immune cell response which have been shown to be dysregulated in previous studies. At day 8 p.i. total BAL CD8+ T cells and NP-specific CD8+IFNγ-producing T cells of the lung were increased in the diet-induced obese mice that exercised in comparison to the obese non-exercised. These results further imply that exercise might be associated with an improved host adaptive immune response to IAV infection when practiced regularly prior to infection.

Materials and Methods:

Mice, Influenza Virus, Exercise Protocol

C57/BL6 mice were purchased (n = 100) from Jackson laboratories at 6 weeks of aged and upon arrival placed on a 10 kcal% fat (Research Diets) or 60 kcal% fat (Research Diets) diet.
At seven weeks of age, half of the mice on each diet were randomly selected to acclimate to a motorized treadmill and undergo an eight week training protocol. All randomly selected mice adapted well to daily treadmill running and gradually the rate of speed and duration of each session was increased from 12 m/minute for 10 minutes until mice were running 45 min/day, five days a week at 18 m/min. Mice were separated into individual cages and body weight was monitored on a weekly basis. One week prior to infection, a subset of 10 mice per group were selected to undergo DXA (dual-energy x-ray) imaging to determine body composition (total body fat % and abdominal fat %) after eight weeks of training and diet consumption. In addition, beginning at 3 days prior to infection, food consumption was monitored. About 24 hours after the last exercise session all mice were anesthetized with aerosolized isoflurane and infected with A/PR/8/34 (approximately 0.002 HAU/mouse). Body weight and food consumption were monitored over the course of the infection as a means of assessing illness severity in each of the groups of mice. Bronchoalveolar lavage fluid (BALF), lungs and blood were collected at days 3 and 8 p.i. BALF was centrifuged to pellet BAL cells and remove debris from supernatants which were stored at -80°C until subsequent testing could be carried out by Multiplex 32-plex (Millipore) plates on the Luminex 200 system (BIO-RAD). An aliquot of BAL from all mice for both day 3 and 8 experiments was tested by qPCR for viral titer along with a portion of the lung tissue from the day 3 experiment. Anatomical left lobe was collected for lung RNA which was isolated using TRIzol Reagent (Invitrogen). Lung RNA was further purified using the RNeasy Micro Kit (QIAGEN), then frozen at -80°C. Subsequently, 1,500 nanograms of purified lung mRNA were used to run a Mouse Interferon α, β Response RT² Profiler™ PCR Array (SABiosciences, Frederick, Maryland). Samples were analyzed using the BioRad iCycler and corresponding MyIQ Optical System Software Version 1.0. At day 8 the
remaining lobes were utilized for the lung activation assay. Blood was collected by heart puncture shortly after euthanization and centrifuged at 3500 rpm for 15 minutes for serum collection, all sera was stored at -80C until further testing until ELISA testing could be carried out.

**Identification Lung Cell Populations by Flow Cytometry**

Cell populations were determined in bronchoalveolar lavage fluid at day 3 and day 8 by staining with the following antibodies, mouse anti-mouse NK-1.1 APC-Cy 7 (rat anti-mouse CD8α APC-Cy7 at day 8), hamster anti-mouse CD11c PE-Cy7 , rat anti-mouse CD3ε PerCP-Cy5.5 , rat anti-mouse Ly6G PE (rat anti-mouse Gr-1 PE at day 8), and rat anti-mouse CD11b Alexa Fluor 700 (rat anti-mouse MHC-II Alexa Fluor 700), rat anti-mouse mPDCA-1 APC (rat anti-mouse CD4 APC at day 8) and rat anti-mouse Ly6c APC followed by analysis using FACSCanto and FlowJo software (version 7.6). Day 3 lung populations identified included neutrophils (SSC<sup>hi</sup> CD11b<sup>hi</sup> CD11c<sup>lo</sup> Ly6g<sup>hi</sup> Ly6c<sup>hi</sup>), NK cells (NK1.1+ CD3−), general macrophages (CD11b<sup>hi</sup> CD11c− autoflourescence<sup>hi</sup>) plasmacytoid dendritic cells (CD11c<sup>int</sup> mPDCA-1+), and TNFα/iNOS producing dendritic cells (CD11b<sup>hi</sup> CD11c<sup>int</sup> Ly6c<sup>hi</sup> Ly6g− MHC II<sup>hi</sup>). At day 8 p.i., the lung populations included in the analysis were general macrophages, neutrophils (SSC+ CD11b+ CD11c<sup>lo</sup> Gr-1<sup>hi</sup>), inflammatory monocytes (SSC− CD11b+ CD11c<sup>lo</sup> Gr-1<sup>hi</sup>), CD4+ T cells (CD3ε+ CD4+ CD8−) and CD8+ T cells (CD3ε+ CD4− CD8+).

Whole lung tissue from day 8 infected mice was homogenized into a single cell suspension by pressing tissue through a strainer into a petri dish, then centrifuged at 1500 rpm for five minutes. Cold RBC lysis buffer was applied for approximately three minutes, cells were then washed two times with cRPMI media. Cell counts were taken and cells were adjusted and
plated at a rate of 2X10^6 cells per well in duplicate per sample and treated with the following: 1μM of NP peptide, NP_{368-374} (WLIFMVY), PMA/Ionomycin as a positive control, or no stimulation (media control) as a negative control for two hours at 37°C. Brefeldin (Golgi Plug) was added for four hours at 37°C. Cells were pelleted by centrifugation at 1500 rpms for five minutes, resuspended in 100 μL BSA 0.1% PBS wash buffer and stained with rat anti-mouse CD8β Alexa Flour 488 and rat anti-mouse CD3ε PerCP-Cy5.5. Cells were then fixed, permeabilized and stained using rat anti-mouse IFNγ APC. After staining each sample was analyzed by flow cytometry on the FACSCanto for CD8β+ IFNγ+ cells activated by the various treatments (PMA/Ionomycin, NP-peptide and no-treatment). FlowJo 7.6 software was used for gating and analysis.

*IgG and IgG2c ELISA*

Mouse serum was tested for influenza specific total IgG and subtype IgG2c antibody by indirect ELISA. ELISA plates (Immulon, Alexandria, VA) were coated with 100uL of Influenza A/PR/8/34 (200HAU/mL) diluted in carbonate coating buffer (pH 9.6) and incubated overnight at 4°C. Plates were blocked with PBS-0.1% BSA-blocking buffer at 37°C for one hour. Plates were washed three times with PBS/0.05% Tween 20 between each step. Serum was diluted and added to the plates in duplicate then incubated for four hours at room temperature. AP-conjugated goat anti-mouse IgG and IgG2c were added to the plate and incubated for eight hours at 4°C. Plates were developed using phosphatase substrate (Sigma Aldrich, St. Louis) for 15 minutes at room temperature. Optical density (OD) of each well was measured using a microtitration plate reader (FluoStar Galaxy) at 405 nm.
Statistical Analysis

All statistical analysis was carried out using SPSS Statistics 17.0. A two-way ANOVA (diet by exercise interaction) was used to establish main effects of diet and exercise on immune measures, including antibody titers, BAL cytokines and chemokines, and cell populations. A mixed ANOVA (diet X exercise, treatment X time) with time as repeated measures (within subjects) variable was used to analyze change in body weight over time. One-way ANOVA was used to determine statistical significance for mucociliary clearance experiment and microarray analysis. Statistical trends were denoted by $p < 0.1$ and significance levels for all tests were set at $p < 0.05$.

Results:

Effect of Exercise on Body Fat Composition and Food Intake

Weekly body weights were collected thru the eighth week treadmill training period (Fig. 1A). The results showed a significant exercise by diet by time interaction suggesting that the rate of weight gain over time differed by diet or exercise treatment. High-fat fed (DIO) mice that did not exercise gained the most body weight. Daily treadmill exercise reduced the amount of body weight gained over the course of the training period in the high-fat fed (DIO-TM-Ex) mice in comparison to the high-fat diet fed non-exercised mice (DIO-No-Ex) ($p < 0.05$), while low-fat diet fed non-exercised (Lean-No-Ex) mice and low-fat fed treadmill-exercised (Lean-TM-Ex) mice gained a similar amount of weight over the 8 week training period ($t*d*e$ for a significant interaction between diet and exercise over time of treatment; $p < 0.05$) (Fig. 1A). After eight weeks of exercise and diet treatment, mice from each treatment group underwent an imaging procedure using a DXA (dual-energy X-ray) scanner to determine total body fat
percentage and abdominal fat percentage. Both high-fat fed groups (exercised and non-exercised) had significantly higher levels of fat percentage in comparison to the low-fat fed mice (d; main effect of diet; $p < 0.05$). Exercise resulted in significant reductions in total and abdominal fat percentage in the high-fat and low-fat fed mice (e; main effect of exercise, $p < 0.05$; Fig. 1B). Exercise attenuated the increase in fat percentage of the high fat diet mice (approximately 18.75% reduction of total fat% in the lean mice versus 27% reduction of total fat% in the DIO mice) leading to a significant diet by exercise interaction (d*e; $p < 0.05$) by two-way ANOVA.

Food intake was compared between treatment groups during the last week of exercise training (at a time point by which we had determined that body fat was significantly greater in high fat diet treated mice). When food intake was assessed using total grams (Fig. 1C, left) it appeared that the DIO mice were consuming less food than the lean mice (d; $p < 0.05$). However, when kilocalories were determined per gram of each diet, kilocalorie intake per day was comparable across lean and DIO groups (Fig. 1C, right). Both DIO and lean exercised mice tended to consume fewer kilocalories in comparison to the DIO and lean non-exercised during this three day time period (e+ trend for a main effect of diet; $p < 0.1$).

**Illness Severity and BAL Viral Load**

Illness severity was assessed using body weight loss each day, total kcal intake, and lung viral titer at day 3 and day 8 p.i. (Fig. 2A-2D). All infected mice lost a significant amount of weight in comparison to non-infected mice over the course of the infection (non-infected were not included in the graphs unless indicated). The results of the body weight analysis until day 8 p.i. showed that diet and exercise treatments differentially altered weight loss (t*d*e - significant
time by diet and time by exercise interactions at \( p < 0.05 \). However, there was a main treatment effect of exercise \( (p < 0.05) \) such that exercised mice (DIO or lean) had attenuated weight loss in response to influenza infection. Total kilocalorie intake up until day 3 p.i. was increased in the both lean and DIO exercised mice in comparison to the lean and DIO non-exercised mice \( (e, \) main effect of exercise \( p < 0.05) \). At day 8 p.i., again exercise treatment was associated with increased total caloric intake in DIO and lean mice \( \) (main effect of exercise, \( p < 0.05) \). BALF and lung tissue were collected in the day 3 p.i. experiment and used to determine viral load in the lungs of the lean and DIO exercised and non-exercised mice. At day 3 post infection, lung viral titer was reduced by exercise \( \) (main effect of exercise), but a diet by exercise significant interaction was found suggesting that the exercise-associated decrease in viral load was of a greater magnitude in the lean mice than in the obese mice. Viral titer in BAL fluid followed a similar pattern, although the exercise effect was not statistically significant \( \) (Fig. 2C). At day 8 p.i., a main effect of exercise was identified in both the lean and DIO exercised mice in comparison to the non-exercised \( (e; p < 0.05) \) showing decreased viral load with exercise \( \) (Fig. 2D).

*Mucociliary Clearance is Enhanced by Exercise in DIO-mice*

Tracheas from DIO and lean mice were collected at day 3 p.i. and stimulated with the beta-agonist isoproterenol to stimulate ciliary beat frequency in epithelial cells. Ciliary beat frequency \( (CBF) \) was quantified per minute and represented in Figure 3. Infection caused reductions \( (* \) significant effect of infection; \( p < 0.05) \) in CBF at day 3 p.i. when non-infected mice were compared to infected mice. There was not a significant effect of exercise on CBF in lean mice, however in DIO mice, CBF was augmented with exercise \( (***) \) significant effect of
exercise, \( p < 0.05 \)). By day 8 p.i. all infected (DIO and Lean) mice had significant epithelial damage such that CBF could not be determined (data not shown). These results show that exercise aids the DIO host in maintaining mucociliary clearance leading to increased lung epithelial cell function for a longer period of time after infection.

Lung Cell Populations at Day 3p.i. and Day 8p.i. are altered by Diet and Exercise.

Bronchoalveolar lavage fluid (BALF) was collected for total cell counts (Fig. 4A), percentage of infiltrating cells (Fig. 4B and 4C), and cytokine and chemokine production at day 3 and 8 p.i. (Fig. 4D and 4E). At day 3 p.i. total cell counts were reduced in the DIO mice in comparison to the lean mice (d; \( p < 0.05 \)). Also at day 3 p.i. BAL cell collection was reduced in the lean exercised mice in comparison to the lean non-exercised, and conversely enhanced in DIO exercised mice in comparison to the DIO non-exercised (d+e, trend for a diet by exercise interaction, \( p < 0.1 \)). Similar to day 3 p.i. results, a significant diet by exercise interaction was reported at day 8 p.i. (d*e, \( p < 0.05 \)). However, at this time point cell infiltration was highest in the DIO-TM-Ex group in comparison to the DIO-No-Ex, yet lower in the Lean-TM-Ex mice compared to Lean-No-Ex.

The composition of lung infiltrating cell populations was assessed by determining the percentage of the various immune cells at day 3 and 8 p.i in BALF (Fig. 4B and 4C). At day 3 p.i., a main effect of diet was found such that lean mice had an increased percentage of macrophages, plasmacytoid dendritic cells (pDCs) and TNFα/iNOS producing DCs (TipDCs) in the BALF. Exercise resulted in an increased neutrophil percentage of the BAL in both lean and obese mice, but a decrease in the percent of BAL NK cells at day 3 p.i. However, exercise had different effects in lean and obese mice with respect to macrophages, pDC’s and TipDC’s.
Exercise reduced the percentage of macrophage, pDC and TipDC in lean exercise mice compared to lean non-exercised, yet increased the percentage of each of these populations in the DIO exercised in comparison to the non-exercised (d*e, p < 0.05). By day 8 p.i. a main effect of diet was found (increased percentage of macrophage, inflammatory monocytes, and neutrophils in DIO mice). In addition, exercise resulted in an increased percentage of macrophages, inflammatory monocytes and CD8+ T cells in comparison to non-exercise mice regardless of type of diet.

CD8+ IFNγ-producing T cells were detected in whole lungs from day 8 IAV infected mice by in vitro stimulation with PMA/ionomycin (P/I), NP-peptide, or media alone (Fig. 4D). Total NP-specific CD8+ IFNγ+ T cells were reduced with exercise in the lean mice and increased with exercise in the DIO mice (d*e; p < 0.05). Additionally, the amount of IFNγ produced by CD8+ T cells in response to no stimulus (media), NP-peptide or P/I was also examined (Fig. 4E). While there were no difference in the amount of IFNγ produced in response to NP-peptide or media on a per cell basis (determined by IFNγ mean fluorescence intensity (MFI)), there was an effect of diet on the amount of IFNγ produced in response to P/I. The high fat diet treated mice had reduced IFNγ MFI per CD8+ cell after stimulation with P/I I regardless of exercise treatment (d+; p<0.1).

The profile of cytokine and chemokine production at day 3 p.i. for each of the groups was similar from protein to protein (Table 4). Significant effects of diet and exercise separately were identified for IFNγ, TNFα, MCP-1, MIP-1α, MIP-1β, KC, and RANTES (d; p < 0.05, e; p < 0.05 and e+; p < 0.1) and diet by exercise interactions were found for these same cytokines along with IL-10 (d*e, p < 0.05) at day 3 p.i.. The general findings for the levels of cytokines were as
follows: exercise reduced the inflammatory proteins at day 3 p.i. in the lean exercised mice compared to non-exercise, and exercise increased the levels of these proteins in the DIO mice. At day 8 p.i. the levels of IFNγ, TNFα, and KC were not different, however IL-10 was increased in the DIO-TM-Ex mice in comparison to the DIO-No-Ex and reduced in the Lean-TM-Ex in comparison to the Lean-No-Ex mice (e+; p < 0.1 and d*e; p < 0.05). Similar results were found for MIP-1α, MIP-1β and RANTES.

*Interferon-Stimulated Genes are Up-Regulated in Response to IAV Infection in DIO Exercised Mice.*

In the lung tissue of the day 3 IAV infected mice differences in the up-regulation of interferon stimulated genes (ISG) were examined in lean compared to DIO mice that did or did not exercise (Table 5). The high fat diet treatment resulted in reductions in mRNA levels of CXCL-10 (difference of ~480 fold), IFNα2 (~10-fold), IFNβ(~118-fold) in DIO-No-Ex compared to Lean-No-Ex mice (d, p<0.05). Also, CXCL-10, IFNβ, Mx1 and OAS1a each were increased at least 40-fold in the DIO mice that exercised in comparison to DIO mice that did not exercise. In contrast to the DIO mice, gene expression of CXCL-10, IFNα, IFNβ, Mx1, Mx2, OAS1a, OAS2 and STAT2 were all reduced in the lean exercised mice in comparison to the lean non-exercised mice.

*IgG2c anti-Influenza Antibody is Increased in Response to IAV Infection and Exercise in Diet-Induced Obese Mice*

The levels of serum anti-influenza IgG antibody were determined at day 8 p.i. in lean versus diet induced obese mice that did or did not exercise (Fig. 6). Serum IgG and IgG2c were higher in all infected groups in comparison to the non-infected mice (data not shown). Total IgG
levels at this relatively early time point after infection were not statistically different between treatment groups. However, IgG2c levels were increased in the exercised lean mice when compared to the lean non-exercised mice, and the same trend was observed for DIO mice as well.

**Discussion:**

This study was carried out to determine whether exercise could restore immune impairments that have been identified in DIO mice infected with Influenza virus. DIO mice tend to have increased mortality, delayed time recovery and wound healing after IAV infection [219, 308, 309]. In our study we demonstrated that regular moderate exercise prior to IAV infection led to a significant reduction in illness severity in both lean and DIO mice. DIO and lean exercised mice lost less weight, had increased caloric intake, and reduced viral titer in the lung tissue at day 3p.i. and in BALF at day 8p.i. in comparison to both DIO and lean non-exercised mice (Fig. 2A-2D). Past studies have shown similar exercise associated improvements in IAV infection in treadmill trained BALB/c mice in comparison to sedentary controls. Exercised reduced illness severity, lung viral titer, and the lung inflammation commonly associated with IAV infection as early as day 4 post-influenza infection. Not surprisingly, similar to the previous studies in exercised BALB/c mice, the lean C57/BL6 mice included in this present study had improvements in the response to influenza infection when they were exercised prior to infection. The improvement in illness for the DIO mice is also significant as this suggest that the immune impairments associated obesity may be attenuated with exercise resulting in improved disease outcome.
In previous studies involving diet-induced obesity and IAV infection, immune cell infiltration, chemokine production, and type-I interferon production in the lungs have been shown to be reduced or delayed in DIO mice in comparison to lean [219, 221]. In our study we were able to show that regular treadmill exercise led to significantly higher total immune cell infiltration in the DIO mice, and an increased percentage of macrophages, pDCs, neutrophils, and TipDCs. This finding, along with the increased cytokine and chemokine (IFNγ, TNFα, MCP-1, MIP-1α, MIP-1β and KC) production measured in BALF of DIO exercised mice, suggests that cell infiltration driven by chemokine production is at least partially restored in the DIO mice with exercise (Table 4). In addition to the increased cell infiltration, type-I interferon lung mRNA expression was elevated in the DIO-TM-Ex mice in comparison to the DIO-No-EX, along with a maintenance of mucociliary clearance at day 3p.i., which to our knowledge had not been examined in DIO mice. The increased percentage of pDCs, known for their production of IFNα along with the increased type-I interferon (IFNα/β) mRNA and the increase in mRNA levels of interferon stimulated genes (Mx1, Mx2, OAS), suggests a restoration of the anti-viral response. These changes in interferon responsiveness might explain the reduced viral titer in the DIO exercised mice in comparison to non-exercised (Table 5). Furthermore, the increased cell infiltration in the exercised DIO mice suggests that they are responding to the IAV infection in such a way that will lead to containment of the virus. Cells such as the TipDC have been shown to be necessary for clearance of the IAV infection and survival and this suggest that an appropriate amount of inflammation needs to be attained for an immune response to be effective [49]. The findings for increased cell infiltration, elevated interferon stimulated genes and improvements in mucociliary clearance at this early time point demonstrate that the early innate
immune response might be restored in the DIO mice with exercise, which may lead to an early clearance of the virus and decreased mortality.

At day 8 p.i. caloric intake was increased and viral titer was decreased in both DIO and lean exercised mice compared to non-exercised. Interestingly at this time point total BALF cell infiltration had increased in the DIO mice in comparison to the DIO non-exercised, yet remained reduced in the exercised lean mice in comparison to the non-exercised. This finding may suggest that exercise associated reduction in viral load in lean mice resulted in reduced cell infiltration, whereas exercise stimulated immune responsiveness in obese mice leading to increased.

Various aspects of the adaptive immune response including the development of the influenza specific CD8+ T cell response have been examined along with influenza specific antibody titers after infection or vaccination in DIO mice [221-223, 310]. In general, these studies find that diet-induced obesity causes reductions in the influenza specific CD8+ T cell response, regardless of whether the studies are examining influenza-specific CD8+ T cells during primary infection or memory CD8+ T cells after infection or vaccination. This memory cell type is altered in such a way that the DIO host in a way that leads to decreased protection and increased mortality during re-challenge. In our study we chose to examine CD4+ and CD8+ cells in the BALF at day 8 p.i., and to stimulate NP-specific CD8+ T cells in a total lung tissue to determine if the exercise could restore some aspect of the influenza specific CD8+ T cell response in the DIO mice. The percentage of CD4+ T cells were not different across the groups, but the percentage of CD8+ T cells detected in the BALF at day 8 p.i. in the DIO and lean exercised mice was significantly increased in comparison to the DIO and lean non-exercised (Fig. 4C). This suggested that perhaps the exercise enhanced CD8+ T cells which could be
beneficial to the DIO host as these cells could be cytotoxic T cells (CTLs) necessary for a successful immune response against IAV infection [17]. Furthermore we were able to show that the total numbers of NP-specific CD8+ IFNγ+ cells per total lung cells collected were higher in the DIO-TM-Ex mice in comparison to the DIO-No-Ex mice. In addition, to the increased influenza specific CD8+ T response, IgG2c was also increased in the serum of DIO-TM-Ex mice (Fig.6). Taken together these results suggest the influenza specific adaptive response might be enhanced with exercise in the DIO mice and could also explain the improvements in illness severity and reduced viral titer at day8 p.i. in the DIO-TM-Ex mice compared to DIO-No-Ex.

The results so far have highlighted reductions in illness severity which seems to be related to improvements in the immune response in diet-induced obese mice that have exercised in comparison to those that have not. However, when we compare the lean non-exercised mice to the DIO non-exercised mice, body weight loss appears comparable between these two groups, but food intake is lower in the lean-No-Ex mice in comparison to DIO-No-Ex. This suggests that the lean mice have a slightly increased level of illness during the influenza infection in comparison to the DIO mice. A possible explanation for the findings is that the lean mice are producing a robust inflammatory response to fight the infection leading to the production of cytokines that also have metabolic roles in the body in addition to their role in the induction of an immune response. These cytokines (TNFα, IL-6, IL-1β) can directly or indirectly cause the loss of appetite and malaise commonly associated with influenza infection [311, 312]. Thus, since we know that DIO mice have a delayed or reduced immune response early after influenza infection, illness severity measured by body weight loss may not be an optimal measure of disease severity in DIO mice [309]. Furthermore, a long term study should be carried out to assess whether the DIO mice compared to lean have increased time to recovery from the infection or
increased mortality. This study would also be helpful to confirm the positive findings that seem to have been identified in the DIO-TM-Ex mice in comparison to the DIO-No-Ex.

The experiments in this study were carried out to determine if exercise could play a role in reversing the immune impairments identified in DIO mice infected with IAV. While these results are still preliminary and future studies are underway it is clear that exercise is altering the immune response in the diet-induced obese host leading to improvements in both innate and adaptive immunity. These results herein seem to show promise for exercise as a possible treatment for obesity and obesity-associated impairments in the human population as well.
Figures Chapter V:

Figure 1A.

![Graph showing total body weight over weeks for different groups.](image-url)
Figure 1B.
Figure 1A-1C. High fat diet leads to increased body weight and higher percent body fat in exercised and non-exercise C57/BL6 mice. 1A) Body weight increases week 1 to week 8 in both high-fat fed and low-fat fed mice even in those that exercise. 1B) Fat percentage is also increased at week 8 of training measured by DXA imager. 1C) Exercise is associated with reduced kilocalorie (kcal) intake over a two day period prior to infection. Sample size (n) equals 24-26 per group.
Figure 2A.

Day 0 to Day 8p.i.

grams weight loss

Lean-No-Ex
Lean-TM-Ex
DIO-No-Ex
DIO-TM-Ex
Figure 2B.
Figure 2C.

Day 3

BAL Lung
log viral titer
1e+6
1e+7
1e+8
1e+9
1e+10
Lean-No-Ex
Lean-TM-Ex
DIO-No-Ex
DIO-TM-Ex

d*e, e

d+
Figure 2A-D. Body weight loss, food consumption and bronchoalveolar lavage fluid (BALF) viral titer at Day 3 and 8 post-influenza infection. Low-fat fed exercised (Lo-TM-Ex) and non-exercised (Lo-No-Ex), and high-fat fed exercised (Hi-TM-Ex) and non-exercised (Hi-No-Ex) mice were infected with A/PR/8/34 until 3 days and 8 days p.i.. Non-infected mice were included as controls and lost no body weight over the course of the infection (data not shown). Body weight loss 2A) is represented in grams and food consumption 2B) shown as total kilocalories consumed until day 3 and day 8 p.i.. 2C) BALF and lung tissue at day 3p.i. and 2D) BALF at day 8p.i. was tested by qPCR for viral NP mRNA as a means of assessing viral load in
the lungs. A two-way ANOVA was used to determine statistical differences (d for a main effect of diet (p<0.05); d+ p<0.1 and e for a main effect of exercise (p<0.05); e+ p<0.1) and interactions between diet and exercise (d*e, p<0.05 and d+e, p<0.1). Day 3 experiments n=6-9 for all groups and at day 8 n=11-14.
Figure 3: Ciliary beat frequency was quantified in the tracheas of day 3 infected diet-induced obese and lean mice that did or did not exercise as a means of assessing mucociliary clearance. Infection effect is indicated by *** and * for a significant effect of exercise n=10 for infected groups and n=3 for uninfected mice.
Figure 4A.
Figure 4B.
Figure 4C.

Day 8

Percentage(%) total BAL cells

Lean-No-Ex
Lean-TM-Ex
DIO-No-Ex
DIO-TM-Ex

%Mac %inf.Monos %Neuts %CD4 %CD8

d,e

d, e
d+e, d
e
Figure 4D.

NP-CD8+IFNγ+ cells

Total cells per lung

Lean-No-Ex  Lean-TM-Ex  DIO-No-Ex  DIO-TM-Ex

0  2e+4  4e+4  6e+4  8e+4  1e+5

Legend: d*e
Figure 4A-E. Infiltrating cell populations were characterized in bronchoalveolar lavage fluid (BALF) at day 3 and 8 p.i.  4A) Total BAL cell numbers were increased significantly with infection and altered by diet and exercise at both time points (d*e, p<0.05 and d+e, p<0.1).  4B) At day 3 p.i. innate immune cell infiltration was determined by showing the percentage of macrophage, pDC, neutrophils, NK cells, and inflammatory monocytes present per total BAL cells.  4C) Percentage of adaptive immune cells (CD4+ and CD8+), inflammatory monocytes and immune cells (macrophage and neutrophils) involved in wound healing were assessed at Day 8 p.i.. BAL from two mice were combined, n=4-7 after combining within groups.
Table 4. Cytokines and chemokines in BALF at day 3 and 8 p.i..

<table>
<thead>
<tr>
<th>Protein</th>
<th>Lean-No-Ex (Mean±SEM)</th>
<th>Lean-TM-Ex (Mean±SEM)</th>
<th>DIO-No-Ex (Mean±SEM)</th>
<th>DIO-TM-Ex (Mean±SEM)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10</td>
<td>5±1</td>
<td>3±1</td>
<td>3±1</td>
<td>6±2</td>
<td>d*e</td>
</tr>
<tr>
<td></td>
<td>8p.i.</td>
<td>175±60</td>
<td>70±25</td>
<td>92±45</td>
<td>101±43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>d*e, e+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>652±221</td>
<td>131±80</td>
<td>72±65</td>
<td>176±77</td>
<td>d*e, d, e+</td>
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<tr>
<td></td>
<td>6356±3000</td>
<td>4290±2599</td>
<td>4687±2500</td>
<td>6005±2800</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>37±8</td>
<td>9±3</td>
<td>5±2</td>
<td>11±3</td>
<td>d*e, d, e</td>
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<tr>
<td></td>
<td>67±50</td>
<td>29±9</td>
<td>27±10</td>
<td>28±6</td>
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<tr>
<td>MCP-1</td>
<td>583±150</td>
<td>154±171</td>
<td>70±60</td>
<td>155±60</td>
<td>d*e, d, e</td>
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<tr>
<td></td>
<td>2305±1700</td>
<td>1353±900</td>
<td>2310±2000</td>
<td>1033±380</td>
<td></td>
</tr>
<tr>
<td>MIP-1α</td>
<td>103±15</td>
<td>43±15</td>
<td>27±12</td>
<td>63±12</td>
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<td></td>
<td>103±22</td>
<td>55±15</td>
<td>62±18</td>
<td>73±17</td>
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<tr>
<td>MIP-1β</td>
<td>280±75</td>
<td>78±45</td>
<td>38±23</td>
<td>94±24</td>
<td>d*e, d, e+</td>
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<td></td>
<td>328±135</td>
<td>135±48</td>
<td>158±68</td>
<td>176±50</td>
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<td>KC</td>
<td>752±180</td>
<td>236±125</td>
<td>110±77</td>
<td>214±84</td>
<td>d*e, d, e</td>
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<td></td>
<td>151±46</td>
<td>160±42</td>
<td>124±38</td>
<td>129±58</td>
<td></td>
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<tr>
<td>RANTES</td>
<td>68±12</td>
<td>30±15</td>
<td>14±7</td>
<td>36±8</td>
<td>d*e, d</td>
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<tr>
<td></td>
<td>97±27</td>
<td>37±12</td>
<td>36±14</td>
<td>40±10</td>
<td>d*e, d, e</td>
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</table>
Table 4. Bronchoalveolar lavage fluid was tested for various cytokines and chemokines in exercised and non-exercised, lean and diet-induced obese mice. Significant diet (d, p<0.05 and d+, p<0.1) and exercise (e, p<0.05 and e+, p<0.1) effects, and interactions between diet and exercise (d*e, p<0.05), were determined by two-way ANOVA; n = 6-9 for day 3 p.i. and n=11-14 for day 8 p.i. experiment.
Table 5. Day 3p.i. mice lung mRNA fold-change relative to uninfected.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Lean-No-Ex</th>
<th>Lean-TM-Ex</th>
<th>DIO-No-Ex</th>
<th>DIO-TM-Ex</th>
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</thead>
<tbody>
<tr>
<td>CXCL-10</td>
<td>512.05±255</td>
<td>157±77 (e)</td>
<td>36±16 (d)</td>
<td>285±100</td>
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<tr>
<td>IFNa2</td>
<td>12±3.41</td>
<td>3.70±2.0 (e)</td>
<td>1.7±1.2 (d)</td>
<td>5.33±2.5</td>
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<tr>
<td>IFNb</td>
<td>136±20</td>
<td>32±15 (e)</td>
<td>8.12±5 (d)</td>
<td>66±35</td>
</tr>
<tr>
<td>Mx1</td>
<td>125±35</td>
<td>64±30</td>
<td>16±9 (d)</td>
<td>112±65</td>
</tr>
<tr>
<td>Mx2</td>
<td>20±5</td>
<td>17±7</td>
<td>5.56±2.5 (d)</td>
<td>11±5.0</td>
</tr>
<tr>
<td>OAS1a</td>
<td>41±10</td>
<td>22±8 (e+)</td>
<td>7.4±3.5(d)</td>
<td>47±20</td>
</tr>
<tr>
<td>OAS2</td>
<td>20±5</td>
<td>12±6</td>
<td>12±6 (d+)</td>
<td>5.61±3.1</td>
</tr>
<tr>
<td>STAT2</td>
<td>12±3</td>
<td>6.71±1.5 (e)</td>
<td>3.60±1.0 (d)</td>
<td>19±8.5</td>
</tr>
</tbody>
</table>

Table 5. Interferon stimulated genes (ISG) measured by PCR microarray in lung mRNA isolated from DIO-mice and lean mice that did and not exercise. Fold-change is relative to non-infected mice. One-way ANOVA was used to assess either diet effects (d significant effects of diet, p<0.05 and d+ p<0.1) in the non-exercised and exercised mice separately or exercise effects (e significant effect of exercise, p<0.05 and e+ p<0.1) in the lean and DIO mice separately. Sample size (n) was 4-5 per group.
Figure 6: Early assessment of anti-influenza IgG and IgG2c at day 8 p.i. Anti-influenza IgGs were detected by indirect ELISA. Two-way ANOVA was used to determine main effects of diet (d, p<0.05 and d+, p<0.1) or exercise (e, p<0.05 and e+, p<0.1), and interactions between diet and exercise (d*e, p<0.05 and d+e, p<0.5) on serum anti-influenza antibody levels. Sample size (n) was 12-14 per group.
Supplemental Figures Chapter V.

Supplemental Figure 1A.

[Graph showing serum D3 and D8 levels across different conditions.]

- Lo-No-Ex
- Lo-TM-Ex
- Hi-No-Ex
- Hi-TM-Ex
- Uninf

Serum Leptin Levels (ng/μL) vs. Serum D3 and D8.
Supplemental Figures 1A and 1B. Comparing serum and BAL leptin levels in day 3 and 8 p.i. DIO and non-obese mice, exercised and non-exercised mice.
SUMMARY AND CONCLUSIONS FOR THE DISSERTATION

Influenza A virus (IAV) is a common respiratory pathogen that infects the epithelial cells of the upper and lower respiratory tract causing morbidity and mortality in human cases of infection. Aged hosts are known to have increased susceptibility to seasonal IAV infection as they are considered an immune compromised individual based upon studies examining the phenomenon of immune senescence. However, young individuals are also at risk of IAV infection as new strains of the virus are constantly emerging. In pandemic years younger individuals have been shown to have a high degree of morbidity and even death over the course of the infection, as pandemic strains tend to be more virulent causing extensive lung immunopathology in younger hosts. In this dissertation we chose to further examine the effect of exercise in young and aged hosts to extend our understanding of the exercise-induced changes associated with IAV infection when exercise is practiced regularly prior to infection. Past studies, have shown reduced illness severity, viral load and attenuated lung lesion scores in exercised young and aged mice in comparison to non-exercised controls. In Chapter II of this dissertation we examined the effect of exercise training prior to influenza infection on the long term (until 6 months post primary infection) anti-influenza specific antibody response, the time to recovery from the infection, and the recall response to IAV 8 days after post secondary challenge. We found significant reductions in anti-influenza IgG and IgG2a at all time points examined, including reductions at 8 days after re-challenge. However, IgG1 was also reduced in young exercised in comparison to non-exercised but only until day 28 p.i.. After re-challenge IgG1 levels were elevated in the young exercised mice in comparison to non-exercised, while
NP-specific CD8+ memory T cell populations tended to be reduced. To our knowledge the effects of exercise prior to initial infection on the long-term response have yet to be determined in mice and humans. These results indicate that the training effect has a greater impact on the host memory response than expected. Additionally, the increased levels of IgG1 discovered in the young exercised after re-challenge indicates that the reduced viral antigen during primary challenge was associated with the development of an alternative B cell population (TM-Ex IgG1>IgG2a and No-Ex IgG2a>IgG1) in comparison to non-exercised mice. Most importantly the study showed that reduced antibody was not associated with increased mortality since exercised mice were not susceptible to illness during re-challenge.

The study presented in Chapter III was carried out to assess differences in the aged versus young mice infected with IAV apart from exercise. In this chapter anti-IFNα neutralizing antibody was used to determine if the role of IFNα if the immune response to IAV was altered between young and aged hosts. Interestingly, some studies have been able to show alterations involving Type-I interferons in aged mice as they respond to influenza infection in comparison to young mice. In these studies, results have suggested that IFNα/β production might be equal in response to Influenza A virus and that certain cell types have increased expression of IFNaR1/2 (receptor for type-I interferon), but impairments in the intracellular signaling mechanisms still exist though the expression of this innate protein and its receptor are intact or increased. Aged and young mice were treated with IFNα until 4 days post-influenza infection. Various immune measures, including illness assessment, BAL cytokines and populations, and serum antibody, were taken at day 4, 8, and 10 p.i.. The most important finding from this study was that aged mice treated with anti-IFNα had reduced symptom severity to the IAV infection and cleared virus more quickly in comparison to the aged untreated controls. Conversely, young anti-IFNα
treated mice had increased symptom severity and prolonged lung viral titers in comparison to the young untreated controls. The study further examined the specific cell populations and aspects of the lung immune response that were altered with anti-IFNα treatment in the aged and young hosts to possibly explain these outcomes. It is possible that after 4 days of neutralizing IFNα that the aged mice may have recovered their adaptive or cell mediated immune function (based upon increased IFNγ, IL-12p70 and IL-10 in the BAL and increased inflammatory monocytes and CD8+ T cells at day 8p.i.) in comparison to the young anti-IFNα treated mice leading to quicker viral clearance. Additionally, we examined the recall response to influenza A virus infection and showed that IFNα was important for the development of the NP-specific CD8+ resident memory T cell population. This population was reduced in the lungs of anti-IFNα treated mice at 11 days after lethal dose re-challenge. In summary, illness severity and viral clearance were altered between the aged and young host. The increased illness or viral titer has been reported in the young mice but the loss of IFNα leading to improvement in the illness in the aged mice has not been reported. This chapter also presents a unique finding for the long lasting effects of anti-IFNα treatment on the development of the memory response. During primary infection anti-IFNα treatment altered the shape of the CD8+ memory T cell population during re-challenge.

In Chapter IV the effect of exercise on dendritic cell function was assessed. This cell is important in both the innate and adaptive responses and has not been fully examined in the exercised host. A thorough investigation of this cell isolated from the exercised host is presented in this chapter as this work has not been completed in the field of exercise immunology. In this study we were able to show an earlier peak of antigen uptake in lung phagocytic populations in the aged and young exercised mice in comparison to non-exercised, along with what appeared to be increased antigen processing in dendritic cells derived from aged mice that exercised
compared to aged non-exercised. Furthermore, dendritic cells numbers were also increased in the lymph nodes of aged and young exercised mice shortly after treatment with antigen via an intranasal route. We believe these results alone indicate an early enhancement of the innate immune response that could potentially limit viral replication early after influenza infection. Possible exercise-induced enhancements in dendritic cell function which would enhance the adaptive immune response were also identified in this study. CD11c+ cells isolated from young exercised mice activated a higher number of CD8+ IFNγ+ cells in an antigen presentation/activation assay in comparison to young non-exercised, while CD4+ IL-2+ cells from aged exercised mice had increased IL-2 per cell in the same assay in comparison to the aged non-exercised. Since CD8+ IFNγ+ cells are a necessity for the clearance of Influenza A virus from the lungs, and an impairment in IL-2 production by CD4+ T cells has been associated with immunosenescence in the aged, these findings indicate a way that exercise leads to adaptive improvements in both young and aged hosts. To our knowledge these findings for enhanced adaptive immunity have not been reported in the literature of aging or exercise immunology.

In Chapter V exercise treatment was applied to diet-induced obese (DIO) mice infected with influenza to determine if the immune impairments in obese mice identified in previous work by other groups could be restored with exercise. Beginning at 7 weeks of age C57/BL6 mice were randomized into two groups and fed either a high fat diet or low fat diet and assigned to a exercised or non-exercised groups. After 8 weeks of training a dual energy x-ray image was used to assess body composition of the mice to ensure than DIO exercised mice maintained a higher percentage of fat in comparison to the lean controls. This was the first report in the DIO studies involving influenza in which the percentage of body fat precisely measured. Kilocalorie intake was also assessed and shown to be reduced in the exercised mice prior to infection in the
lean and DIO mice, to our knowledge this also has not been reported in the literature. Our results showed improvements in lung cell infiltration early after infection, and increased chemokines in the lungs of DIO mice that had exercised. Also, mucociliary clearance was maintained at 3 days after infection in diet-induced obese mice who exercised in comparison to diet-induced obese mice that did not exercise. Additionally, at day 8 p.i. mice that had exercised (lean or DIO) had significant increases in CD8+ T cells and inflammatory monocyte influx into the lungs in comparison to mice that did not exercise. Furthermore, NP-specific cells were increased in lung tissue of DIO exercised mice in comparison to the non-exercised at day 8 p.i.. These results, taken together with the reduced viral titer and illness severity identified in the DIO (and lean) exercised mice, suggests that both the innate and adaptive arms of the immune response were enhanced by exercise. To our knowledge, this is the first study to report regular moderate exercise as a means of correcting immune impairments associated with diet-induced obesity.

In conclusion, this dissertation further confirms the benefits of exercise in the aged and young host. More specifically, this work advances our understanding of the exercise-associated alterations on the antibody response in young exercised mice and on the respiratory dendritic cell in the young and aged mice. We were also able to show that the exercise-associated immune benefits could be extended to the diet-induced obese host as well. Finally, a unique age-associated alteration in the immune response to influenza was also identified in the experiments involving IFNα.
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