Nutritional Modulation of the Innate Immune Response to Influenza Infection

A Thesis

Sumitted to the Faculty

of

Drexel University

by

Barry W. Ritz

in partial fulfillment of the

requirements for the degree

of

Doctor of Philosophy

June 2007

© Copyright 2007
Barry W. Ritz. All Rights Reserved.

Dedications

This thesis is dedicated to my wife Kendall for her loving support. Kendall, you have truly been an inspiration to me. You are the epitome of beauty and intellect, of grace and strength. The family we are building and the dreams we share make everyday an exciting adventure. I wish everyone could experience what we share.

Acknowledgments

So many people have provided me with support, encouragement, and counsel throughout this journey that it would be impossible to adequately acknowledge each person by name. Therefore, I hope I have expressed my appreciation along the way and that it is abundantly clear that I recognize the value of every relationship I have formed, both personally and professionally, that has made this possible. A few prominent examples require specific mention.

First, I must thank my advisor Dr. Elizabeth Gardner for accepting me as her first doctoral student and, thereby, providing me with this opportunity. Liz, I could not have asked for a better situation. I came to you in search of a research project that would blend my interest in basic science with my background in nutrition. As a result of working with you, I have become impassioned by the field of nutritional immunology and believe that I have a future in this area. You granted me a great deal of freedom to pursue this work, so that I could develop confidence as a scientist, yet I always felt that I had your full support and that you were my ally. We make a great team and, I suspect, will continue a long, fruitful relationship.

Next, I thank the remaining members of my dissertation committee: Dr. Donna Murasko, Dr. Stephen Jennings, Dr. Peter Lelkes, and Dr. Peter Oelkers. It has been my privilege and good fortune to interact with such a knowledgeable, dynamic, and encouraging group of professionals. Dr. Murasko, as my chair you have been flexible and available, and as my “grand-mentor” you have taught me so much. Every time I interact with you I learn something.

To all of the faculty and my peers in the Department of Bioscience & Biotechnology, thank you. You have provided me with a breadth of understanding in various fields in the life sciences, as well as some invaluable experiences. It is truly an exciting time to be affiliated with this department. Dr. Mary K. Howett, you are an incredible scientist, a generous administrator, and a professional and personal inspiration. I have the greatest level of respect for you.

I am also thankful to all of the members of the Gardner and Murasko labs, those at present and those who have come and gone. I have worked with some amazing people, and no one can say that we did not have fun. In fact, we were sometimes accused of having too much fun. Thank you for putting up with my constant singing, but also for pouring so much effort into our work. Shoko, I especially thank you for your friendship and for being such a hard worker and a joy to interact with on a daily basis. I have never met anyone with quite so many skill sets; it’s clear that you will be a great success.

Last but certainly not least, I thank my family for their unconditional love and support. To my parents Barre and Carol, I know you sometimes wonder where I came from, but I can assure you that everything I have, everything that I have achieved is a direct result of your teaching. From how to have a successful marriage and interact with people to the work ethic and drive required to complete my PhD, you’ve provided the example. Kendall, thank you for all that you’ve done, all that you’ve sacrificed to allow me to go back to graduate school to pursue my dream of earning my PhD. I love you all.

Table of Contents

LIST OF TABLES vii

LIST OF FIGURES ix

ABSTRACT xii

1. SCOPE OF THE RESEARCH 1

2. REVIEW OF THE LITERATURE 3

 2.1 Influenza virus 3

 2.2 The innate immune response to influenza infection 10

 2.3 Aging and the immune response to influenza infection 23

 2.4 Caloric restriction 28

 2.5 Malnutrition and energy restriction differentially affect viral immunity ... 37

3. EXPERIMENTAL DATA 53

 3.1 Natural killer cells control lung virus in young and aged mice during influenza infection 53

  3.1.1 Abstract 53

  3.1.2 Introduction 54

  3.1.3 Materials and Methods 57

  3.1.4 Results and Discussion 61

 3.2 Caloric restriction increases early severity of influenza infection in young adult C57BL/6 mice 73

  3.2.1 Abstract 73

  3.2.2 Introduction 74

  3.2.3 Materials and Methods 76

  3.2.4 Results 88

3.2.5 Discussion 125

3.3 Supplementation with active hexose correlated compound increases the innate immune response of young mice to primary influenza infection 130

3.3.1 Abstract 131

3.3.2 Introduction 132

3.3.3 Materials and Methods 134

3.3.4 Results 139

3.3.5 Discussion 152

4. DISCUSSION 156

4.1 Introduction 156

4.2 Natural killer cells control influenza infection in young and aged mice . 157

4.3 Caloric restriction increases severity of influenza infection 162

4.4 Supplementation with AHCC increases the innate immune response to influenza infection 178

4.5 Conclusions and Limitations 181

4.6 Closing statement 183

5. LIST OF REFERENCES 184

APPENDIX I: Animal diets 201

APPENDIX II: PCR sequences 203

APPENDIX III: Supplementation of aged mice with AHCC 206

VITA 207

List of Tables

2.1.1 Confirmed human H5N1 influenza infections and fatalities since 2004 8

2.2.1 Toll-like receptors and their ligands 20

2.4.1 Reported effects of caloric restriction (CR) in animal models 30

2.5.1 Comparitive definitions of terms that are often misused in the literature..40

2.5.2 Reported effects of aging, protein-energy malnutrition, and energy restriction on immune function 42

3.1.1 Percent and number of NK1.1+ lymphocytes in young and aged lungs following influenza infection 66

3.1.2 Lung virus titer ($TCID_{50}$) in young, aged, and young NK cell-depleted mice during influenza infection 71

3.1.3 Lung virus titer (ng/lung) in young, aged, and young NK cell-depleted mice during influenza infection 72

3.2.1A Lung virus determined by MDCK ($TCID_{50}$/mL).....................................92

3.2.1B Lung virus determined by real-time qPCR (ng/lung) 92

3.2.2 Percent and number of total, NK1.1+, and CD8+ lymphocytes in the lungs of AL and CR mice 101

3.2.3 Percent and number of total, NK1.1+, and CD8+ lymphocytes in the spleens of AL and CR mice 103

3.2.4 IFN-γ in the lung homogenates of AL and CR mice during influenza infection 117

3.2.5 Percent and number of high side-scatter events, CD11b+, and CD11c+ cells in the lungs of AL and CR mice 119

3.2.6 IL-6 in the plasma of AL and CR mice during influenza infection 122

3.2.7 Percent and number of CD11c+ cells expressing B220 in the lungs of AL and CR mice 123

3.3.1 Lung virus titers in control and AHCC-treated mice following influenza infection 142

3.3.2 NK cell activity in control and AHCC-treated mice lungs following influenza infection 145

3.3.3 NK cell activity in control and AHCC-treated mice spleens following influenza infection 146

3.3.4 Percent and number of NK cells in control and AHCC-treated mice lungs following influenza infection 147

3.3.5 Number of lymphocytes and T cells in control and AHCC-treated mice lungs following influenza infection 149

3.3.6 Number of CD11b+ macrophages in control and AHCC-treated mice lungs following influenza infection 150

4.3.1 Summary of flow cytometry data from the lungs of CR mice compared to AL at baseline and 3 days p.i.: total and NK1.1+ lymphocytes 165

4.3.2 Summary of flow cytometry data from the lungs of CR mice compared to AL at baseline and 3 days p.i.: CD11b+ cells and pDCs.....................168

4.3.3 Effects of leptin deficiency and administration in mice 174

List of Figures

2.1.1 The structure of influenza A virus 5

2.2.1 The innate immune response to influenza infection in mouse lung....14

2.2.2 Natural killer cell activating cytokines and their effector functions....15

2.2.3 The induction of antiviral effects by IFN-α/β....17

2.2.4 NK cell-DC interactions 19

2.5.1 Weight loss occurs in mice with influenza infection due to a decrease in energy intake and an increase in energy demands....50

3.1.1 Natural killer cell cytotoxicity in young and aged mice 63

3.1.2A Percent of NK cells in young and aged mice....64

3.1.2B Percent of NK cells in young and aged mice....65

3.1.3 Confirmed NK cell depletion and loss of cytotoxicity in anti-NK1.1 antibody-treated young mice....69

3.1.4 Weight loss....70

3.2.1 Animal diets....77

3.2.2 Intranasal infection....78

3.2.3 Schematic of flow cytometry methods 81

3.2.4 Decreased survival in young adult CR mice during influenza infection ...88

3.2.5 Increased weight loss in CR mice during influenza infection 90

3.2.6 Decreased food intake in CR mice at baseline and during influenza infection 91

3.2.7 Representative H&E staining of lung tissues at baseline and 4 days following infection with influenza virus in AL and CR mice 95

3.2.8 Increased lung pathology in CR mice at baseline and during influenza virus infection 96

3.2.9 Decreased influenza-induced NK cell cytotoxicity in the lungs and spleens of CR mice 98

3.2.10 Representative flow cytometry data of the percentage of NK1.1+ and CD8+ lymphocytes in the lungs of AL and CR mice 102

3.2.11 Representative flow cytometry data of the percentage of NK1.1+ and CD8+ lymphocytes in the spleens of AL and CR mice 104

3.2.12 Intracellular perforin and granzyme B production by NK1.1+ lymphocytes in the lungs of AL and CR mice in response to influenza infection 107

3.2.13 Decreased IFN-α/β expression in the lungs of CR mice during influenza infection 108

3.2.14 Increased systemic antiviral activity in CR mice during influenza infection 109

3.2.15 Altered IL-12 production in the lungs of CR mice during influenza infection 110

3.2.16 Increased expression of CD25 on NK1.1+CD8- lymphocytes in CR mice during influenza infection 112

3.2.17 Increased expression of B220 on NK1.1+CD8- lymphocytes in the lungs of CR mice during influenza infection....................113

3.2.18 Representative flow cytometry data of the percentage of NK1.1+CD8- lymphocytes producing IFN-γ in the lungs of AL and CR mice during influenza infection....................115

3.2.19 Increased percentage of IFN-γ-producing NK1.1+CD8- lymphocytes in the lungs of CR mice during influenza infection....................116

3.2.20 Increased percentage of CD11b+ cells in the lungs of CR mice during influenza infection....................120

3.2.21 Increased percentage and MFI of CD11c+ cells expressing B220 in the lungs of CR mice during influenza infection....................124

3.3.1 Percent survival of control and AHCC-supplemented young mice (6-8 wk) from infection with 100 HAU influenza A/PR8 through d 10 post-infection 140

3.3.2 Body weights of control and AHCC-supplemented young mice following infection with 100 HAU influenza A/PR8 through d 10 post-infection 141

3.3.3 Representative H&E staining of lung tissue from control and AHCC-supplemented young mice at baseline and d 10 post-infection with 100 HAU influenza A/PR8 144

3.3.4 Representative IHC staining of macrophages in lung tissue from young control mice at baseline and d 7 post-infection with 100 HAU influenza A/PR8 151

4.2.1 Natural killer (NK) cells and the immune response to influenza infection in young and aged mice 158

4.2.2 Adoptive transfer model 160

4.3.3 The study of innate immunity includes an evaluation of NK cell percentage and number, NK cell activation, NK cell effector functions, and cytokine production by accessory and inflammatory cells 164

4.3.4 Natural killer cell-activating cytokines and effector functions in the lungs of young adult CR mice in response to primary influenza infection 166

4.3.5 Proposed metabolic mechanism for the decreased innate immune response of young adult CR mice to influenza infection 170

4.4.6 The chemical structure of the active compound in AHCC 179

Abstract

Nutritional modulation of the innate immune response to influenza infection

Barry W. Ritz, MS

Elizabeth M. Gardner, PhD

Natural killer (NK) cells play an important role in controlling virus infections and provide a potential target for nutritional modulations that may alter the innate immune response to viruses. Data presented here provide direct evidence that 1) NK cells limit influenza virus in the lung early following infection, 2) an age-associated defect in inducible NK cell cytotoxicity contributes to the increased susceptibility of aged mice to primary influenza infection, and 3) NK cells respond, both positively and negatively, to nutritional interventions. Caloric restriction (CR) is a nutritional intervention that has been shown to extend lifespan in mice and postpone age-related changes in immunity. However, in our studies, aged (22 mo) CR mice exhibited increased mortality, impaired viral clearance, and reduced natural killer (NK) cell activity following influenza infection compared to aged *ad-libitum* (AL) mice. To determine if these detrimental effects of CR occur independently of advanced age, young adult (6 mo) CR and AL C57BL/6 mice were infected with 10^4 $TCID_{50}$/100 HAU of influenza A virus (H1N1, PR8). The CR mice exhibited increased mortality (*P*<0.05), weight loss (*P*<0.01), lung virus titers (*P*<0.05), and lung pathology (*P*<0.001) compared to young AL controls. Also, CR mice exhibited a decrease in total (*P*<0.001) and NK1.1+ lymphocytes (*P*<0.05) compared to AL in response to infection, as well as a reduction in influenza-induced NK cell cytotoxicity in both lung (*P*<0.01) and spleen (*P*<0.05). These data are the first to describe an age-independent and detrimental effect of CR on the innate immune response to influenza infection. In a separate study, young (6-8wk) C57BL/6 mice were

supplemented with 1g/kg body weight of active hexose correlated compound (AHCC), a fermented mushroom extract, for 7 days prior to and throughout infection with 100 HAU of influenza A virus (H1N1, PR8). Supplementation increased survival and decreased weight loss ($P<0.001$) in response to influenza infection. Further, supplementation increased NK cell activity in lung at 1 day p.i. ($P<0.05$) and 4 days p.i. ($P<0.01$) and in spleen at 2 days p.i. ($P<0.01$). In conclusion, NK cells are important in controlling primary influenza infection, and both aging and nutritional status alter this response.

CHAPTER 1: SCOPE OF THE RESEARCH

Influenza and related secondary pneumonias represent a leading cause of death in persons over the age of 65 in the United States, and it has been proposed that the increased incidence of infectious disease in the elderly is related to an overall dysregulation in immune function. The elderly demonstrate a reduced ability to mount a protective antibody response to influenza vaccination, illustrating an age-associated decline in immunity that has been studied extensively in both humans and rodents. Influenza virus also provides a useful, albeit underutilized, model for the study of the immune response to primary virus infection, meaning viruses to which we have had little or no previous exposure.

The study of primary virus infection is particularly relevant in this era of newly emerging influenza strains, such as highly-virulent H5N1 avian influenza, and the threat of using influenza as an agent of bioterrorism. Therefore, a major focus of our laboratory's research has been to characterize the age-related decline in the immune response to primary influenza infection using a mouse model. An age-related defect in adaptive immunity, characterized by a delay in the expansion of influenza-specific CD8+ T cells in lung that is paralleled by a decrease and delay in maximal cytotoxic T lymphocyte (CTL) activity and lung virus clearance, has been demonstrated. However, there remains a paucity of data on the innate immune response to primary influenza infection and, specifically, the role of natural killer (NK) cells in controlling virus replication at the site of infection, i.e., the lungs. Interest in the innate immune response to influenza infection was prompted by two observations that suggest an important role of

NK cells in controlling virus burden prior to a CTL response and the potential for an age-associated defect in this process: 1) aged mice exhibited a decrease in inducible NK activity, and 2) aged mice that were subjected to 40% caloric restriction (CR) succumbed quickly to intranasal (i.n.) influenza infection. The latter observation was in contrast to myriad reports in which CR, in the absence of malnutrition, extended lifespan in mice and maintained various aspects of immune function with increased age, including the antibody response to influenza vaccination. Importantly, these previous studies that suggested an increase in immunity in aged CR mice did not expose mice to primary virus infection.

In Chapter 3 of this thesis, three independent but related studies are described that provide examples of how age and nutrition influence the innate immune response to influenza infection. The first study establishes the critical importance of NK cells in controlling lung virus and the severity of influenza infection in both young and aged mice. The next series of experiments clearly demonstrate that there is an age-independent and detrimental effect of CR on the innate immune response during primary influenza infection. This is followed by a final study showing an increase in the innate immune response to influenza infection in young mice supplemented with active hexose correlated compound (AHCC), a fermented mushroom extract of Japanese origin that is commercially available in the U.S. and promoted as a nutraceutical for immune enhancement. To our knowledge, this thesis is the first to concurrently evaluate the effects of age and nutrition on the innate immune response to primary influenza infection and to provide both positive and negative examples of how nutritional interventions can influence innate immunity.

CHAPTER 2: REVIEW OF THE LITERATURE

2.1 Influenza virus

> *Influenza was about to become interesting. For the virus had not disappeared. It had only gone underground, like a forest fire left burning in the roots, swarming, and mutating, adapting, honing itself, watching and waiting, waiting to burst into flames…*
>
> *Over the next year it flourished… It killed more people in twenty-four weeks than AIDS has killed in twenty-four years, more people in a year than the Black Death of the Middle Ages killed in a century.*
>
> John M. Barry, *The Great Influenza*, 2004

Influenza virus infects the epithelial cells of the respiratory tract, resulting in acute and contagious respiratory infections known collectively as *influenza* or *the flu*. Influenza viruses are responsible for seasonal epidemics, as well as occasional pandemics. Epidemics of acute respiratory illness have been documented throughout history, including an early recording made by Hippocrates in 413 BC (Wright 2001). The name influenza comes from the ancient Greek for *influence*, because the epidemics were thought to result from astrological or supernatural influences. Influenza virus itself was not identified as the causative agent of influenza until the 20th century. An influenza pandemic, known as the Spanish Flu, killed as many as 50 million people worldwide

between the years of 1918-1919, described in the citation above. The severity of this outbreak accelerated influenza research and resulted in the isolation and identification of influenza virus as the causative agent of influenza infections (Wright 2001). A number of species serve as natural reservoirs for influenza virus, including birds, swine, seals, whales, mink, dogs, and equine species. Among these, birds and swine have been linked to human infection (Wright 2001, Beigel 2005), and aquatic birds, like ducks, may be the ancestral source of all influenza viruses.

The influenza viruses belong to the *orthomyxoviridae* family, which are defined as enveloped viruses with a segmented, negative single-stranded RNA (ssRNA) genome. The *orthomyxoviridae* are so named because of their ability to bind to sialic acid residues in mucus (from the Greek *myxa*) and are designated as *ortho* (meaning standard or correct) to distinguish them from a related group of negative-stranded RNA viruses called the *paramyxoviridae* (from *para* meaning alternate), a family which includes Sendai virus and Newcastle disease virus. The orthomyxoviridae family includes influenza A, B, and C viruses, as well as the genus *Thogotovirus*, tick-borne viruses that are genetically and structurally similar to the influenza viruses (Lamb 2001). Influenza A viruses are further differentiated by their surface phenotype, which is comprised of a combination of one of 16 known hemagglutinin (HA) glycoprotein subgroups and one of nine neuraminidase (NA) subgroups (Briedis 2007). Utilizing this nomenclature, for example, the common circulating H1N1 subtype is differentiated from newly-emergent, highly-virulent H5N1 avian influenza virus. Finally, individual strains are conventionally identified by the geographic location where they were first identified, the strain number, and the year of

isolation. Accordingly, influenza A/PR/8/34 was the eighth A strain isolated in Puerto Rico in 1934.

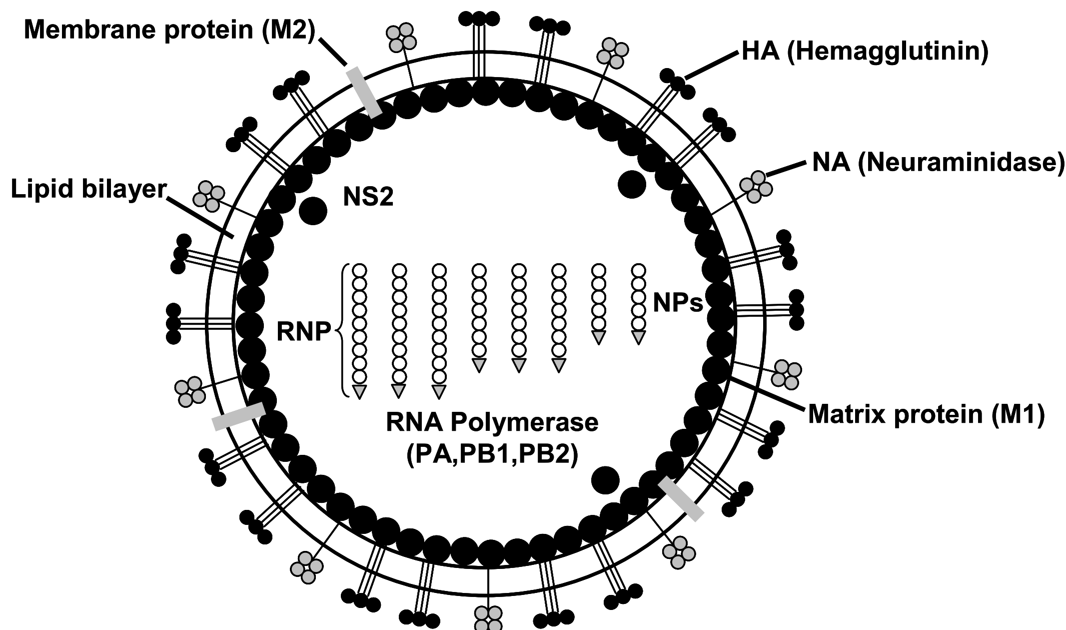


Figure 1. The structure of influenza A virus.

The influenza A and B viral genomes each contain eight ssRNA segments, which encode 10 known proteins responsible for virion structure, infectivity, or transcription. Influenza C virus, which does not cause illness in humans, does not express NA on its surface, and therefore contains only seven ssRNA segments (Lamb 2001). Influenza A viruses encode HA, NA, viral matrix protein (M1), integral membrane protein (M2), nucleocapsid protein (NP), the RNA polymerase complex (PA, PB1, and PB2), and non-structural proteins (NS1 and NS2) (**Figure 1**). Hemagglutinin and NA are the major

surface glycoproteins responsible for antigenic determination and are encoded by RNA segments 4 and 6, respectively. Hemagglutinin outnumbers NA on the virus surface by approximately 4:1 to 5:1 and is the major virus antigen against which neutralizing antibodies are produced (Lamb 2001). The M1 protein is the most abundant protein in the virion and is believed to lie interior to the lipid envelope to provide structure to the virion. The M1 protein appears to associate with the cytoplasmic tails of HA and NA, as well as interact with the ribonucleoprotein (RNP) complexes. Following replication, M1 is necessary for nuclear export of RNPs (Boulo 2007). The use of M1 protein expression as a means of quantifying virus replication is described in Chapter 3.1. The M2 protein forms an ion channel essential for the uncoating of the virus and the release of NPs from the virion. Both M1 and M2 are encoded by RNA segment 7. Nucleocapsid protein, encoded by RNA segment 5, is a major structural protein and interacts with the RNA segments and the RNA polymerase complex (RNA segments 1-3) to form each RNP. The NS1 and NS2 proteins are encoded by RNA segment 8. NS1 expression has only been identified in infected cells, while NS2 is present in purified virions. The NS2 protein is also referred to as a nuclear export protein (NEP), because it is thought to be important for export of the RNP from the nucleus (Boulo 2007).

Briefly, the viral life cycle begins with the binding of HA to the sialic acid-containing receptors on the epithelial cell surface (Lamb 2001). The virion then enters the cell via receptor-mediated endocytosis, and acidification of the endosome results in two essential steps necessary for transport of RNPs from the virus into the cellular cytoplasm. First, protonation of the HA results in a conformational change in which the viral membrane becomes irreversibly fused to the endosomal membrane. Secondly, the

interior of the virion becomes acidified, which allows for the disassociation of M1 from the RNPs. The M2 H+ ion channel is then able to mediate cleavage of the viral RNPs into the cellular cytoplasm (Boulo 2007). Unlike most RNA viruses, influenza virus replicates in the nucleus and utilizes host RNA primers to synthesize positive-stranded copies of the virus RNA. The RNA polymerase complex then generates a complete set of eight virus negative-stranded ssRNA segments. From this, virus HA, NA, and M2 proteins are then synthesized, inserted in the cellular endoplasmic reticulum, and transported to the cell surface via the Golgi apparatus. Although the role of NA in virus replication remains unclear, this protein may be responsible for cleaving the sialic acid-HA interactions to allow virion export (Lamb 2001). Virus assembly occurs at the cell surface in association with lipid rafts where virion budding takes place, attaining its lipid envelope from the host membrane.

Influenza A viruses undergo continuous genetic variations that periodically result in the emergence of new strains. New strains result from either *antigenic drift* (the accumulation of point mutations) or *antigenic shift* (re-assortment of the viral genome resulting from the mixing of two or more viral subtypes). Antigenic drift occurs in both the HA and NA glycoproteins and is principally responsible for yearly variations in circulating strains of influenza virus. Examples of antigenic shift include the emergence of H2N2 in 1957 (Asian Flu), the emergence of H3N2 in 1968 (Hong Kong Flu), and the re-emergence of H1N1 in 1977 (Russian Flu) (Wright 2001). More recently, an H5N1 influenza A virus subtype emerged in poultry. The first cases of human infection with H5N1 virus were reported in Hong Kong in 1997, following direct contact with infected poultry. Eighteen confirmed influenza infections resulted in six deaths (Beigel 2005). An

outbreak of H5N1 among poultry followed in Asia in late 2003-2004, killing approximately 150 million birds. Since 2004, confirmed human infections with highly virulent H5N1 avian influenza virus have totaled 266, with a combined mortality rate of over 50% (**Table 1**). In addition to countries reporting human infections, poultry infections have occurred in Malaysia, Nigeria, Iran, India, Greece, Italy, Austria, Germany, and France (www.who.int).

Table 1. Confirmed human H5N1 avian influenza infections and fatalities since 2004.

Country	**Number of confirmed cases**	**Number of deaths (%)**
Azerbaijan	8	5 (63%)
Cambodia	7	7 (100%)
China	24	15 (63%)
Egypt	34	14 (41%)
Indonesia	79	61 (77%)
Iraq	2	2 (100%)
Thailand	25	17 (68%)
Turkey	21	4 (19%)
Vietnam	66	22 (33%)
TOTAL	**266**	**147 (55%)**

Compiled from data available at www.who.int.

The emergence of new viruses, like H5N1 avian influenza virus, underscores the need for a comprehensive understanding of the immune response to primary virus infection. It is unknown whether traditional vaccine strategies will be effective against H5N1 avian influenza virus or if effective vaccines can be produced quickly enough to respond to a pandemic threat. Further, analysis of H5N1 viruses isolated from both poultry and human samples have demonstrated some resistance against currently available antiviral drugs (www.cdc.gov). While neutralizing antibodies to HA and NA surface glycoproteins are generated during the immune response to influenza viruses and protect against subsequent exposure to the same virus, CD8+ T cells are essential for the eradication of a primary virus infection (Murasko 2005). Prior to the adaptive immune response, however, natural killer (NK) cell-mediated innate immunity plays an important, yet under-studied, role in controlling virus early during the immune response to primary virus infection, including influenza.

2.2 The innate immune response to influenza infection

Originally described as “null” lymphoid cells, natural killer (NK) cells were later discovered as a distinct population of lymphocytes with spontaneous cytolytic activity against tumor cells that could be further induced with interferon (Herberman 1981, DiSanto 2006, O’Connor 2005). Natural killer cells are now recognized as potent innate effector cells with not only anti-tumor, but also anti-microbial and anti-viral activities (Andoniou 2006, DiSanto 2006). These important lymphocytes are typically identified in mice as CD3-NK1.1+ and comprise approximately 5-20% of total lymphocytes in secondary lymphoid organs. NK cell-mediated innate immunity appears to play a significant role in controlling virus burden early in infection until an antigen-specific T cell response can be achieved (Biron 2001a, Neff-La Ford 2003, Solana 2000, Janeway 2005). Most NK cells confer direct cytolysis upon target cells and secrete a variety of cytokines, while a minority may function as an immunoregulatory subset mediating effects exclusively through the production of cytokines (Ferlazzo 2004). Upon activation, NK cells respond rapidly, peaking within hours to the first few days of infection, and produce interferon (IFN)-γ (Biron 2001a, Nguyen 2002, Hunter 1997). In turn, IFN-γ induces increased MHC class I and II expression on a wide variety of cells and further increases the cytotoxicity of both NK cells and cytotoxic T lymphocytes (CTLs) (Biron 2001b). These CTLs are principally responsible for killing virus-infected cells, culminating in recovery from infection (Neff-La Ford 2003, Bender 1995, Bender 1992, Moskophidis 1998). Thus, although NK cells alone are unlikely to mediate recovery from virus infection, they actively participate in the induction of adaptive immunity and may be critical in controlling infection prior to an antigen-specific response.

The importance of NK cells in controlling virus infections has long been demonstrated (Bukowski 1983). According to a recent review (Andionou 2006), NK cells limit the replication of viruses, including hepatitis C virus, human immunodeficiency virus (HIV), dengue virus, and yellow fever virus. Further, NK cells are important in controlling the severity of certain viral infections, such as herpes simplex virus (HSV), hepatitis C virus, HIV, and cytomegalovirus (CMV) in humans and HSV, murine CMV (MCMV), lymphocytic choriomeningitis virus (LCMV), Sendai virus, and Kunjin virus in mice. However, early studies did not clearly identify a role for NK cells in controlling influenza virus (Stein-Streilein 1988, Suttles 1986, Bot 1996). As a result of these inconsistent data and an emphasis on immunization, the potential role of NK cells in the immune response to primary influenza infection has been largely ignored. Experiments described in Chapter 3.1 of this thesis examine primary influenza infection and virus titers in the lungs of immunocompetent mice following confirmed NK cell-specific depletion. These data provide direct evidence linking NK cells with resistance to influenza infection and suggest that NK cells are important for controlling virus titer in the lung in response to influenza infection.

In lieu of antigen-specific receptors, NK cells express a combination of activating and inhibitory receptors that function to allow NK cells to directly identify pathogens without prior recognition. Predominantly, these receptors are classified as CD94/NKG2 or killer immunoglobulin-like receptors (KIR) in mice and humans, or Ly-49 receptors in mice. Members of all three receptor families can function as activating or inhibitory receptors (Andoniou 2006, O’Connor 2005, Gasser 2006). In general, NK inhibitory receptors recognize MHC class I on the surface of healthy host cells, delivering an

inhibitory signal that overrides signals provided by activating receptors. As virally-infected cells or tumor cells express altered or reduced levels of MHC class I, the inhibitor signal ceases, resulting in NK cell activation.

Additionally, NK cell activation may occur indirectly, following pathogen recognition by accessory cells (Newman 2007). Natural killer cells form receptor-mediated interactions with accessory cells, such as macrophages and dendritic cells (DCs), resulting in NK cell activation through the transmission of activating signals and the exchange of cytokines. Both direct and indirect NK cell activation result in NK cell cytotoxicity and cytokine production, as well as regulatory functions mediated by NK cell-accessory cell interactions. The relative importance of direct and accessory cell-dependent activation of NK cells in response to influenza infection remains under investigation. Some evidence suggests that influenza infection may actually increase NK cell inhibitory receptor binding as an evasion tactic, which may increase dependence on DC-mediated activation (Achdout 2003).

Initially, the immune response to primary influenza infection is mediated by non-cellular components, such as proinflammatory cytokines and chemokines (**Figure 1**). These soluble factors are produced by infected macrophages and endothelial cells and are involved in host defense both systemically and at the site of infection by initiating inflammation, activating NK cells, or recruiting leukocytes to the site of infection (Neuzil 1996, Price 2000, Conn 1995, Hennet 1992, Dawson 2000). *In vitro* studies, as well as *in vivo* human and animal studies, suggest that Type I interferon (IFN-α/β), tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-12, IL-18, MCP-1 (CCL2), and MIP-1α/β (CCL3/4) are among the major cytokines and chemokines produced in response to

influenza infection (Brydon 2005, Biron 2001b, Julkenen 2001, Van Reeth 2000, Neuzil 1996, Hennet 1992, Liu 2004, Pirhonen 1999, Saraneva 1998). These cytokines are produced rapidly in lung following influenza infection, and pulmonary cytokines generally exceed systemic cytokine levels, suggesting a predominantly local response (Van Reeth 2000, Orange 1996). The cytokines TNF-α, IL-1β, and IL-6 act as proinflammatory, endogenous pyrogens and upregulate MHC I expression, DC migration, and IFN-γ production in reponse to viral infection (Biron 2001b). Interferon-α/β, IL-12, and IL-18 activate cells of innate immunity, as described in detail below. Cytokines and chemotactic proteins, such as MCP-1 and MIP-1α/β, produced by macrophages and activated NK cells, promote the activation of resident macrophages and recruit neutrophils (polymorphonuclear cells), dendritic cells, and additional macrophages and NK cells to the lung to amplify the immune response (Brydon 2005, McKenna 2005).

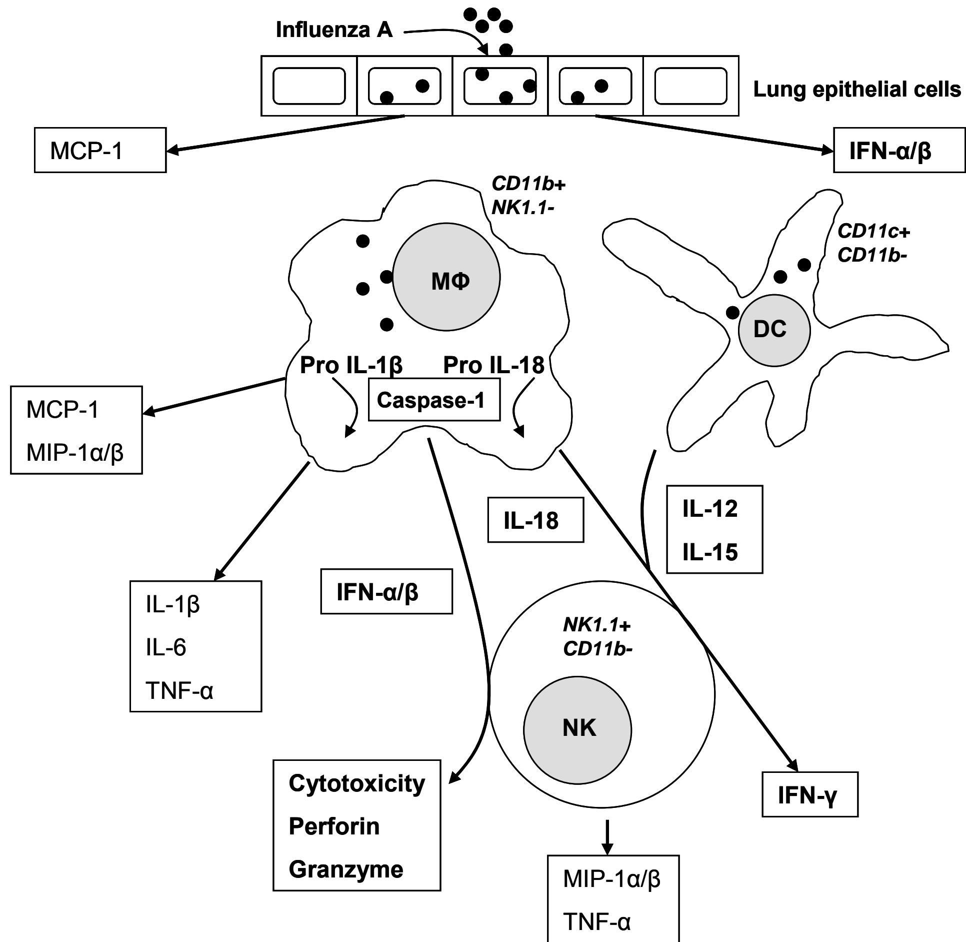


Figure 1. The innate immune response to influenza infection in mouse lung.

Although the exact mechanism of increased NK cell cytotoxicity in response to individual cytokines has not yet been elucidated, there is considerable information regarding the roles of IFN-α/β, IL-12, IL-15, and IL-18 as endogenous regulators of NK cell responses early after virus infections (Biron 2001a, Biron 2001b, Julkenen 2001, Nguyen 2002, Cousens 1997, Pien 2000). Interleukin-12 and IL-18 are sometimes referred to as *NK cell stimulating factor* and *IFN-γ inducing factor*, respectively, and both cytokines are produced by virus-infected macrophages and dendritic cells (Trinchieri 2003, Pirhonen 1999, Saraneva 1998).

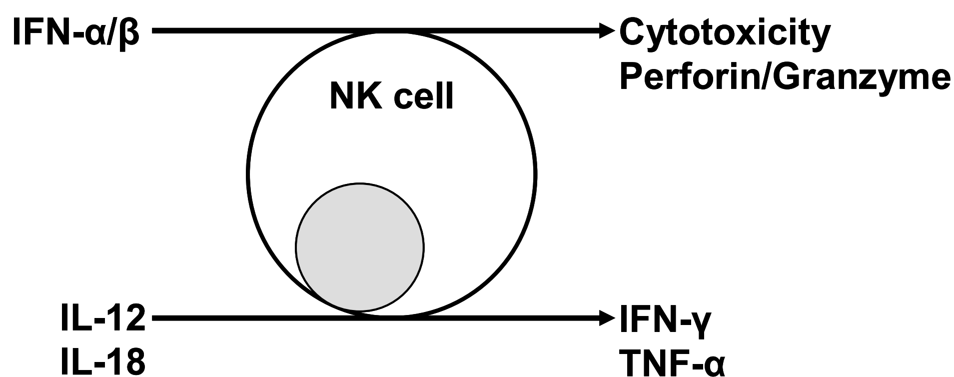


Figure 2. Natural killer cell activating cytokines and their effector functions.

Kinetic studies in mice infected with MCMV have shown that, in addition to IFN-α/β, the endogenous production of IL-12 and IL-18 acts to induce NK cell activity and contributes to peak IFN-γ production by NK cells (**Figure 2**), thus providing an important link to adaptive immunity by stimulating the activity of CTLs (Nguyen 2002, Cousens 1997, Pien 2000, Orange 1996). Although neither cytokine appears to be essential for NK cell activation, receptors for IL-12 and IL-18 are constitutively expressed by NK cells (Hyodo 1999, French 2006), and IL-12 and IL-18 independently and synergistically

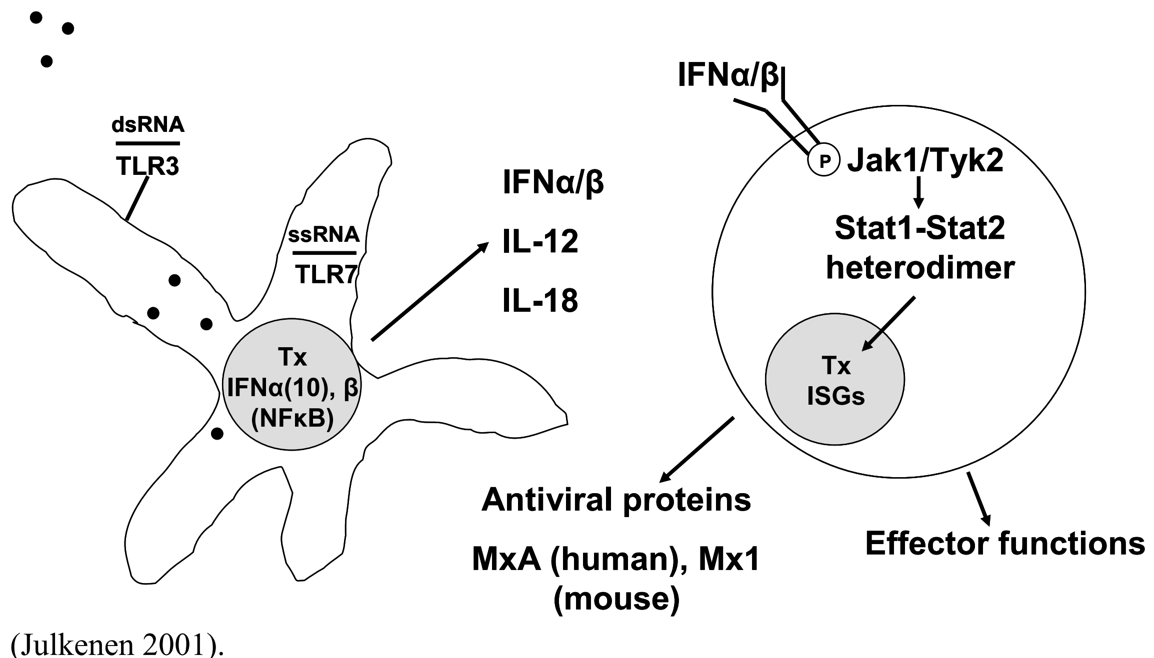
augment perforin-mediated NK cell cytotoxicity and induce NK cells to produce IFN-γ (Hyodo 1999, Walzer 2005).

The significance of IL-18 in the immune response to influenza infection is still under investigation. Interleukin-18 is structurally related to IL-1β and, like IL-1β, it is produced by activated macrophages in response to influenza virus infection by a caspase-1-dependent mechanism (Pirhonen 1999, Saraneva 1998). In one study, IL-18 deficient mice exhibited increased virus, increased IL-12, and decreased IFN-γ in lung following intranasal infection with influenza virus (10^3 plaque-forming units, PR8) (Liu 2004). However, in a separate study, IL-18 deficient mice exhibited increased viral titer but normal IFN-γ production when infected with a low dose of influenza A virus (10 $TCID_{50}$, PR8) (Van der Sluijs 2005).

Interleukin-15, described as an *NK cell survival factor*, enhances NK cell function *in vitro* and enhances the proliferation response (Ferlazzo 2004). Also, IL-15 may be required for NK cell development and for survival of mature NK cells *in vivo*, a process which appears to require expression of the IL-15Rα by accessory cells (Prlic 2007, Koka 2007, DiSanto 2006). While IL-18 alone does not appear to stimulate the proliferation or cytotoxicity of isolated NK cells *in vitro*, IL-18 augmented IL-15-dependent NK cell proliferation in culture (French 2006) and increased both proliferation and cytotoxicity in combination with IL-2 (Son 2001).

Interferon-α/β has also demonstrated critical importance in host defense to virus infection (Biron 2001b). It induces an antiviral state, inhibits virus replication in host cells, increases major histocompatibility complex (MHC) class I expression and antigen presentation in all cells, and activates NK cells during virus infection (Garcia-Sastre

2006, Nguyen 2002, Samuel 1991). Briefly, IFN-α and IFN-β bind to transmembrane Type I IFN receptors on target cells, resulting in the activation of Janus-activated kinase 1 (Jak1) and tyrosine kinase 2 (Tyk2) (**Figure 3**). Phosphorylation follows and drives the formation of the Stat1:Stat2 heterodimer, which translocates to the nucleus and activates the transcription of greater than 100 IFN-stimulated genes (ISGs) (Garcia-Sastre 2006). Gene products with antiviral activities include Mx1 protein in mice and MxA protein in humans, which inhibit the replication of multiple viruses, including influenza virus



(Julkenen 2001).

Figure 3. The induction of antiviral effects by IFN-α/β.

Interferon-α/β is also required for the induction of NK cell cytotoxic activity, in part by stimulating the upregulation of perforin and granzyme B (Van Dommelen 2006, Johnson 2003, Liang 2003, Wright 1983). Conversely, IFN-α/β may inhibit the expression of IL-12, as well as IL-12-mediated IFN-γ production by NK cells (Biron

2001b, Garcia-Sastre 2006). IFN-α/β may also control the production of proinflammatory cytokines, as Stat1-/- mice unable to produce IFN-α/β exhibited increased pulmonary inflammation and increased pathology following infection with influenza virus (Durbin 2000). On a clinical note, specific IFN-α and IFN-β subtypes are under investigation as potential adjuvants to improve the efficacy of influenza vaccination (James 2007).

Innate accessory cells, in particular DCs, are also key components of the innate immune response by way of cytokine production and NK cell activation. Dendritic cells act as the sentinels of the innate immune system, continuously sampling antigens in the periphery and delivering them slowly to the lymph nodes. Upon activation and maturation, DCs migrate more quickly to the lymph nodes and express high levels of MHC and costimulatory molecules, a process described as *licensing*, in preparation for increased antigen presentation (Walzer 2005). A naïve T cell, upon identification of its rare cognate antigen, is then believed to form a stable interaction with the DC, at which point the T cell receives activating signals. In contrast, NK cells appear to form longer-lasting interactions with DCs, even in the absence of antigen, during which the NK cells receive survival signals from the DCs in the form of IL-15 (Bajenoff 2006) (**Figure 4**).

In 1999, Fernandez and colleagues first described NK cell activation mediated by direct contact with DCs, as *in vitro* co-culture of NK cells and DCs resulted in increased NK cell cytotoxicity and IFN-γ production (Fernandez 1999). Further, *In vitro* studies clearly demonstrate that DCs activate NK cells through a combination of direct cell-to-cell contact and cytokine production. Receptor-mediated NK cell-DC interactions might enhance NK cell activation, in part, by improving the delivery of NK cell-activating

cytokines from the DC to the NK cell (Newman 2007). Moreover, recent evidence suggests that NK cell-DC interactions actually perform bi-directional functions. In other words, in addition to DC-mediated NK cell activation, receptor-mediated signaling between an NK cell and a DC may also result in either NK cell-mediated DC lysis or DC maturation. A low ratio of activated NK cells to immature DCs appears to result in DC maturation, including upregulation of MHC class I, thereby conferring protection against autologous NK cell-mediated cytolysis (Piccioli 2002, Walzer 2005). *In vitro* evidence suggests that DC maturation is also dependent on IFN-γ and TNF-α, which are produced by activated NK cells (Ferlazzo 2002) (**Figure 4**). *In vivo*, NK cell-DC interactions are important for controlling viral replication and resistance to infection, as demonstrated in multiple models (Walzer 2005, Andrews 2002). For example, DC depletion resulted in impaired NK cell activation and increased susceptibility to HSV-1 infection in mice (Kassim 2006). To date, however, the role of DCs in NK cell activation in response to influenza infection has not been clearly described.

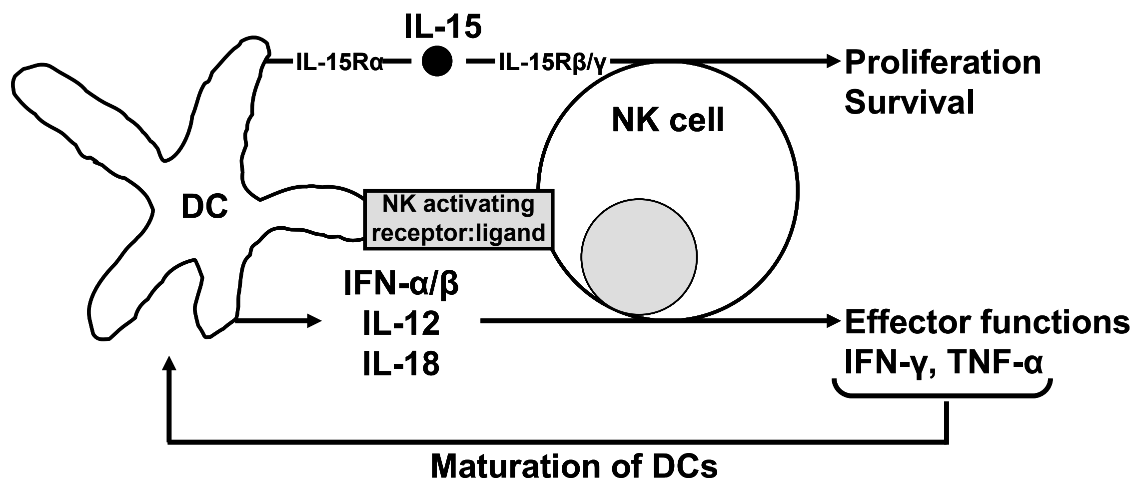


Figure 4. NK cell-DC interactions.

The ability of NK cells and accessory cells to recognize virus infection is mediated, in part, by toll-like receptors (TLRs), which are innate pattern recognition receptors that recognize conserved pathogen-associated molecular patterns (PAMPs) common to bacteria or viruses (**Table 1**). The interaction between a TLR and its ligand signals such events as the production of antiviral Type I IFN and pro-inflammatory cytokines, such as IL-6 and TNF-α. Natural killer cells, as well as accessory DCs and macrophages, express a number of TLRs important in the identification of influenza virus and its products.

Table 1. Toll-like receptors and their ligands.

TLR	Ligand
1	Gram + bacteria, yeast cell wall components
2	Gram + bacteria, yeast cell wall components
3	dsRNA, bacterial flagellin
4	LPS, Gram - bacteria
5	dsRNA, bacterial flagellin
6	Gram + bacteria, yeast cell wall components
7	ssRNA, dsRNA
8	ssRNA
9	dsRNA

LPS, lipopolysaccharide; ds, double-stranded; ss, single-stranded.

(O'Connor 2005, Renshaw 2002)

Toll-like receptor 3 is perhaps the best characterized TLR regarding NK cell activation. The consensus among immunologists is that NK cells express TLR3, which recognizes dsRNA motifs common to viral replication (O'Connor 2005). Activation of NK cells, as well as DCs, by polyinosinic-polycytidylic acid (Poly I:C), a dsRNA analogue, is mediated by this receptor (Verdijk 1999).

Toll-like receptors 7, 8, and 9 are intracellular TLRs expressed on the phagocytic endosome that recognize ssRNA or dsRNA viral patterns. In addition to TLR3, recent data suggest that NK cells may also express TLR7 and TLR8 (O'Connor 2005, Moretta 2006). Although human NK cells have also been observed to express TLR9, evidence at this time does not support the expression of TLR9 by murine NK cells.

Plasmacytoid dendritic cells (pDCs), identified as CD11b-CD11c+B220+, are the major producers of IFN-α/β after virus infection (Walzer 2005, Garcia-Sastre 2006, McKenna 2005). Type I IFN production in response to influenza infection appears to follow the recognition of ssRNA by TLR7 (Diebold 2004), which is predominantly expressed on pDCs. Virus particles from HSV and MCMV may be predominantly recognized by TLR9, while influenza virus and vesicular stomatitis virus (VSV) are recognized by TLR7 (Lee 2007, Honda 2006). Interestingly, both UV-irradiated and live influenza virus or HSV infections induce Type I IFN production by the pDCs, whereas the introduction of heat- or UV-killed VSV or respiratory syncytial virus (RSV) do not generate such a response (Lee 2007).

As outlined above, the innate immune response to primary virus infections involves a complex interaction of cellular and soluble mediators. An understanding of the coordinated activities of NK cells and accessory cells in recognizing and controlling

virus infection is only beginning to emerge. Given the role of innate immunity in controlling virus infection prior to the initiation of an antigen-specific adaptive response, innate immunity may be a particularly important defense against newly emergent viruses. As such, the innate immune response to primary influenza infection requires further exploration, especially in the context of those at an increased risk for morbidity and mortality from infectious disease, such as the elderly.

2.3 Aging and the immune response to influenza infection

"Old age isn't so bad when you consider the alternative."

Maurice Chevalier, *New York Times*, October 9, 1960

The elderly, defined as those individuals aged 65 years and older, represent the fastest growing segment of the U.S. population and are the major users of our health care resources. Predominant health conditions within this population include cancer, heart disease, diabetes, Alzheimer's disease, autoimmune disease, and an increased incidence of infectious disease. The elderly demonstrate increased rates of infection, as well as increased morbidity and mortality in response to infectious diseases, such as RSV (Han 1999), severe acute respiratory syndrome (SARS) (Donnelly 2003), and influenza virus (MMWR 2004). It is estimated that influenza is related to greater than 200,000 hospitalizations and as many as 36,000 deaths annually, 90% of which occur in the elderly (Thompson 2003, Thompson 2004). Despite a substantial increase in vaccination rates, influenza-related hospitalizations and deaths have continued to rise over the past two decades, and thus, influenza infection is recognized as a major ongoing public health burden (Simonsen 2005, Jefferson 2006).

It has been hypothesized that the increased incidence of infectious disease in the elderly is related to an overall dysregulation in immune function, although a limited number of studies have directly assessed the relationship between age-associated changes in immunity and increased infectious disease (Murasko 2005, Meyer 2001). The reduction in the immune response to influenza vaccination in the elderly is corroborated by numerous studies demonstrating an inability to mount effective antibody titers,

decreased T cell proliferative response, reduced cytotoxic T cell (CTL) activity, and altered cytokine production following immunization (Murasko 2003, Murasko 2002, Murasko 1990, Murasko 1987, Bernstein 1999, Webster 2000, Govaert 1994, Gardner 2002). Fewer studies, however, have investigated age-related changes in the immune response to influenza infection, and it has been suggested that immunization studies can no longer remain the cornerstone of aging immunology research (Murasko 2005). The pandemic threat of H5N1 avian influenza virus, the emergence of relatively new viruses, such as Ebloa virus and SARS, as well as the recognition that new or adapted viruses could be used as agents of bioterrorism, mandate the need for a comprehensive understanding of the immune response to primary viral infections, meaning those viruses to which we as humans have had no previous exposure.

Influenza virus is well studied, and thus, has been instrumental in elucidating age-related changes in the immune response to primary viral infections. Of course, studies involving the infection of elderly humans with live virus are not ethical because of the lack of adequate antiviral therapies. Instead, the investigation of age-related changes in the immune response to viral infections must be conducted in animal models, such as mice (Mestas 2004). Studies in aged mice have demonstrated reduced antibody responses, impaired CTL responses, and increased lung virus titers after infection with influenza A virus (Murasko 2005). Impaired CD8+ T cell function was associated with increased lung virus and prolonged virus shedding, indicating the importance of CTLs in the recovery from influenza infection (Po 2002, Moskophidis 1998, Bender 1991, Bender 1995, Effros 1983). Following influenza infection, aged mice exhibited a decreased and delayed expansion of influenza-specific CD8+ T cells that was paralleled by a decrease in

the magnitude and kinetics of the CTL response, resulting in delayed virus clearance from lung and suggesting that the adaptive immune response is not entirely absent in aged mice, but functioning at a lower level than in young mice (Po 2002).

While it is clear that antigen-specific CTLs are principally responsible for killing virus-infected cells and, therefore, are critical for recovery from a variety of virus infections, including influenza, MCMV, and HSV, NK cell-mediated innate immunity may also play a significant role in controlling virus burden early in infection to prevent mortality until an antigen-specific T cell response can be achieved (Andoniou 2006, Biron 2001a, Solana 2000, Janeway 2005). Upon activation, NK cells respond rapidly, peaking within hours to the first few days of infection, and produce IFN-γ, which further increases the cytotoxicity of both NK cells and CTLs (Biron 2001a, Nguyen 2002, Liu 2004).

The increased incidence of cancers and infectious diseases in the elderly and the importance of NK cells in the eradication of tumors and in eliminating virus-infected cells prompted the examination of age-related changes in NK cells. Although most evidence from humans (Kutza 1996) and animal models (Plett 1999, Plett 2000, Albright 2004) indicates no change in the percentage or number of NK cells with advancing age, age-related changes in both basal and inducible NK cell activity have been observed. Basal NK cell activity measured *ex vivo* in peripheral blood samples gathered from elderly donors does not decrease, and possibly increases, with age (Kutza 1995). In mice, however, there is a developmental change in NK cell activity, such that young (6-8 week) mice demonstrate higher basal NK cell activity than young adult (6 month) or aged (22-24 month) mice (Saxena 1984, Provinciali 1989). Although basal NK activity does not

differ between adult and aged mice, there is an age-related decrease in cytokine-inducible NK cell activity. IFN-α/β is an important inducer of NK cell cytotoxicity during virus infection (Garcia-Sastre 2006). Both *in vitro* and *in vivo* IFN-α/β treatment increased the NK cell activity of 6 month-old mice to levels seen in 2 month-old mice, whereas there was no increase in the NK cell activity of 22-24 month-old mice (Plett 1999, Provinciali 1989), reflecting an age-associated decrease in inducible NK cell cytotoxicity, which has also been observed in elderly humans (Solana 1999). The mechanism behind the decrease in inducible NK cell activity in aging has not been determined but may include an alteration in IFN-α/β receptor expression, an increased rate of NK cells undergoing apoptosis, or a shift in the balance between NK cell stimulating and inhibiting receptors (Plett 2000, Lutz 2005). Some studies also suggest that the production of IFN-α/β in response to virus infections decreases in aged compared to young mice, which would be expected to limit the ability of aged mice to induce NK cell cytotoxicity *in vivo* (Murasko 2005).

The role of NK cells in controlling influenza virus replication and limiting the severity of influenza infection remains largely unknown and has not been studied in the context of the age-associated decline in inducible NK cell activity. However, NK cells are known to limit viral replication and control the severity of infections in response to multiple other viruses, including HSV and MCMV (Andoniou 2006). Data presented in Chapter 3.1 of this thesis are the first to suggest that pulmonary NK cells play a similar role in response to influenza virus following intranasal infection. Further, a reduction in influenza-induced NK cell cytotoxicity appears to accompany the delay in virus clearance, as well as the reduced and delayed CTL response that has been documented in

aged mice following influenza infection. These data suggest that NK cells play an important role in controlling virus titers in lung in response to influenza infection and that an age-associated defect in inducible NK cell activity may contribute to the increased susceptibility to influenza infection in aging.

The effects of aging on immunity are highly heterogeneous among humans, including among the healthy elderly. For example, some elderly individuals maintain the ability to mount a protective antibody response to influenza vaccination, while others do not (Murasko 2003). Therefore, additional variables that may influence immunity, such as nutritional status, may explain differences in the incidence and pathology of infection in the elderly (Mazari 1998, Lesourd 2004). Indeed, malnutrition or nearly any nutritional deficiency, if severe enough, will negatively impact immune status. Studies that have attempted to correlate micronutrient status with antibody response to influenza vaccination in the elderly, however, have produced conflicting results (Hara 2005, Gardner 2000, Pozzetto 1993). These varied outcomes suggest that some degree of immune senescence occurs in the elderly independent of alterations in nutritional status. Perhaps some immune changes that occur with aging cannot be avoided. For example, the increase in memory T cells associated with aging may result from lifelong antigenic stress and likely contributes to the age-associated decrease in proliferative ability (Lesourd 2004). Meanwhile, innate immunity, which occurs very early in the course of infection and is highly variable among the elderly (Lesourd 2004), could be more closely related to nutritional status. Further study into the role of innate immunity in controlling virus burden may reveal opportunities for the study of nutritional interventions to alter the immune response to primary virus infection.

2.4 Caloric restriction

In the study of aging, caloric restriction (CR), in the absence of nutrient deficiencies, has emerged as the only known intervention capable of extending maximal lifespan (Speakman 2007, Yamaza 2002, Masoro 2005, Wanagat 2000). The extension of lifespan in rodents by CR without malnutrition was first demonstrated in rats by McCay and colleagues in 1935 (McCay 1935). Since then, diets ranging from 30-70% CR have been shown to increase median and maximal lifespan by up to approximately 65% and 50%, respectively, over AL diets (Barger 2003). Similar results have been obtained in multiple rodent species and strains, dogs, and non-mammalian species, like fish and flies (Masoro 2005). Ongoing studies of rhesus and squirrel monkeys maintained at 30% CR indicate the likelihood of a comparable decrease in morbidity and mortality rates (Lane 2002), although it is premature to determine the effects of CR on maximal lifespan in non-human primates.

The effects of CR on metabolism and immunity, and their relationship to the extension of lifespan, have been studied extensively (Sohal 2002, Avula 2002, Heilbronn 2003, Yamaza 2002). Metabolic changes induced by CR, consistent in both rodents and non-human primates, include decreased weight and abdominal obesity, decreased oxidative stress, a decrease in body temperature, and a transient decrease in metabolic rate (**Table 1**). Metabolic rate appears to normalize when adjusted for decreased body weight in CR animals. Caloric restriction is also associated with changes clinically related to a decrease in age-associated disease pathology and disease-specific mortality, including reduced blood pressure, lowered triglycerides, and improved insulin sensitivity (Yamaza 2002, Speakman 2007, Heilbronn 2003, Roth 2001, Masoro 2005, Gredilla

2005). Caloric restriction is generally acknowledged to delay the development of immunity and preserve immune function at an advanced age (Pahlavani 2000). Caloric restriction reduces the incidence of spontaneous tumors and cancers in aged rodents (Lane 2001, Weindruch 1989, Weindruch 1986) and maintains T cell proliferation, cytokine production, and cytotoxic T lymphocyte activity (Messaoudi 2006, Nikolich-Zugich 2005). The overall changes in immune response in CR animals are reviewed in greater detail in Chapter 2.5 of this thesis.

Table 1. Reported effects of caloric restriction (CR) in animal models.

		Species[1]	References[2]
↑	**Lifespan**	r	**Speakman 2007**, **Yamaza 2002**, Goonewardene 1995, Fernandes 1976
↓	**Lean body mass**	r,p	**Speakman 2007**, **Heilbronn 2003**, Verdery 1997
↓	**Body temperature**	r,p	**Heilbronn 2003**, Lane 1996
↓	**Energy expenditure**	r,p	**Speakman 2007**, Lane 1996
↓	**Metabolic rate**[3]	r,p	**Speakman 2007**, **Heilbronn 2003**
↑	**Immune function**	r,p	**Nikolich-Zugich 2005**, **Pahlavani 2000**
↓	Tumor formation	r,p	**Lane 2001**, **Weindruch 1989**
↑	T cell proliferation	r,p	**Messaoudi 2006**, **Nikolich-Zugich 2005**, Umezawa 1990, Goonewardene 1995, Tian 1995
↑	Antibody responses	r	**Nikolich-Zugich 2005**, Effros 1991
↓,=	Antibody responses	p	**Nikolich-Zugich 2005**, Roeker 1996

↑	**Insulin sensitivity**	r,p	**Heilbronn 2003**, **Lane 2001**
↓	**Cardiovascular risk factors**	r,p	**Roth 2001**, Guo 2002, Verdery 1997
↓	Blood pressure	r,p	**Roth 2001**
↓	Total cholesterol	r	**Roth 2001**, Choi 1988, Masoro 1983
↓,=	Total cholesterol	p	**Roth 2001**, Verdery 1997
↑	HDL	r,p	**Lane 2001**, Verdery 1997
↓	Triglycerides	r,p	**Lane 2001**, Verdery 1997, Choi 1988, Masoro 1983
↑	**Stress (corticosterone)**	r	**Masoro 2005**
↓	**Oxidative stress**	r,p	**Gredilla 2005**, **Masoro 2005**, **Heilbronn 2003**, Guo 2002, Tian 1995

[1] Species: r, rodents; p, primates.

[2] Scientific reviews were included when available and are indicated in bold.

[3] CR animals may experience an absolute decrease in metabolic rate; however, when adjusted for decreased body mass, the decrease in metabolic rate is not significant (Masoro 2005).

It is likely that a combination of mechanisms contribute to life extension in CR animals (Masoro 2005). Leading hypotheses include a reduction in oxidative damage, alterations in stress response, decreased body temperature, and increased insulin sensitivity. Each of these mechanisms may be associated with alterations in gene expression in CR (Han 2005, Warner 2005).

The broad anti-aging effects of CR align well with the oxidative stress hypothesis of aging, which suggests that the accrual of oxidative damage, induced by reactive oxygen species (ROS), promotes the aging process and results in the loss of physiological functions (Sohal 2002, Yu 1996). Caloric restriction is associated with both a decrease in metabolic rate and, according to most reports, an increase in antioxidant defenses. The restriction of calories results in a shift away from pro-oxidative mitochondrial activity with advanced age, reduced production of reactive oxygen species, less protein and DNA oxidative damage, and decreased H_2O_2-induced lymphocytosis (Gredilla 2005, Rebrin 2003, Sohal 2002, Avula 2002, Merry 2004).

Aging is associated with an increase in glucocorticoids and a decrease in stress responses that have been implicated in the age-associated impairment in immunity and loss of cognition (Berner 2004, Patel 2002, Yu 2001). The hypothesis that CR may yield anti-aging effects by attenuating age-related hyperadrenocorticism was dismissed with the recognition that CR results in a prolonged, mild increase in free plasma corticosterone levels (Sabatino 1991). It was then proposed that CR might extend lifespan by inducing a chronic state of low-level stress, thus increasing the preparedness of rodents to respond to acute stress and immune challenge (Masoro 1998). The term *hormesis* has been applied to a protective low-level stress that would be expected to yield detrimental effects at a

higher level. It has further been suggested that despite an increase in corticosterone, positive effects of CR, such as decreased plasma glucose, attenuated oxidative stress, and increased expression of stress-response genes, may result in a net protective state (Berner 2004, de Cabo 2003, Patel 2002, Yu 2001).

Similarly, there is some debate over whether body temperature may have an impact on lifespan, as a reduction in core body temperature is consistently observed in CR (Lane 1996). Recent data generated in transgenic mice suggest that a modest, sustained reduction in core body temperature prolongs life, independent of any dietary alterations (Conti 2006).

Finally, CR is associated with a decrease in fat mass, an increase in insulin sensitivity, and altered neuroendocrine function. A decrease in fat mass, independent of CR, may have a potent anti-aging effect, as demonstrated in long-lived, fat-specific insulin receptor knockout mice (Bluher 2003). Recent evidence suggests that positive alterations in insulin and glucose metabolism associated with longevity in CR may be mediated by activation of the SIRT1 gene in adipose tissue and its effects on peroxisome proliferator-activated receptor (PPAR)-γ (Bordone 2005).

While the majority of studies have assessed the effects of lifelong CR, some studies of short-term CR have also demonstrated increased lifespan and changes in metabolic and immune parameters. Adult-onset of CR in rodents (12 months of age) resulted in an increase in mean and maximal lifespan of 10-20%, as well as a decrease in spontaneous tumor formation and an increase in mitogen-stimulated T cell proliferation (Weindruch 1995, Weindruch 1982). Adult male rhesus monkeys subjected to 30% CR demonstrated decreased triglycerides and improved insulin sensitivity in as little as 6

months (Lane 2000). These data suggest that lifelong restriction is not required to demonstrate positive effects and, therefore, may be more relevant to the potential use of CR in humans.

The attraction of CR in humans is understandable, as up to a 50% increase in maximal lifespan has been demonstrated in rodents (Barger 2003). If a similar 50% increase in lifespan could be achieved in humans, this would translate into an increase in human life expectancy from 78 to 117 years in men and from 83 to 124 years in women (Speakman 2007). However, lifelong restriction of 40% of total caloric intake, as implemented in animal trials, may not be possible in humans, and while adult onset of restriction is expected to confer some of the reported benefits of CR, the degree of such benefits would be proportionately reduced. Further, most estimates conclude that the extension of human lifespan by CR would be relatively small (Phelan 2005). Mathematical modeling suggests that a 30% reduction in energy intake implemented by a male at age 40, for example, might be expected to increase lifespan by 4.8 years. If 30% CR is begun at age 48, the same person might expect to increase lifespan by a modest 2.4 years (Speakman 2007).

No evaluations have been made on the expectation of quality of life, nor are the potentially adverse effects of CR in humans adequately addressed in the current literature (Dirks 2006). Nonetheless, groups of humans have voluntarily initiated CR in an effort to extend lifespan (www.calorierestriction.org). Those following voluntary CR diets were reported to consume approximately 1200-2000 kcal per day or roughly half the caloric intake of age-matched controls (Fontana 2004). Such a regimen is generally maintained by a repetitive diet composed primarily of raw vegetables, fruits, nuts, dairy

products, egg whites, wheat and soy proteins, and some lean meat, along with careful attention to micronutrient intake in order to avoid potential deficiencies.

One of the earliest and most comprehensive studies of CR in healthy adults occurred by happenstance in Biosphere 2, a 3.15 acre, enclosed ecological chamber in which 4 men and 4 women were expected to subsist for 2 years without outside influence. Due to a crop shortage, food availability was decreased, and the subjects consumed a CR diet of approximately 1750-2100 kcal, despite a high level of physical requirements. The diet consisted mostly of vegetables, fruits, nuts, grains, and legumes, with some dairy, eggs, and meat, providing 77% of energy as carbohydrate, 12% as protein, and 11% as fat. During this time, several physiological changes were observed that agreed with observations made in CR rodents, including significant weight loss, decreased blood pressure, decreased total cholesterol (although decreased HDL, as well), and increased cortisol (Walford 2002). Prior to this study, work by Keys and colleagues in the 1950s suggested that healthy, lean men subjected to a 50% reduction in food intake for 24 weeks demonstrated a decrease in weight, metabolic rate, and energy expenditure (Heilbronn 2003). More recently, studies of CR in healthy, non-obese humans have indicated a decrease in body weight; a decrease in cardiovascular risk factors, such as decreased blood pressure, decreased total cholesterol, and increased HDL; a decrease in fasting insulin; decreased metabolic rate; reduced energy expenditure; and a reduction in oxidative stress (Fontana 2004, Heilbronn 2006, Civitarese 2007, Mendoza-Nunez 2005).

To understand the potential benefits and limitations of CR, the National Institute on Aging initiated the Comprehensive Assessment of Long-term Effects of Reducing Intake of Energy (CALERIE), a series of human clinical trials to assess body

composition, metabolic adaptations, oxidative damage, and biomarkers of aging, but not lifespan per se, in response to interventions consisting of 10-30% CR with or without exercise. The trials began in 2002, and early reports suggest a decrease in body weight, as expected, loss of fat mass, a decrease in fasting insulin, decreased core body temperature, and decreased oxidative stress in CR subjects with and without exercise, as compared to controls (Heilbronn 2006).

Thus far, human studies have not provided any direct insight into the effects of CR on the susceptibility to infectious disease. Mice are housed in pathogen-free cages, and the response of CR mice to infection had not been the focus of previous investigations. However, recent evidence of an increased susceptibility of aged CR mice to influenza infection (Gardner 2005) warrants a detailed investigation into the effects of CR on the immune response to primary virus infection. Data presented in Chapters 3.1 and 3.2 of this thesis confirm that both aging and CR independently increase susceptibility to influenza infection. This is of particular interest, because the elderly exhibit increased morbidity and mortality from infectious diseases, including influenza, and are also at high risk for CR or malnutrition resulting from social, physical, economic, and emotional obstacles to eating.

2.5 Malnutrition and energy restriction differentially affect viral immunity (Published as Ritz 2006a)

Barry W. Ritz and Elizabeth M. Gardner

Department of Bioscience & Biotechnology, Drexel University, Philadelphia, PA 19104

ABSTRACT Protein-energy malnutrition (PEM) is associated with a decrease in immunity and an increase in infectious disease. Both of these effects are exacerbated in aging. Conversely, energy restriction (ER) without malnutrition extends lifespan in animals and retards the age-related decline in various parameters of immune function. Recent evidence suggests, however, that aged ER mice exhibit an increased mortality in response to primary influenza infection compared to age-matched controls. Underweight may contribute to this outcome due to an inability to meet the energy demands associated with the immune response to primary viral infection. The energetic costs of immune responsiveness must be considered in the undernourished aging population and emerging studies of ER in humans.

KEY WORDS: protein-energy malnutrition, caloric restriction, influenza, underweight, aging

Abbreviations used: AL, *ad-libitum*; BMI, body mass index; CTL, cytotoxic T lymphocyte; DTH, delayed-type hypersensitivity; ER, energy restriction; NK, natural killer; PEM, protein-energy malnutrition

The observed interaction between nutrition and immunity predates both nutrition science and immunology as fields of study. Interest in the influence of undernutrition on the susceptibility to viral infection emerged over 200 years ago with the observation that malnutrition appeared to lead to increased infection in some instances and increased resistance to infection in others (Sprunt 1956). In time, it was generally accepted that nutritional adequacy in the host could fuel viral replication, while any nutritional limitation to the host would interfere with the metabolism of the virus itself (Sprunt 1956). More recent studies have further explored this host-pathogen interaction, confirming that host nutritional status not only influences host immunity and viral replication, but in doing so also directs the viral genome and, thus, can influence selective virulence (Beck 2004). Attention to nutritional status, therefore, is relevant to all aspects of infectious disease: from infection, through the course of disease and recovery, and on to subsequent infections.

Fifty years ago, Sprunt and Flanigan concluded that "…the effect of malnutrition on the resistance of an animal is dependent upon the state of the animal's nutritional reserves at the time of infection." (Sprunt 1956) According to their data, an increase in the duration and severity of nutritional depletion, as well as a decrease in fat stores, were correlated with an increase in the susceptibility of mice to influenza infection. Indeed, nearly any experimental deficiency, if severe enough, will result in impaired immunity and an increased incidence of infection (Scrimshaw 1997).

The focus here is on the role of reduced energy intake on immunocompetence, which is a poorly understood process due, in part, to inconsistent definitions. **Table 1** provides a list of relevant terms, typically applied to both the nutritional disturbance and

the physiologic condition that results. Although protein-energy malnutrition (PEM) and starvation are generally associated with a decrease in immunity and an increased incidence of infectious disease, moderate undernutrition may have less or even an opposite effect on certain aspects of immune function. For example, energy restriction (ER) without malnutrition reduces body weight, extends lifespan in animals, and retards the age-related decline in a number of general indices of immune function (Pahlavani 2000), including the antibody response to influenza vaccination (Effros 1991).

This review emphasizes the paucity of data regarding the effect of ER on the immune response to infection. This is important, because recent evidence suggests that ER may be detrimental to host defenses when superimposed with a primary influenza infection (Gardner 2005), possibly related to low body weight and insufficient energy stores to meet the metabolic demands of the immune response to infection. We focus on influenza as an experimental model, because influenza is one of the best characterized viruses with regard to the effects of age and nutrition on the immune response to viruses. Influenza also remains a significant public health threat among children and the elderly (Meyer 2001). In general, innate and cell-mediated immunity are more sensitive than humoral immunity to both age (Lesourd 2004) and nutrition (Scrimshaw 1997), and as a result, are reviewed in greater detail.

Table 1

Comparative definitions of terms that are often misused in the literature[1]

Term	Definition
Undernutrition	Inadequate intake of ***energy, protein, or other nutrients*** resulting in weight loss, usually associated with poor health
Malnutrition	Failure to meet nutrient requirements due to alterations in intake, digestion and/or absorption, metabolism, excretion, and/or the metabolic requirements of dietary ***energy, protein, or other nutrients***; characterized by unintentional weight loss and poor long-term protein status, always associated with poor health
Protein-energy (calorie) malnutrition	Long-term inadequate intake of ***protein and energy***
Starvation	Long-term inadequate intake of ***protein, energy, or both***
Energy Restriction (Caloric Restriction)	Long-term inadequate intake of ***energy*** while maintaining adequate intake of protein, vitamins, and minerals; "undernutrition without malnutrition"
Food restriction	Non-specific restriction of ***food*** intake
Dietary restriction	Non-specific restriction of ***any dietary component(s)***

[1]Definitions adapted from (Insel 2002) and (Shils 2005).

Aging. A proper examination of the effects of PEM and ER on the immune response to viral infection first requires a brief review of the changes in immunity that are associated with aging (Lesourd 2004, Murasko 2003, Murasko 2005), since aging has been the focus of the most consistent and extensive studies on immune dysfunction, and because studies of PEM and ER must be considered in the context of age-matched controls (**Table 2**). Studied extensively in our laboratory, aged humans exhibit a decrease in antibody titers to influenza vaccination (Gardner 2001). Aging is associated with a slight decrease in total lymphocyte number and an age-associated shift from naïve (CD45RA+) to memory (CD45RO+) CD4+ and CD8+ T cells, likely to limit the inducible T cell response (Lesourd 2004, Murasko 2003, Miller 1997). In mice, basal natural killer (NK) cell function appears to remain intact with advanced age, while the inducible NK response decreases (Plett 2000). This effect is not consistently observed in humans (Murasko 2003). It has been postulated that decreased immunity is the reason for the observed increase in morbidity and mortality resulting from infectious agents in the elderly (Meyer 2001, Murasko 2003). However, the effects of aging on immunity are highly heterogeneous among humans, including among the healthy elderly. For example, some elderly individuals maintain the ability to mount a protective antibody response to influenza vaccination, while others do not (Murasko 2003). Therefore, additional variables that may influence immunity, such as nutritional status, may explain differences in the incidence and pathology of infection in the elderly (Lesourd 2004).

Table 2

Reported effects of aging, protein-energy malnutrition, and energy restriction on immune function[1]

Variable	**Species**[2]	**Aging**	**PEM (wasting)**	**ER**	**References**[3]
Lifespan	r	NA	↓	↑	**Gavazzi 2004** **Masoro 2005** **Barger 2003** **Yamaza 2002**
Infection	h,r	↑	↑	-	**Murasko 2005** **Gavazzi 2004**
Lymphocyte	h,r	↓ or =	↓		**Murasko 2003** Ingram 1995
number or %	r			↓	**Nikolich-Zugich 2005**
	p	=		↓ or =	**Lane 2002** **Nikolich-Zugich 2005**
Lymphocyte	h,r	↓	↓		**Pahlavani 2000** **Lesourd 2004** Ingram 1995 **Webster 2000**
proliferation	r	↓		↓	**Pahlavani 2000** **Nikolich-Zugich 2005**
	p			↑ or ↓	**Pahlavani 2000** **Nikolich-Zugich 2005**
Cytokine production	h,r	altered (↓IL-2)	altered (↓IL-2)		**Pahlavani 2000** **Lesourd 2004**

	r,p			altered (↑ or = IL-2)	**Murasko 2003** **Lesourd 1997** **Webster 2000** **Pahlavani 2000** **Lane 2002** **Nikolich-Zugich 2005**
Antibody response	h,r	↓	↓		**Pahlavani 2000** **Lesourd 2004** **Murasko 2003** Gardner 2001
to vaccination	r			↑	**Lesourd 1997** **Pahlavani 2000** Effros 1991
	p			-	**Nikolich-Zugich 2005**
CTL activity	r	↓	↓	↑	**Pahlavani 2000** **Murasko 2003** **Murasko 2005**
Antigen presentation	r	↓ or =	↓ or =	↑ or =	**Lesourd 1997** **Pahlavani 2000** **Murasko 2003** Redmond 1995
Naïve:memory T cells	h,r	↓	↑		Zhang 2002 **Lesourd 2004** **Murasko 2003** **Murasko 2005** Miller 1997 **Lesourd 1997** Woodward 1999

	r,p	↓		↑	**Nikolich-Zugich 2005** Spaulding 1997
NK activity: basal	h,r	=	↓		**Lesourd 2004** **Murasko 2003** **Murasko 2005**
	r			↓	Plett 2000 Ingram 1995 Weindruch 1983
NK activity: inducible	r	↓	↓	↑ (Poly I:C) ↓ (Influenza)	**Pahlavani 2000** Gardner 2005 **Murasko 2005** Plett 2000 Ingram 1995 Weindruch 1983
	h	↓ or =	↓		**Lesourd 2004** **Murasko 2003** **Murasko 2005**

[1]Effects of PEM and ER are compared to age-matched controls.

[2]Species: r, rodents; h, humans; p, primates.

[3]Scientific reviews were included when available and are indicated in bold.

NA, not applicable; -, insufficient data; =, no change; ns, this result was not statistically significant (P>0.05).

Protein energy malnutrition (PEM). PEM is a precipitating factor in the incidence of infectious disease, including influenza infection, and infection, in turn, has an adverse bearing on nutritional status (Scrimshaw 1997). PEM is the most common cause of immunosuppression worldwide, and the increase in infectious disease associated with malnutrition is believed to be related to decreased immunity (Gavazzi 2004, Redmond 1995, Ingram 1995). Like in aging, PEM is characterized by a lymphopenia, reduced lymphocyte proliferation in response to mitogenic or antigenic stimulation, decreased cytotoxic T lymphocyte (CTL) activity, altered cytokine production, and reduced antibody response to vaccination (Scrimshaw 1997, Lesourd 2004, Lesourd 1997, Zhang 2002) (Table 2). PEM is associated with a shift toward increased naïve CD4+ and CD8+ T cells (Woodward 1999). PEM also has a strong negative influence on innate immunity that is exacerbated in elderly humans (Lesourd 2004) and aged mice (Ingram 1995).

Protein restriction can lead to compromised immunity, decreased viral clearance from the lungs, and increased mortality in influenza infected mice (Jakab 1981). PEM often results in wasting (involuntary weight, muscle, and tissue losses), associated with a decrease in NK activity in both humans and mice (Ingram 1995). However, NK activity appears to be somewhat resistant to PEM if wasting is avoided (Ingram 1995). Likewise, in a study of experimental PEM without wasting, mice exhibited normal lymphocyte proliferation and antigen presentation (Redmond 1995). These observations suggest the possibility that weight loss is a critical aspect in PEM-related immune dysfunction. Refeeding, with an emphasis on protein and/or micronutrients, has produced favorable results in terms of T cell proliferation, IL-2 production, DTH, antibody response, NK

activity, and most important, a decreased rate of infection (Meyer 2001, Lesourd 2004, Lesourd 1997).

Energy restriction (ER). ER, the phenomenon described as "undernutrition without malnutrition," (Mendoza-Nunez 2005) retards aging and extends average and maximal lifespan, as first demonstrated in rats by McCay, et al., in 1935 (McCay 1935). While this extension in lifespan has been correlated to total energy intake regardless of nutritional composition (Masoro 2005), it is important to note that ER diets are nutritionally enhanced to avoid malnutrition or deficiency. An ER diet (typically 40% restricted in mice) is gradually achieved by underfeeding an isocaloric diet supplemented with protein, vitamins, minerals, and salts, usually at the expense of carbohydrate. Lifelong ER was shown to increase the mean and maximal lifespan of mice by up to approximately 65% and 50%, respectively, compared with a diet consumed *ad libitum* (AL) (Barger 2003). Ongoing studies of non-human primates predict a comparable decrease in morbidity and mortality rates (Lane 2002, Nikolich-Zugich 2005), although it is premature to determine the effects on long-term disease outcome and lifespan.

The extension of lifespan and a reduced incidence of spontaneous tumors in ER rodents promulgated early interest in the potential preservation of immune function by ER (Weindruch 1989). ER is generally acknowledged to delay the development of immunity and maintain its function later in life (Pahlavani 2000). Most studies suggest that ER rodents maintain mitogen-stimulated T cell proliferation, cytokine production, antibody response, and inducible NK cell activity at an advanced age (Pahlavani 2000, Gardner 2005) (Table 2). Certain outcomes, such as the lymphopenia and enhanced

antibody response to vaccination that are observed in mice subjected to ER (Effros 1991, Miller 1997), have not been confirmed in non-human primates (Nikolich-Zugich 2005). ER results in a consistent increase in naïve:memory T cell subpopulations, possibly related to an increased proportion of functional T cells (Miller 1997, Woodward 1999, Nikolich-Zugich, Spaulding 1997).

ER has been described as an effective modality for increasing the immune response to influenza (Meyer 2001, Webster 1989); until recently, however, studies were limited to challenge by influenza vaccine (Effros 1991). We discovered an anomaly in which aged ER mice were unable to withstand primary influenza infection and died within 4-7 days post-infection (Gardner 2005) (**Figure 1**). Due to the early time course and an observed decrease in NK activity in aged ER mice when infected with influenza (Gardner 2005), we suspect that ER mice do not possess the innate immunity to control primary infection while mounting a specific CD8+ T cell response, a possible effect of ER masked by the limitations of vaccine studies. Previously, ER was shown to diminish basal splenic NK cell activity in mice by about 50%, although NK response to Poly I:C injection was increased to a percentage of cytotoxicity comparable to that in young controls (Weindruch 1983).

Underweight. Infection generates a significant energy demand (Scrimshaw 1997); therefore, the availability of energy is likely to play a critical role in the immune response to infection. Infections are associated with the catabolism of protein for the production of energy via gluconeogenesis (Scrimshaw 1997). The activation of cell-mediated immunity includes an increase in glucose uptake, glycolysis, and protein synthesis by T

cells in order to support proliferation, differentiation, cytokine production, and effector function (Matarese 2004). However, while ER has been reported to promote gluconeogenesis and decrease glycolysis (Yamaza), ER studies to date have ignored the metabolic costs of infection.

Influenza infection results in an anorexia that is believed to be mediated, at least in part, by the cytokine and chemokine milieu (Van Reeth 2000). As such, weight loss and recovery serve as useful indicators of the severity and course of infection. Our observations suggest that young and aged mice can lose up to 35% of baseline body weight and still recover from influenza infection. Any additional weight loss is not compatible with recovery. Kinetic analyses of weight loss during sub-lethal influenza infection indicate that the recovery of weight is concomitant with a maximal CD8+ T cell response and viral clearance (Murasko 2005).

There are clearly stated health risks associated with underweight in humans (BMI <18.5 kg/m^2), including compromised immunity (Dirks 2006). Indeed, a history of weight loss is associated with a poor clinical prognosis in hospitalized elderly, including increased infections (Sullivan 1990). Prospective and retrospective studies suggest that low or even normal body weight predicts mortality in the elderly, while increased weight may have a protective effect (Mendoza-Nunez 2005). If underweight when infected with influenza virus, energy stores might not be sufficient to withstand the combined reduction in energy intake and increased energy demand associated with the infection. Aged ER mice are underweight compared to AL mice, such that weight loss during the first four days of influenza infection resulted in an average body weight equal to the critical weight that predicts mortality in aged AL mice (Gardner 2005) (Figure 1). Voluntary ER in

humans also results in underweight in some cases (Fontana 2004), although the potential influence of ER on the immune response to viral infection in humans remains entirely unknown.

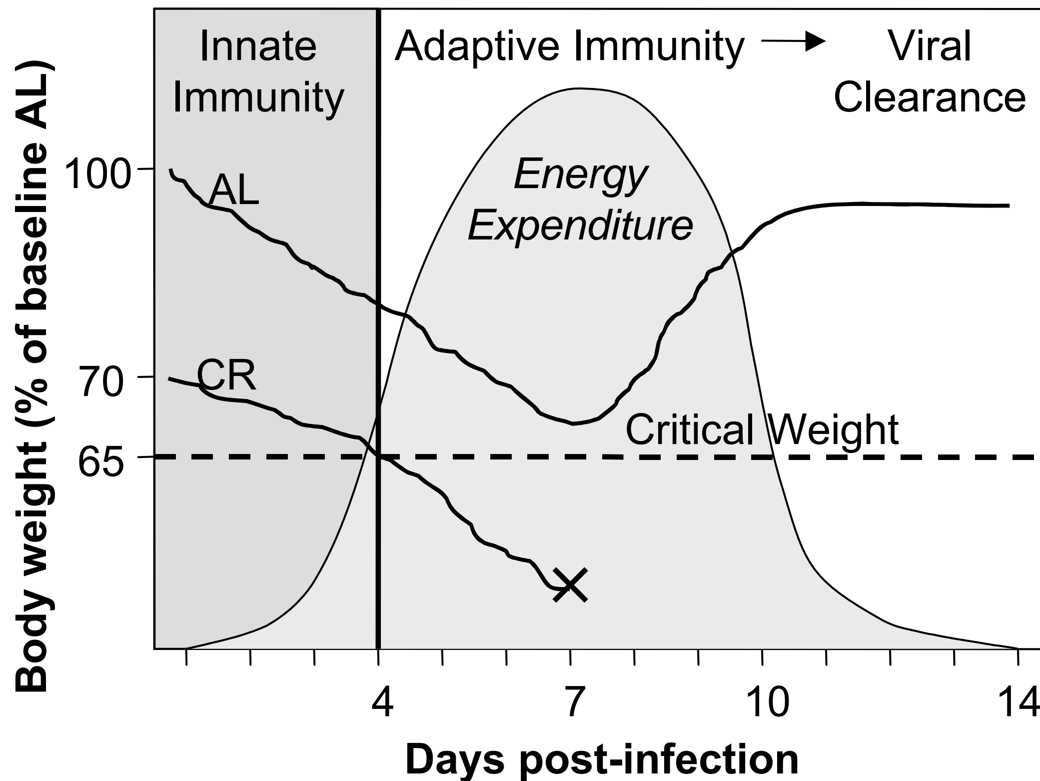


Figure 1. Weight loss occurs in mice with influenza infection due to a decrease in energy intake and an increase in energy demands. Young and aged mice can lose up to 35% of their body weight and recover from infection, which suggests a critical weight indicative of sufficient energy reserves to recover from infection. Lifelong ER results in a 30% decrease in starting weight that may be only marginally above this critical threshold. In the case of aged ER mice, 100% mortality was observed by day 7 (X), prior to an adaptive T cell response, suggesting a primary influence of underweight on innate immunity.

Summary. Aging and PEM with wasting are associated with similar and cumulative defects in innate and cell-mediated immunity and an increased incidence of infection. Although the preponderance of evidence suggests that ER maintains immune function at an advanced age, including in response to immunization, more recent data indicate impairment in the immune function of aged ER mice following primary influenza infection. This observation supports the notion that immunization can no longer serve as the sole indicator of the immune response to viruses (Murasko 2005). Further, if applicable to the human circumstance, these data have clear implications for elderly individuals at high risk for reduced energy intake resulting from social, physical, economic, and emotional obstacles to eating (Pirlich 2001). Infection is associated with both an increase in energy demands and an anorexia that decreases energy intake. Underweight, therefore, may contribute to a poor prognosis in infection by exacerbating this energy deficit, thus negating the spectrum of health benefits attributed to ER and the maintenance of a low body weight. The potential consequences of underweight in response to infection must be addressed in future proposals on the therapeutic benefits of ER in humans (Sullivan 1990, Heilbronn 2003) and in consideration of the Comprehensive Assessment of Long-term Effects of Reducing Intake of Energy (CALERIE), a series of human clinical trials initiated by the National Institute on Aging in 2002. Immediate action is warranted to determine the metabolic, physiologic, and immune changes associated with ER that may affect the outcome to primary viral infection. Future studies should evaluate the kinetics of innate and cell-mediated immunity, viral clearance, and recovery in ER mice and investigate the effects of

refeeding prior to infection to delineate the roles of weight and energy status on the immune response to primary viral infection.

ACKNOWLEDGEMENTS

The authors thank Drs. Irene Olsen and Donna H. Mueller for their comments and Emilia Ralston for her assistance.

CHAPTER 3: EXPERIMENTAL DATA

3.1 Natural killer cells control lung virus in young and aged mice during influenza infection

Barry W. Ritz*, Shoko Nogusa*, Sadik H. Kassim[†], Stephen R. Jennings[†], Elizabeth M. Gardner*

*Drexel University, Department of Bioscience and Biotechnology

[†]Drexel University College of Medicine, Department of Microbiology and Immunology

3.1.1 Abstract

The importance of Natural killer (NK) cells in controlling influenza infection, before the initiation of an antigen-specific response, has not been clearly illustrated, nor has the role of altered NK cell activity in the age-associated decline in immunity been investigated in the context of a primary virus infection. Young and aged C57BL/6 mice were infected intranasally with influenza A virus (PR8), and NK cell-mediated cytotoxicity was determined in lung and spleen by ^{51}Cr-release in YAC-1 target cells. Young mice exhibited an increase in influenza-inducible NK activity not seen in aged mice, as well as an increase in the percentage and number of NK1.1+ cells in response to infection. Young mice depleted of NK cells by rabbit anit-NK1.1 (PK136) exhibited increased weight loss and lung virus titers, determined by MDCK assay and M1 expression by RT-qPCR. Natural killer cells are important for controlling influenza virus infection, and this process is negatively influenced by aging.

3.1.2 Introduction

Influenza infection and related secondary pneumonias represent the 4th leading cause of death in persons over the age of 65 in the U.S. (MMWR 2004, Gross 1995, Thompson 2003). It has been proposed that increased infectious disease in the elderly is related to an overall dysregulation in immune function (Meyer 2001). While the age-associated decline in the immune response to influenza vaccination has been well studied, information regarding age-associated changes in the immune response to influenza infection is lacking. Further, the study of primary viral infections, meaning those viruses to which we have had little or no previous exposure, is relevant in this era of threats from highly virulent (hv) H5N1 avian influenza and bioterrorism (Murasko 2005).

Natural killer cell-mediated innate immunity provides the first line of defense against virus infection, before the engagement of an antigen-specific response. Our lab (Po 2002) and others (Bender 1995, Effros 1983) have previously demonstrated the importance of CTL activity in recovery from influenza virus infection and an age-related impairment in CD8+ T cell function that is associated with increased lung virus and prolonged virus shedding. However, less is known regarding the potential association between altered NK cell activity in aged lungs and lung virus early following influenza infection, before the generation of an influenza-specific CTL response.

Although basal NK cell activity does not differ between adult (6 months) and aged (22 months) mice (Provinciali 1989), an age-related decrease in cytokine-inducible NK cell activity has been demonstrated (Plett 2000). Both *in vitro* and *in vivo* IFN-α/β treatment increased the NK cell activity of adult mice to levels seen in young (6-8 weeks) mice, whereas there was no increase in the NK cell activity of aged mice (Provinciali

1989). These data reflect an age-associated decrease in inducible NK cell cytotoxicity, which has also been observed in aging humans (Solana 1999, Kutza 1996).

The importance of NK cells in controlling virus infections has long been acknowledged. Natural killer cell depletion has resulted in increased virus titer and pathology in C3H/St mice infected with MCMV, MHV, and vaccinia virus, but not LCMV, suggesting that the relative importance of NK cells in the immune response varies by virus (Bukowski 1983). Mice of the E26 strain, deficient in both NK and T cells, has demonstrated an increase in virus in lung 3 days post-infection (p.i.) and an increase in virus in brain 10 days p.i. with HSV-1 compared to T cell knockout and C57BL/6 controls (Adler 1999). This study suggested that NK cells are able to limit HSV-1 infection, without T cell involvement.

In contrast, early studies did not clearly identify a role for NK cells in controlling influenza virus infection. Intravenous treatment with rabbit anti-Asialo GM1 24 hours prior to intranasal (i.n.) infection with a 50% lethal dose of influenza A (PR8) increased lung virus and mortality in B6D2F1 and nude (*nu/nu*) mice (Stein-Streilein 1988). However, GM1 is expressed by NK cells, CTLs, and macrophages, resulting in indiscriminate depletion (Suttles 1986, Keller 1983) and limiting its use in more recent studies. Influenza infection resulted in an increase in NK cell percentage and number in the lungs of *scid* mice (NK cells, but no B or T lymphocytes) when mice were immunized and then infected by aerosol with a lethal dose of PR8. However, in the same study, depletion of NK1.1+ cells, confirmed only in spleen, did not alter survival following immunization and secondary influenza challenge (Bot 1996). As a result of

these inconsistent data and an emphasis on immunization, the potential role of NK cells in the immune response to primary influenza infection has been largely ignored.

To our knowledge, the current study is the first to examine primary influenza infection in the lungs of immunocompetent mice following confirmed NK cell-specific depletion. We further demonstrate a decrease in NK cell cytotoxicity in response to primary influenza infection and an increase in virus titers as determined by both MDCK assay and real-time quantitative PCR (RT-qPCR) analysis of M1 gene expression in the lungs of aged compared to young mice. These data provide direct evidence linking NK cells with resistance to influenza infection. Natural killer cells appear to play an important role in controlling virus titer in the lung in response to influenza infection, and an age-associated defect in inducible NK cell activity contributes to the increased susceptibility to influenza infection in aging.

3.1.3 Materials and Methods

Mice and influenza infection

The protocol was approved by the Drexel University Institutional Animal Care and Use Committee. Young (6-8 week) and aged (22 month) male C57BL/6 mice were purchased from the National Institute on Aging colony of Charles River Laboratories (Wilmington, MA). Mice were housed in micro-isolator cages in the AAALAC-accredited barrier facility at Drexel University and acclimated for at least 1 week before use. Mice with tumors were eliminated from the study. Influenza A/Puerto Rico/8/34 (H1N1, PR8) was propagated in specific pathogen-free eggs (B & E Eggs) and cell-free supernatants were stored at -36°C until use. At baseline (Day 0), mice were anesthetized by i.p. injection with Avertin (2,2,2-tribromoethanol, Sigma) and infected i.n. with 10^4 $TCID_{50}$/100 HAU of PR8.

Lymphocyte isolation

Briefly, mice were asphyxiated by CO_2, and spleens and lungs were aseptically removed. As previously described (Po 2002), spleens were homogenized and resuspended in RPMI-1640 (Mediatech). A lobe of each lung was frozen in TRI reagent (Molecular Research Center) and saved for RNA extraction. Remaining lungs were minced, incubated at 37°C for 2 h in 3mg/mL Collagenase A and 80 Kuntz units of DNAse I/mL (Roche) in IMDM (Mediatech), passed through a 40 μm nylon mesh (Fisher), and centrifuged at 500 x g for 5 min. Supernatants were aliquoted and stored at -36°C for analysis of lung virus by MDCK assay. Pellets were washed, and mononuclear cells were isolated from cell suspensions layered on Histopaque-1083 (Sigma) gradient and centrifuged at 1400 x g for 20 min.

NK cell cytotoxicity

A standard ^{51}Cr-release assay with YAC-1 target cells was employed to assess NK cytotoxicity, as previously described (Plett 2000). Briefly, Yac-1 cells were incubated with 200 μCi $Na^{51}CrO_4$ (PerkinElmer) for 2 h, washed in RPMI-1640, and plated in triplicate in v-bottom 96-well plates with lung and spleen cell preparations at an E:T of 50:1. Following a 4-h incubation at 37°C, supernatants were harvested onto UniFilter microplates (PerkinElmer), and radioactivity was quantitated by a γ-counter (Packard TopCount) and reported as counts per minute (CPM). Spontaneous release was determined in medium alone, and maximum release in 5% Triton X-100 (Sigma). Spontaneous release was always <5% of maximum release. Percent cytotoxicity was then calculated as follows:

$$\% = \frac{(\text{Experimental CPM} - \text{Spontaneous CPM})}{(\text{Maximum CPM} - \text{Spontaneous CPM})} \times 100$$

Flow cytometry

Following multiple washes, 5×10^5 cells from lung and spleen were resuspended in PBS (Mediatech) containing fluorochrome-conjugated antibodies (eBioscience) to CD8 (APC or PE-Cy5.5) and NK1.1 (PE or PE-Cy7) and incubated on ice in the dark for 30 min. Cells were washed 3 times, resuspended in 1% paraformaldehyde (Sigma). Samples were acquired on a FACSCanto flow cytometer (BD) and analyzed using FlowJo software (Tree Star).

NK cell depletion

Young mice were injected i.p. with a total of 600µL/mouse of rabbit anti-NK1.1 IgG antibody (PK136) or saline vehicle control, administered 2 days and 1 day prior to infection with influenza virus, adapted from a published protocol (Carroll 2001). NK cell depletion was confirmed by FACS analysis as the absence of NK1.1+ lymphocytes in lungs and spleens prior to and during infection.

MDCK assay

Supernatants from lung homogenates were serially diluted and used to infect Madin-Darby canine kidney (MDCK) cells. After incubation at 37°C for 24 h, 0.002% TPCK-treated trypsin (Sigma) was added, followed by a 72-h incubation. Chicken red blood cells (B&E Eggs) were prepared at 0.05% in PBS and added to the cultures. Virus titer was determined as hemagglutination, as previously described (Po 2002), and reported as the 50% tissue culture infectious dose ($TCID_{50}$).

RT-qPCR

Viral load in lungs was also determined as M1 protein mRNA expression using real-time quantitative PCR (RT-qPCR) as previously described (Ward 2004). Briefly, viral RNA was isolated from harvested lung tissues using a QIAamp viral RNA kit and reverse transcribed using an Omniscript RT kit, according to manufacturer's instructions (Qiagen). Reactions were primed with 1µM random hexamers, 10 units of RNAse inhibitor, and 10µM of M1-specific primer in a total volume of 20µL in nuclease-free water (Gibco). Reactions were incubated at 42°C for 60 min, heated at 95°C for 5 min, and cooled to 4°C in a 2720 Thermal Cycler (Applied Biosystems). The PCR reaction mixture contained 2µL of cDNA, 1X TaqMan Universal Master Mix (Applied

Biosystems), 900 nM of each primer, 225 nM of probe in a total volume of 25 μL. Forward and reverse primers and probe, tagged with FAM (6-carboxyfluorescencein) reporter dye on the 5' end and TAMRA (6-carboxytetramethylrhodamine) quencher dye on the 3' end, were purchased from IDT. The matrix protein gene sequence used in Reverse Transcription reactions was 5' TCT AAC CGA GGT CGA AAC GTA 3'. The sense and anti-sense primer sequences used in amplification of cDNA were sense: 5' AAG ACC AAT CCT GTC ACC TCT GA 3' and antisense: 5' CAA AGC GTC TAC GCT GCA GTC C 3'. The M1 influenza A specific probe sequence was 5' TTT GTG TTC ACG CTC ACC GT 3'. Virus titer was calculated by comparing M1 expression in samples to a standard curve of M1 expression in PR8 stock and reported as ng of virus per lung.

Statistics

Statistical analyses were performed using GraphPad InStat 3 software. Comparisons between and within groups were analyzed by ANOVA with Tukey-Kramer multiple comparisons. Mann-Whitney *U*-tests were used when data was not normally distributed. Pearson's correlation was used to investigate the relationship between weight loss and M1 expression. Statistical significance was accepted at $P<0.05$.

3.1.4 Results and Discussion

Aged mice demonstrate reduced influenza-induced NK cell cytotoxicity

Young and aged C57BL/6 mice were infected i.n. with 10^4 $TCID_{50}$/100 HAU of influenza A virus (H1N1, PR8). NK cell cytotoxicity was determined in lungs and spleens of young and aged mice from baseline (Day 0) through 3 days p.i. (**Figure 1**). Basal NK cell activity in lungs and spleens did not differ between young and aged mice, as recently reported (Gardner 2005). Although there was a small decrease in basal NK cell activity in aged splenocytes compared to young, this difference was not significant (**Figure 1B**). These observations are in contrast with earlier reports in which young (8 week) mice demonstrated higher basal splenic NK cell activity than young adult (6 month) or aged (22 month) mice (Saxena 1984, Provinciali 1989). Following infection, influenza-induced NK cell activity in lungs from young mice was increased at 2 days p.i. and was approximately 4-fold higher than NK cell activity in aged mice on the same day (**Figure 1A**). Importantly, NK cell activity in aged mice did not increase over baseline following influenza infection, in agreement with earlier studies demonstrating an age-associated impairment in cytokine-inducible NK cell activity (Plett 2000). Similar results were generated in the spleens of the same young and aged mice following influenza infection (**Figure 1B**).

Aged mice exhibit decreased NK cell percentage and number following influenza infection

The role of increased NK cell number in response to viral infection has previously been demonstrated (Po 2002, Biron 1983, Dokun 2001). The percentage of NK1.1+ in total

lymphocytes in the lungs and spleens of young and aged mice were assessed by flow cytometry on days 0-3 p.i. (**Figure 2**). The number of NK1.1+ lymphocytes in young and aged lungs was then calculated based on the total number of pulmonary lymphocytes isolated from each animal. There were no differences in the percentage or number of NK cells in the lungs or spleens of young and aged mice at baseline. In response to i.n. infection with influenza virus, both the percentage and the number of NK1.1+ cells in lungs of young mice increased significantly at 2 days p.i. (**Table I**), which corresponded with maximal NK cell cytotoxicity in lung. In contrast, there was no significant increase in either the percentage or number of NK cells in the lungs of aged mice. Further, both the percentage and the total number of NK1.1+ cells in the lungs of young mice were significantly higher than aged mice at 2 days p.i. Similarly, the percentage (**Figure 2B**) and number ($7.4x10^5 \pm 0.7x10^5$ vs. $1.8x10^5 \pm 0.1x10^5$, mean ± SEM, $P<0.001$) of NK1.1+ splenocytes were increased in young compared to aged mice at 2 days p.i., again corresponding to peak NK cell cytotoxicity in spleen. These data suggest that reduced NK cell activity in aged mice may reflect an inability to expand the NK cell population in response to influenza infection.

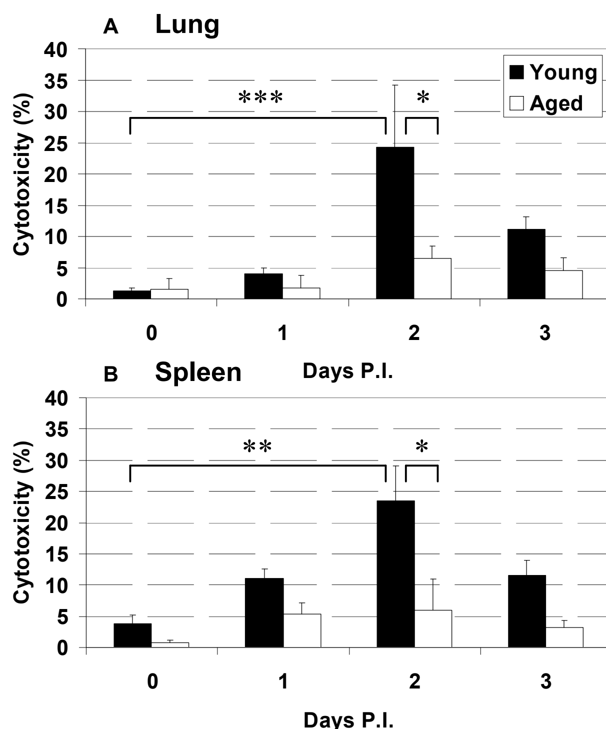


Figure 1. Natural killer cell cytotoxicity in young and aged mice. Young (6-8 week) and aged (22 month) C57BL/6 mice were infected i.n. with 10^4 TCID50/100 HAU of mouse-adapted PR8 influenza A virus, and lymphocytes were isolated at days 0-3 post-infection (p.i.). Lymphocytes were incubated with ^{51}Cr-labeled Yac-1 cells at an E:T of 50:1, and % cytotoxicity was determined by Cr release. Influenza-induced NK cell cytotoxicity peaked in young mice and was higher than in aged mice at 2 days p.i. in both lung (A) and spleen (B). Values represent mean ± SEM, n = 4 mice per day per group, *P<0.05, **P<0.01, ***P<0.001. Repeated a total of 3 times.

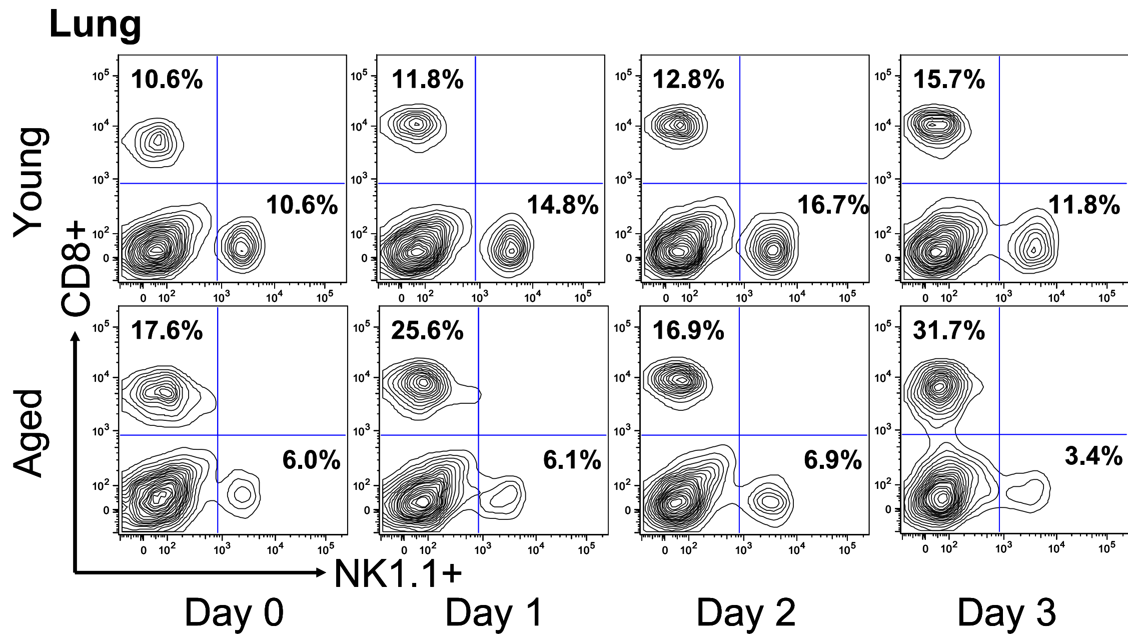


Figure 2A. Percent of NK cells in young and aged mice. The percentage of NK1.1+CD8- lymphocytes was determined in young and aged mice from days 0-3 p.i. with PR8. Young mice exhibited an increase in the percent of NK1.1+ cells on day 2 post-infection in lung, corresponding with increased NK cell cytotoxicity. Representative data with mean values are shown, n = 4 mice per day per group. Repeated a total of 3 times.

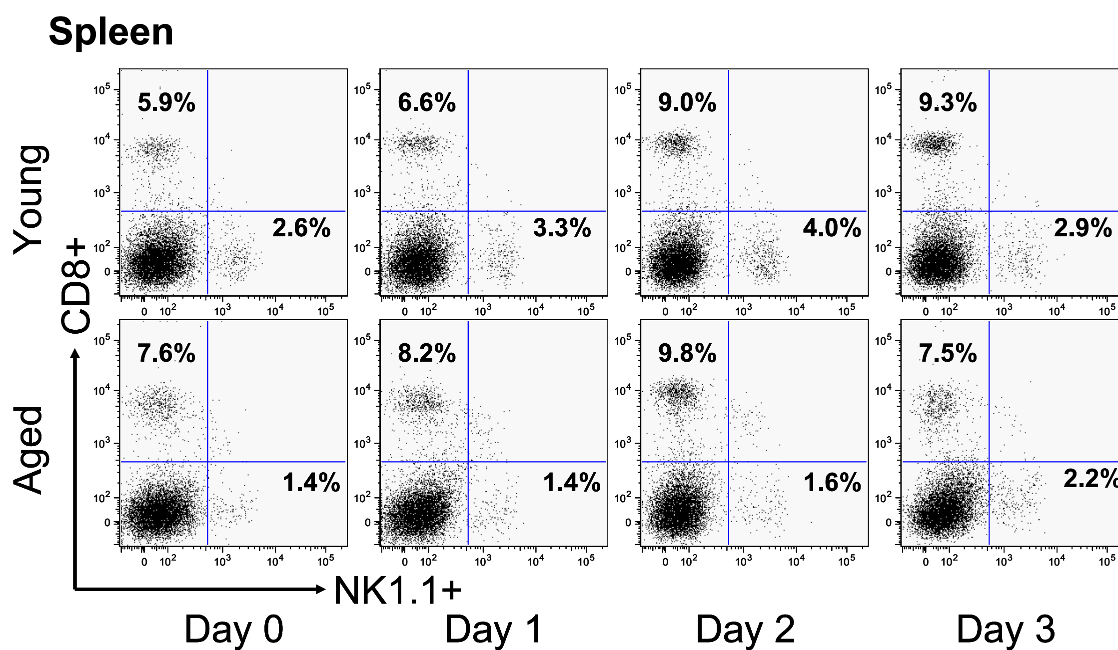


Figure 2B. Percent of NK cells in young and aged mice. The percentage of NK1.1+CD8- lymphocytes was determined in young and aged mice from days 0-3 p.i. with PR8. Young mice exhibited an increase in the percent of NK1.1+ cells on day 2 post-infection in spleen, corresponding with increased NK cell cytotoxicity. Representative data with mean values are shown, n = 4 mice per day per group. Repeated a total of 3 times.

Table I. *Percent and number of NK1.1+ lymphocytes in young and aged lungs following influenza infection.*

		Days post-infection			
		0	**1**	**2**	**3**
Percent	**Young**	10.6±0.7[a]	14.8±0.2[a],***	16.7±1.8[b],***	11.8±0.7[a],***
	Aged	6.0±1.8	6.1±1.2	6.9±0.5	3.4±0.7
Number ($x10^5$)	**Young**	4.3±0.8[a]	9.2±2.4[a]	14.3±1.8[b],**	8.6±1.6[a]
	Aged	3.3±0.5	7.6±2.5	4.4±0.4	5.7±1.5

Analysis by ANOVA with Tukey's multiple comparisons; Values represent mean ± SEM, n = 4 mice per day per group.

Different letters indicate significant differences between days within the same age group, *P*<0.05.

Asterisks indicate significant differences between young and aged mice on the same day, ***P*<0.01, ****P*<0.001. Repeated a total of 3 times.

NK cell depletion and aging increase lung virus following influenza infection

A group of young mice was injected i.p. with two divided doses of anti-NK1.1 antibody (PK136) at both 2 days and 1 day before infection. Young, aged, and young NK cell-depleted (NK-) mice were then infected i.n. with 10^4 $TCID_{50}$/100 HAU of PR8 and assessed from days 0 through 4 p.i. Natural killer cell depletion (**Figure 3A**) and a loss of NK cell cytotoxicity (**Figure 3B**) were confirmed in the lung and spleen of young NK- mice at baseline and 2 days p.i..

To determine if the absence of NK cells (young NK- mice) or altered NK cell activity (aged mice) increased susceptibility to influenza virus infection, weight loss and lung virus titers were measured through 4 days p.i. Although mice in all 3 groups lost weight during the course of infection, weight loss was most dramatic in young NK cell-depleted mice (**Figure 4A**). Also, daily food intake was less for NK- mice, and these mice showed more severe signs of infection, including lack of grooming and lethargy, as compared to young or aged mice (data not shown).

Next, to further assess susceptibility to influenza infection, lung virus titers were determined in lung homogenates from young, aged, and young NK- mice from days 0-4 p.i. using an MDCK infectivity assay. Virus was detected in the lungs of aged and young NK- mice by day 1 p.i., but not until day 2 in young controls **(Table II)**. Lung virus titers appeared to remain elevated in aged and young NK- mice compared to young mice at 4 days P.I, although these differences were not significant.

A limitation of the MDCK infectivity assay in this and previous studies has been low sensitivity, and therefore, it was not possible to clearly determine the relationship between NK cell activity and lung virus utilizing MDCK infectivity alone. To address

this problem, RT-qPCR was employed to measure mRNA expression of M1 protein using a modification of a previously published method (Ward 2004). Since M1 protein is conserved among mouse-adapted influenza A viruses and is an internal protein within the viral envelope, M1 gene expression is expected to correlate with virus levels in infected tissues. Further, we have validated this methodology by assessing intra- and inter-assay variation and directly comparing results obtained by RT-qPCR with those from the standard MDCK assay in the same animals (Unpublished data, Nogusa & Gardner 2007). Using the same samples, we observed that aged mice accumulated influenza virus in lungs earlier than young mice, as indicated by elevated M1 expression at day 1 p.i. (**Table III**). Further, young NK- mice exhibited elevated M1 protein expression in lungs compared to young mice at 3 and 4 p.i.

Finally, across all three groups, weight loss was weakly but significantly correlated with lung virus, as determined by M1 mRNA expression (**Figure 4B**, $r = 0.76$, $r^2 = 0.58$, 95% CI 0.33-0.93, $P = 0.004$). The correlation was strengthened by the omission of aged mice (**Figure 4C**, $r = 0.85$, $r^2 = 0.73$, 95% CI 0.37-0.97, $P = 0.007$). These data clearly suggest that NK cells are important in controlling influenza virus early in the course of infection.

Taken together, these data provide conclusive evidence that NK cells limit influenza virus at the site of infection, i.e., the lungs, early during the course of infection, such that a loss of NK cell-mediated killing was associated with an increase in virus and the severity of infection, determined as increased weight loss. A failure to expand the NK cell population in response to infection and impaired NK cell cytotoxicity both contributed to the age-associated increase in the susceptibility to influenza infection.

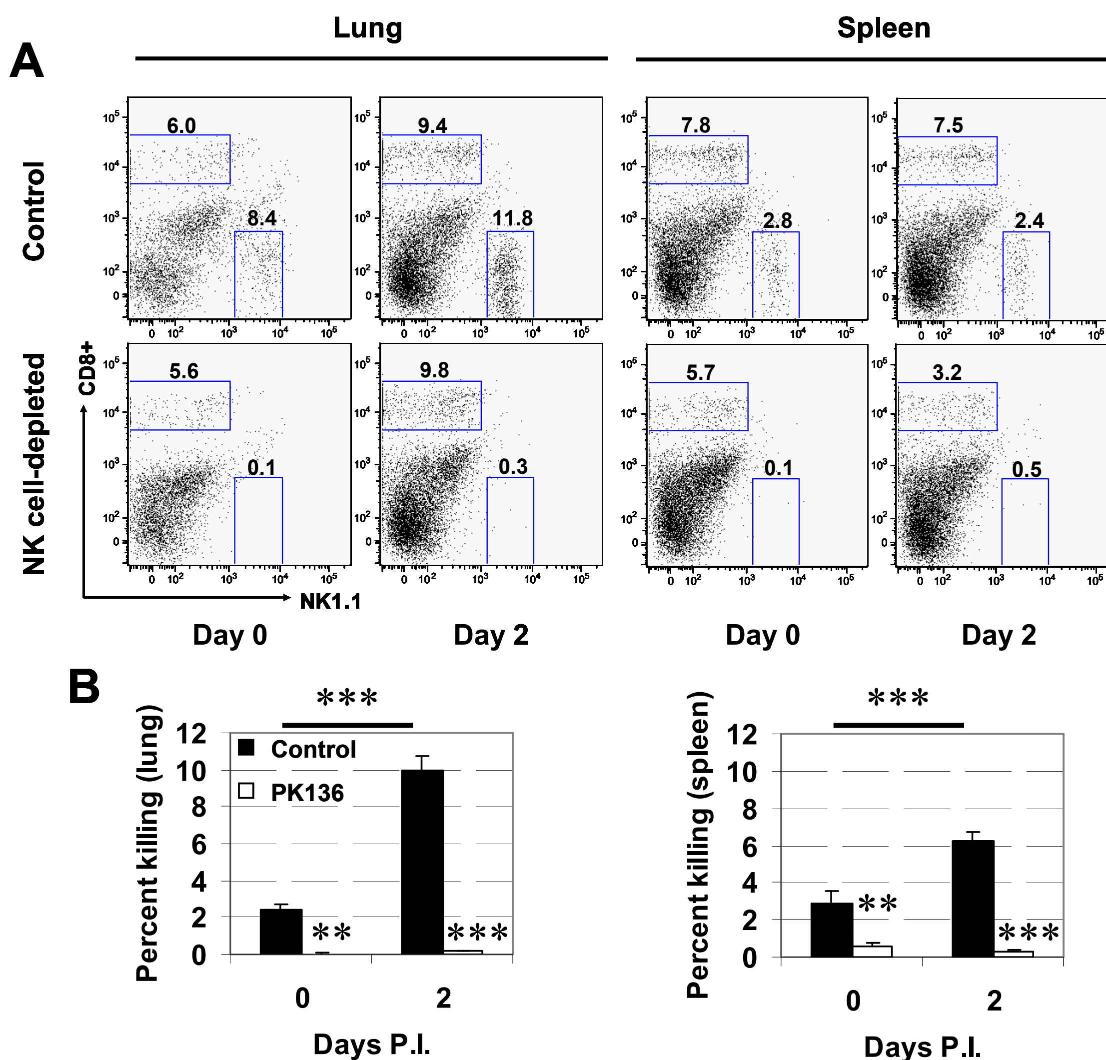


Figure 3. Confirmed NK cell depletion and loss of cytotoxicity in anti-NK1.1 antibody-treated young mice. Young mice were depleted of NK1.1+ lymphocytes by i.p. injection with anti-NK1.1 antibody (PK136). Depletion was confirmed in lung and spleen at baseline and at 2 days p.i. Representative data with mean values are shown (A). Loss of influenza-induced NK cell cytotoxicity was confirmed in lung and spleen at 2 days p.i. (B), n = 4 mice per day per group, ***P*<0.01, ****P*<0.001. Repeated a total of 2 times.

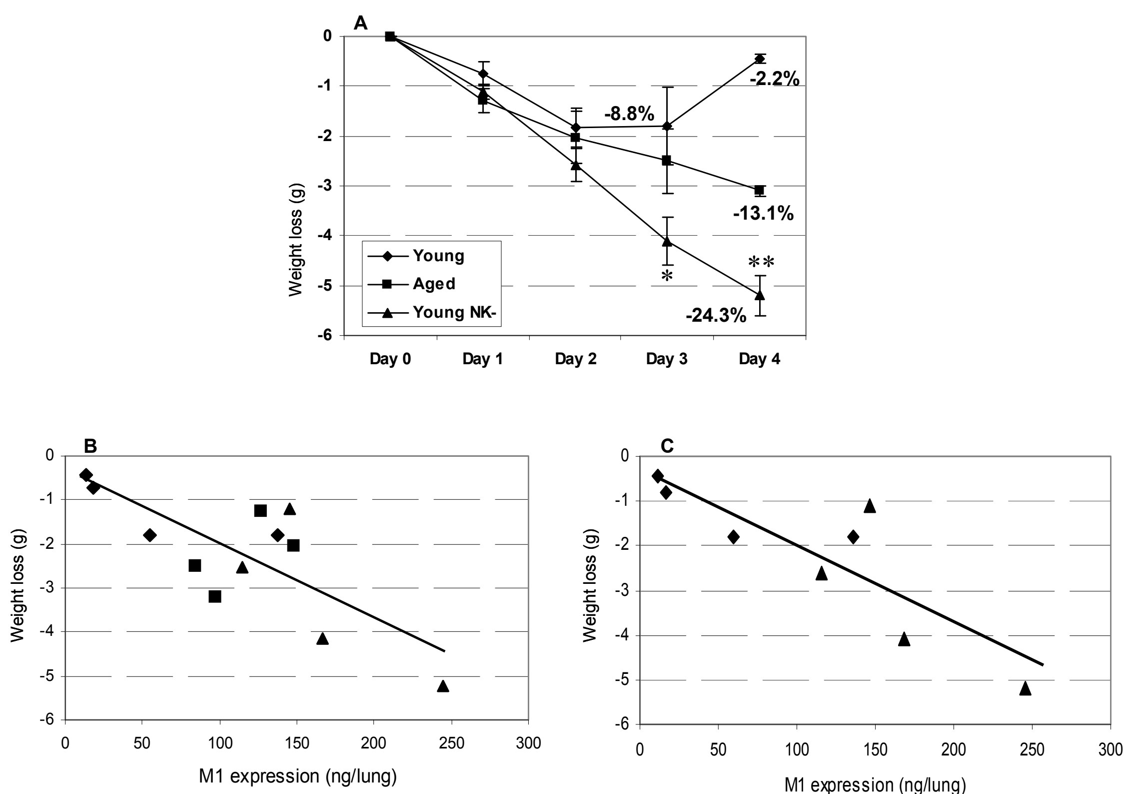


Figure 4. Weight loss. Young, aged, and young NK cell-depleted (NK-) mice were infected with PR8, and weight loss was evaluated from 0-4 days p.i. as an indicator of the severity of infection. A) Young mice (diamond) stopped losing weight by day 2, while aged (square) and young NK- (triangle) continued to lose weight through 4 days p.i. Data points represent the mean body weight ± SEM, n = 3 mice per day per group, *P<0.05, **P<0.01 compared to young on the same day. Percent of weight loss from baseline is also indicated. Repeated a total of 2 times. B) Mean weight loss was significantly correlated with mean M1 expression across all three groups from 1 through 3 days p.i., r = 0.76, r^2 = 0.58, P = 0.004. C) Mean weight loss was significantly correlated with mean M1 expression in young and young NK- mice from 1 through 3 days p.i., r = 0.85, r^2 = 0.73, P = 0.007.

Table II. *Lung virus titer ($TCID_{50}$) in young, aged, and young NK cell-depleted mice during influenza infection.*

	Lung virus ($TCID_{50}$ per lung)[1]		
Days post-infection	**Young**	**Aged**	**Young NK-**
0	ND	ND	ND
1	ND	2.92 ± 0.22	3.67 ± 0.80
2	3.25 ± 0.46	3.08 ± 0.38	1.58 ± 1.52
3	2.25 ± 0.85	2.63 ± 1.05	2.17 ± 0.58
4	0.58 ± 1.5	4.08[2]	2.58 ± 0.14

[1] $TCID_{50}$ was calculated per mL in lung homogenates using an MDCK infectivity assay.

[2] One independent value shown. Statistical analysis was not performed due to a limited number of mice.

Analysis by ANOVA with Tukey's multiple comparisons; Values represent mean ± SEM, n = 3 mice per day per group; ND, not detected. Repeated a total of 2 times.

Table III. *Lung virus titer (ng/lung) in young, aged, and young NK cell-depleted mice during influenza infection.*

Days post-infection	Lung virus (ng per lung)[1] Young	Aged	Young NK-
0	ND	ND	ND
1	16.24[2]	*123.68 ± 14.67	146.16 ± 133.47
2	135.94 ± 64.75	147.90 ± 84.13	116.02 ± 68.06
3	59.38 ± 24.24	86.05 ± 74.08	*168.68 ± 39.58
4	11.28 ± 10.38	193.6[2]	*245.29 ± 39.64

[1] M1 protein mRNA expression was calculated per lung in each animal using RT-qPCR. Lung virus titer was then determined by comparison of M1 expression in samples to a standard curve of M1 expression in PR8 virus stock and reported as ng per lung.

[2] One independent value shown. Statistical analysis was not performed due to a limited number of mice expressing a detectable level of M1.

Analysis by ANOVA with Tukey's multiple comparisons; Values represent mean ± SEM, n = 3 mice per day per group; ND, not detected.

Asterisks indicate significant differences compared to young on the same day, *$P<0.05$. Repeated a total of 2 times.

3.2 Caloric restriction increases early severity of influenza infection in young adult C57BL/6 mice

3.2.1 Abstract

Caloric restriction (CR) without malnutrition extends lifespan in mice and postpones age-related changes in immunity. However, in our previous study, aged (22 month) CR mice exhibited increased mortality, impaired viral clearance, and reduced natural killer (NK) cell cytotoxicity following influenza infection compared to aged *ad-libitum* (AL) mice. To determine if the detrimental effects of CR in response to influenza infection occur independently of advanced age, young adult (6 month) CR and AL C57BL/6 mice were infected with 10^4 $TCID_{50}$/100 HAU of influenza A virus (H1N1, PR8). Young adult CR mice exhibited increased mortality ($P<0.05$), weight loss ($P<0.01$), and lung virus titers ($P<0.05$) and pathology ($P<0.001$) compared to young AL controls. Also, CR mice exhibited a decrease in total ($P<0.001$) and NK1.1+ lymphocytes ($P<0.05$) in response to infection, as well as a reduction in influenza-induced NK cell cytotoxicity in both lung ($P<0.01$) and spleen ($P<0.05$). A decrease in the percentage of NK1.1+ cells in the mixed lymphocyte population can result in a decrease in NK cell cytotoxicity; therefore, NK cell-activating cytokines in lung, as well NK cell activation and function, were further evaluated. In response to influenza, expression of IFN-α/β mRNA (PCR, $P<0.001$) and production of IL-12 (ELISA, $P<0.05$) were reduced in the lungs of CR mice compared to AL mice. Natural killer cells from AL mice exhibited a 2-fold induction in the mean fluorescence intensity of intracellular perforin and granzyme B at 2 days p.i. ($P<0.001$). Importantly, CR mice demonstrated an increase in granzyme B, but

no induction in perforin, resulting in a decrease in perforin in the NK cells of CR mice compared to AL (*P*<0.01). Late in the innate immune response to influenza infection (days 3 and 4 p.i.), the percentage of NK cells expressing activation markers B220 and CD25 in CR mice, as well as the intracellular production of IFN-γ, exceeded that in AL mice (*P*<0.001). Further, increased CD11b+ cellular infiltration in the lung (*P*<0.05) and IL-6 in the serum in CR mice suggest the possibility of a hyper-inflammatory response to influenza infection. These data are the first to describe an age-independent and detrimental effect of CR on the innate immune response to influenza infection.

3.2.2 Introduction

The study of aging in multiple species has revealed that dietary caloric restriction (CR) is the only known intervention capable of extending maximal lifespan (Speakman 2007, Masoro 2005, Yamaza 2002, Wanagat 2000). Extension of both median and maximal lifespan in rodents by CR without malnutrition, was first demonstrated by McCay and colleagues in 1935 (McCay 1935). Since then, diets ranging from 30-70% CR have been shown to increase median and maximal lifespan by up to approximately 65% and 50%, respectively, over AL diet (Barger 2003). Caloric restriction has also been shown to reduce the incidence of spontaneous tumors and cancers in rodents, suggesting positive effects on immune function (Effros 1991, Weindruch 1986, Weindruch 1989). Caloric restriction is now generally acknowledged to delay the development of immunity, as well as to preserve various aspects of immune function with advanced age, including T cell proliferation, cytokine production, and cytotoxic T lymphocyte activity (Ritz 2006, Messaoudi 2006, Nikolich-Zugich 2005, Pahlavani 2000). Further, the decreased

incidence of spontaneous tumors, abrogation of the age-related decline in mitogen-stimulated T cell proliferation, and moderately increased lifespan were observed even when CR was induced in previously AL-fed rodents in late adult or in old age (Weindruch 1982, Weindruch 1995), suggesting that lifelong restriction is not required to demonstrate positive effects on immunity.

Improvement in general indices of immune responsiveness prompted the examination of the effects of CR on age-related changes in the response to antigen-specific stimulation, such as influenza. Multiple investigators have clearly demonstrated positive effects of CR on cell-mediated and antibody responses of aged mice to influenza vaccination, including an increase in memory T cell proliferation and cytotoxicity, relative to aged AL-fed mice (Effros 1991, Fernandes 1990, Weindruch 1983). Importantly, in these studies live virus was given intraperitoneally (i.p.), a protocol which induces immunization, and influenza-specific responses were assessed in the spleen. However, the effects of CR on age-related changes in the immune response to immunization may not necessarily reflect those seen during a primary virus infection, particularly at the site of infection, the lung.

Thus, although the preponderance of evidence suggests that CR maintains immune function at an advanced age, the effect of CR on the immune response to a primary virus infection has not been adequately considered. Such studies are warranted because influenza infection remains a significant public health treat, despite vaccination, infecting approximately 20% of the U.S. population annually (Simonsen 2005, Jefferson 2006). Influenza infection accounts for approximately 200,000 hospitalizations and as many as 36,000 deaths, 90% of which occur in the elderly (Thompson 2004, Thompson 2005).

Further, the elderly are also at an increased risk for reduced energy intake resulting from social, physical, economic, and emotional obstacles to eating (Pirlich 2001). Finally, the study of primary virus infections is relevant among all ages in this era of threats from highly-virulent H5N1 avian influenza and the potential use of new or adapted viruses as agents of bioterrorism (Murasko 2005).

Our laboratory has previously observed an increase in the severity of influenza infection in aged CR mice following intranasal (i.n.) inoculation, which produces infection in the lung (Garnder 2005). Aged CR mice exhibited a reduction in influenza-induced NK cell cytotoxicity, as well as an increase in lung virus. However, since the study did not include young CR mice, it could not be determined whether or not CR alone, or CR in combination with advanced age, accounted for the inability to mount an effective innate immune response against influenza virus infection. Therefore, in the current study, young AL and young CR mice were challenged i.n. with influenza virus to determine the effects of CR alone, independent of advanced age, on the innate immune response to influenza virus infection.

3.2.3 Materials and Methods

Animals and diets

The protocol was approved by the Drexel University Institutional Animal Care and Use Committee. Specific pathogen-free young adult (6 month) AL and CR male C57BL/6 mice were purchased from the National Institute on Aging colony maintained by Charles River Laboratories (Wilmington, MA). Calorically-restricted mice from the colony are weaned and fed an increasingly restricted diet beginning at age 14 weeks and reaching

40% CR at age 17 weeks, according to published protocols (Turturro 1999). Mice achieve energy balance within 30 days, comparable to approximately 2.5 years in humans, such that 6-month old CR mice are weight stable (Speakman 2007). Mice were housed in micro-isolator cages in the AAALAC-accredited barrier facility at Drexel University and acclimated for at least 1 week before use, during which time mice were weighed daily to monitor energy balance. The AL mice were fed an NIH-31 diet and consumed an average of 4.3 g of food daily, providing an average energy intake of 17.3 kcal/d. The CR mice were fed an isocaloric NIH-31/NIA-fortified diet and received a single 2.7g "cookie," providing 10.7 kcal/d (**Figure 1**). As a result, CR mice were maintained on a diet sufficient in micronutrients, but restricted in total energy intake by approximately 40%. (See **Appendix I** for more information on the composition of each diet.)

Figure 1. Animal diets. Young adult (6 month) CR mice were fed one 2.7g "cookie" daily (left). Age-matched control mice were fed *ad-libitum* (AL, right).

Influenza infection

Mouse-adapted influenza A/Puerto Rico/8/34 (H1N1, PR8) was propagated in specific pathogen-free eggs (B & E Eggs) and cell-free supernatants were stored at -36°C until use. At baseline (Day 0), mice were anesthetized by intraperitoneal (i.p.) injection with Avertin (2,2,2-tribromoethanol, Sigma) and infected intranasally (i.n.) with 10^4 $TCID_{50}$/100 HAU of PR8 (**Figure 2**). All mice were weighed daily to monitor their ability to control infection.

Figure 2. Intranasal infection. Young adult AL and CR mice were infected i.n. with 10^4 $TCID_{50}$/100 HAU of mouse-adapted influenza A virus (H1N1, PR8) in saline.

Lymphocyte isolation

The isolation of mononuclear cells from spleens and lungs has been described in detail (Po 2002). Briefly, mice were asphyxiated by CO_2, and spleens and lungs were aseptically removed. Spleens were homogenized by dounce and resuspended in RPMI-1640 (Mediatech). A lobe of each lung was frozen in TRI reagent (Molecular Research Center) and saved for RNA extraction. Remaining lungs were minced and incubated at

37°C for 2 h in 3mg/mL Collagenase A and 80 Kuntz units of DNAse I/mL (Roche) in Iscove's Modified Dulbecco's Medium (IMDM, Mediatech). Digested lungs were passed through a 40 µm nylon mesh (Fisher), and centrifuged at 500 x g for 5 min. Supernatants were aliquoted and stored at -36°C for analysis of lung virus by MDCK assay and cytokine analysis by ELISA. The pellets were washed twice with 5% FBS in IMDM. Cell suspensions from spleens and lungs were layered on Histopaque-1083 (Sigma) and subjected to density gradient centrifugation at 1400 x g for 20 min. Cells from each tissue were resuspended at appropriate concentrations for either NK cell cytotoxicity assay or antibody staining for analysis by flow cytometry.

NK cell cytotoxicity

A standard ^{51}Cr-release assay with YAC-1 target cells was employed to assess NK cytotoxicity, as previously described (Plett 2000). Briefly, YAC-1 cells were incubated with 200 µCi $Na^{51}CrO_4$ (PerkinElmer) for 2 h, washed in RPMI-1640, and plated in triplicate in v-bottom 96-well plates with lung and spleen cell preparations at an E:T of 50:1. Following a 4-h incubation at 37°C, supernatants were harvested onto UniFilter microplates (PerkinElmer), and radioactivity was quantitated by a γ-counter (Packard TopCount) and reported as counts per minute (CPM). Spontaneous release was determined in medium alone, and maximum release in 5% Triton X-100 (Sigma). Spontaneous release was <10% of maximum release. Percent cytotoxicity was then calculated as follows:

$$\% = \frac{(\text{Experimental CPM} - \text{Spontaneous CPM})}{(\text{Maximum CPM} - \text{Spontaneous CPM})} \times 100$$

Flow cytometry

Following multiple washes in PBS/1% FBS (Mediatech), 5 x 10^5 cells from lung or spleen were resuspended in PBS containing various combinations of the following fluorochrome-conjugated antibodies (eBioscience) at concentrations ranging from 1:100 to 1:300: CD4 (Fitc), CD8 (PE-Cy5 or APC), NK1.1 (PE or PE-Cy7), CD11b (Fitc), CD11c (PE), CD25 (APC), B220 (APC), IFN-γ (APC), perforin (Fitc or PE), and granzyme B (Fitc or PE). Cells were incubated in staining cocktails on ice in the dark for 30 min. Cells were then pelleted, washed 3 times, and resuspended in 1% paraformaldehyde (Sigma). Alternatively, intracellular staining was performed after surface staining utilizing reagents provided in the Fixation & Permeabilization kit (eBioscience). Briefly, cells were resuspended in fixation buffer (containing 4% paraformaldehyde), incubated for 20 min on ice, and washed in 1X permeabilization buffer (containing 0.1% saponin and 0.09% sodium azide). Cells were incubated in the appropriated intracellular staining cocktails on ice in the dark for 30 min. Cells were then pelleted, washed 3 times in permeabilization buffer, and resuspended in 1% paraformaldehyde. Samples were then acquired on a FACSCanto flow cytometer (BD) and analyzed using FlowJo software (Tree Star). A generalized schematic for the analysis of flow cytometry data is provided (**Figure 3**).

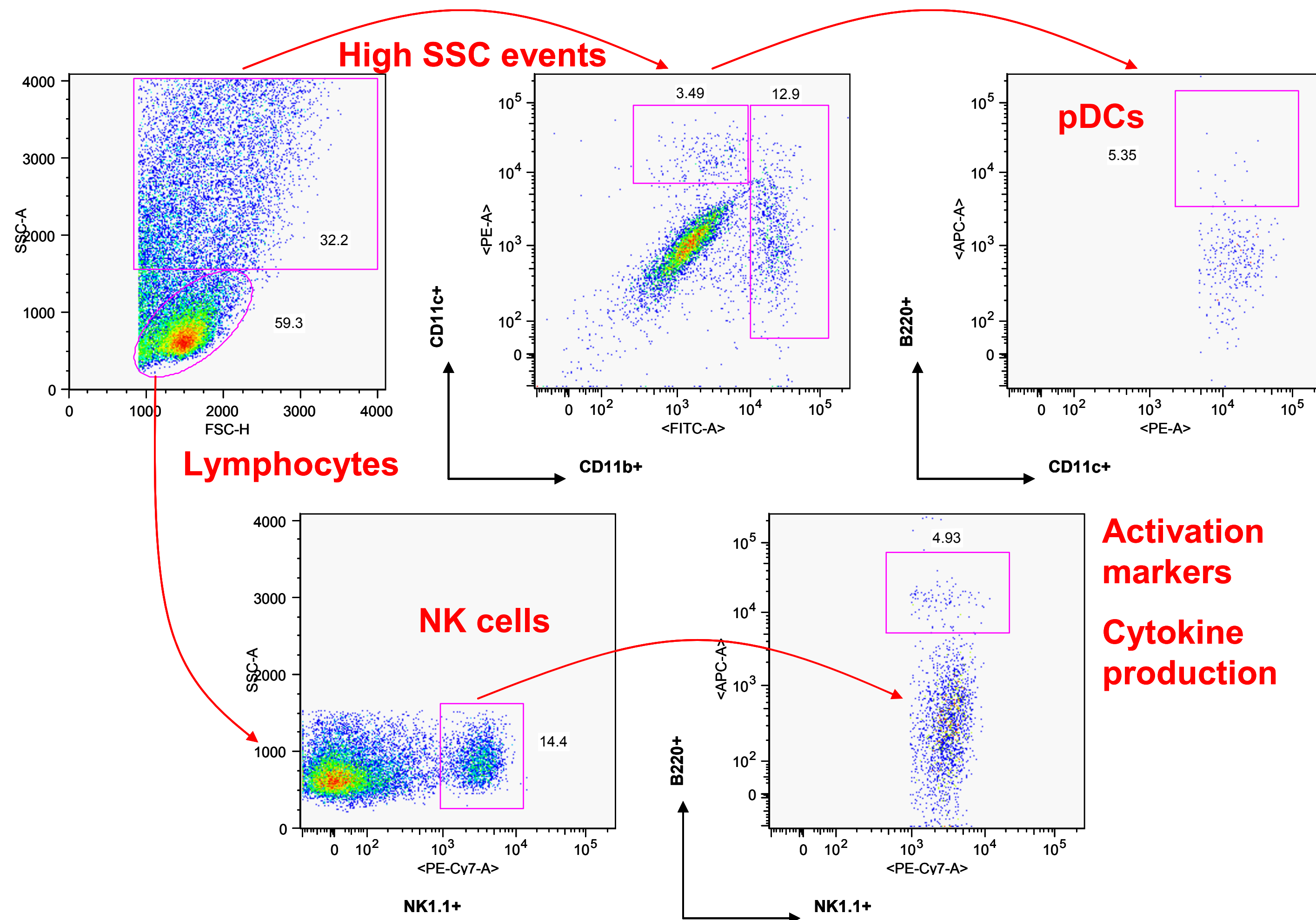


Figure 3. Schematic of flow cytometry methods. Total live events in samples from lung or spleen were analyzed for high side-scatter (SSC) events and lymphocyte populations, as shown.

Lung virus titer by MDCK assay

As previously described (Ritz 2006b), supernatants from lung homogenates were serially diluted and used to infect Madin-Darby canine kidney (MDCK) cells. After incubation at 37°C for 24 h, 0.002% TPCK-treated trypsin (Sigma) was added, followed by a 72-h incubation. Chicken red blood cells (B&E Eggs) were prepared at a concentration of 0.05% in PBS and added to the cultures. Virus titers were then determined based on the presence or absence of hemagglutination, as previously described (Po 2002), and reported as the 50% tissue culture infectious dose ($TCID_{50}$).

Lung virus titer by RT-qPCR

Viral load in lungs was also determined as M1 protein mRNA expression using real-time quantitative PCR (RT-qPCR), as previously described (Ward 2004). Briefly, viral RNA was isolated from harvested lung tissues using a QIAamp viral RNA kit and reverse transcribed using an Omniscript RT kit, according to manufacturer's instructions (Qiagen). Reactions were primed with 1μM random hexamers, 10 units of RNAse inhibitor, and 10μM of M1-specific primer in a total volume of 20μL in nuclease-free water (Gibco). Reactions were incubated at 42°C for 60 min, heated at 95°C for 5 min, and cooled to 4°C in a 2720 Thermal Cycler (Applied Biosystems). The PCR reaction mixture contained 2μL of cDNA, 1X TaqMan Universal Master Mix (Applied Biosystems), 900 nM of each primer, and 225 nM of probe in a total volume of 25 μL. Forward and reverse primers and probe, tagged with FAM (6-carboxyfluorescencein) reporter dye on the 5' end and TAMRA (6-carboxytetramethylrhodamine) quencher dye on the 3' end, were purchased from IDT. The matrix protein gene sequence used in Reverse Transcription reactions was 5' TCT AAC CGA GGT CGA AAC GTA 3'. The

forward (sense) and reverse (antisense) primer sequences used in amplification of cDNA were forward: 5' AAG ACC AAT CCT GTC ACC TCT GA 3' and reverse: 5' CAA AGC GTC TAC GCT GCA GTC C 3'. The M1 influenza A specific probe sequence was 5' TTT GTG TTC ACG CTC ACC GT 3'. Virus titer was calculated by comparing M1 expression in samples to a standard curve of M1 expression in PR8 virus stock and reported as ng of virus per lung.

Cytokine analysis by RT-qPCR

Changes in the expression of IFN-α/β due to infection were determined using RT-qPCR, as previously described (Hunzeker 2004), due to a lack of available reagents for the reproducible analysis of Type I IFN by other methods, such as ELISA. Briefly, RNA was isolated from harvested lung tissues using a QIAamp viral RNA kit and reverse transcribed using an Omniscript RT kit, according to manufacturer's instructions (Qiagen). Reverse transcription reactions were primed with 1μM random hexamers and incubated at 42°C for 60 min, heated at 95°C for 5 min, and cooled to 4°C in a 2720 Thermal Cycler (Applied Biosystems). The PCR reaction mixtures contained 2μL of cDNA, 1X Taqman Universal Master Mix (Applied Biosystems), 900 nM of each primer, and 100 nM of probe, and brought to a final volume of 25 μL in nuclease-free water (Gibco). Forward and reverse primers and probe, tagged with FAM (6-carboxyfluorescencein) reporter dye on the 5' end and TAMRA (6-carboxytetramethylrhodamine) quencher dye on the 3' end, were purchased from IDT. Primers and probe sequences were as follows:

IFN-α, forward primer: 5' TGC AAC CCT CCT AGA CTC ATT CT 3'

IFN-α, reverse primer: 5' CCA GCA GGG CGT CTT CCT 3'

IFN-α, probe: 5’ CTG CAT CAG ACA GCC TTG CAG GTC ATT 3’

IFN-β, forward primer: 5’ TGA ATG GAA AGA TCA ACC TCA CCT A 3’

IFN-β, reverse primer: 5’ CTC TTC TGC ATC TTC TCC GTC A 3’

IFN-β, probe: 5’ AGG GCG GAC TTC AAG ATC CCT ATG GA 3’

As IFN-α contains more than 20 isoforms, primer and probe sequences were chosen based on a published consensus (Hunzeker 2004). The levels of mRNA for GAPDH were also determined for each sample and were used to normalize gene expression during influenza infection. Forward and reverse primer sequences were forward: 5’ GCA GTG GCA AAA GTG GAG ATT G 3’ and reverse: 5’ CCA TTC TCG GCC TTG CTG T 3’. The probe sequence was 5’ TGA CTC CAC TCA CGG CAA ATT CAA CG 3’. (See **Appendix II** for a reference of additional primer and probe sequences.)

Data are expressed in each group as a fold induction over day 0. Fold increase is calculated as follows, where C_T refers to the threshold count:

$$\Delta C_T = C_T\,[\text{target}] - C_T\,[\text{reference}]$$

$$\Delta\Delta C_T = \Delta C_T - \Delta C_T\,[\text{day 0}]$$

$$\text{Fold increase} = 2^{-\Delta\Delta CT}$$

There were no significant differences in IFN-α/β mRNA expression between young AL and CR mice at baseline.

Cytokine analysis by ELISA

Cytokines, including IL-12 (p40) and IFN-γ in lungs, as well as IL-6 in plasma, were quantitated by enzyme linked immunosorbent assay (ELISA) according to the manufacturer’s protocols (eBioscience). Briefly, 96-well plates were coated with the appropriate capture antibody (purified anti-mouse), sealed, and incubated overnight at

4°C. Following multiple washes in PBS/1%FBS (Mediatech), lung homogenates or plasma samples were added, and the plates were again sealed and incubated overnight at 4°C. Standards were prepared using the appropriate recombinant cytokine. Samples were then discarded, and plates were thoroughly washed before adding a biotin-conjugated primary antibody for 1 hr. Following multiple washes, an avidin horse radish peroxidase-conjugated secondary antibody with fluorescent probe was added for 30 min. Again the plates were washed, and 1X TMB substrate solution was added for 15 min to induce the color change. Finally, 1M H_3PO_4 (Sigma) was added to stop the reaction, and results were read at an emission wavelength of 450nm, normalized to 570nm (Multiskan Spectra). The concentration of each cytokine was determined against a standard curve and reported as ng/mL or pg/mL.

Antiviral bioassay

Serum was obtained from mice at baseline and during influenza infection for the analysis of IFN antiviral activity, as previously described (Jiang 2005). Murine epithelial L929 cells (ATCC) were grown to confluence in Dulbecco's modified eagle's medium (DMEM, Mediatech), supplemented with 10% FBS, 1% non-essential amino acids (100X, CellGro), 1% gentamicin (Sigma), and 1% sodium pyruvate (Sigma). Serum samples and murine IFN standard (WHO International) were serially diluted in 96-well plates, 1.0×10^5 cells/mL were added to each well, and plates were incubated overnight at 37°C with 5% CO_2 and humidity. At 24 hr, encephalomyocarditis virus (EMC, 1:2000, kind gift of Donna M. Murasko) was added to each well. Plates were again incubated overnight, and the cytopathic effect of the virus was determined at the time point when the serial dilution of the standard demonstrated 50% protection from the virus, defined as

1 unit. Results for each sample were then reported as units, which were determined as the inverse of the dilution of the sample that resulted in 50% protection against the cytopathic effect of the virus and corrected for the standard.

Lung pathology

Formalin-fixed lung tissue was paraffin embedded, cut into 4μm sections, and stained using the hematoxylin-eosin Y (H&E) method, as previously described (Ritz 2006b). Briefly, slides were baked at 65°C for 30 min and deparrafinized by xylene wash. Rehydration of tissue was carried out through a graded alcohol series (100%, 95%, and 80%). Slides were then stained with hematoxylin (Harleco), rinsed, and counterstained with eosin Y (1% alcoholic, Harleco). Pathology was scored on a semi-quantitative scale from 0 (no pathology) to 4 (100% pathology), as described by (Smith 2007, Nelson 2001). Scoring criteria were as follows:

0 = no infiltration (0%)

1 = infiltration of alveoli, bronchioles clear (<25%)

2 = increased infiltration of alveoli, some infiltration of bronchioles (50%)

3 = Bronchioles infiltrated (75%)

4 = Total infiltration of alveoli and bronchioles (100%)

Two to three slides were prepared from the lung tissue obtained from each mouse, and the slides were scored blindly by three different people. All scores were combined for final analysis.

These scoring methods were validated in a preliminary experiment in which lung tissue was obtained from young uninfected mice (0 HAU) or mice infected i.n. with 10 or 100 HAU of PR8 and evaluated at 7 days p.i. Lung pathology scores (mean ± SEM)

increased in a virus dose-dependent fashion from 0-100 HAU as follows: 0.96 ± 0.2, 1.65 ± 0.1, and 2.55 ± 0.4.

Statistics

Statistical analyses were performed using GraphPad InStat 3 software. Survival data were analyzed using the Kaplan-Meier test with censoring. Comparisons between and within groups were analyzed by ANOVA with Tukey-Kramer multiple comparisons. Only where noted, Student's *t*-tests were used to compare mean values between two groups at a single time point. Mann-Whitney *U*-tests were used when data was not normally distributed. Statistical significance was accepted at $P<0.05$.

3.2.4 Results

Decreased survival in young adult CR mice during influenza virus infection

The initial objective of this study was to determine the susceptibility of CR mice to influenza infection, independent of advanced age. Therefore, young adult AL and CR mice were infected i.n. with 10^4 $TCID_{50}$/100 HAU of PR8 and monitored for 7 days post-infection (p.i.) or until mice were moribund. Based on preliminary studies, moribund was defined as weight loss greater than 30% from baseline in AL mice and greater than 15% from baseline in CR mice (Ritz 2006a). Additional weight loss was not compatible with recovery from infection in these animals, and therefore, mice were euthanized accordingly. Following infection, CR mice exhibited increased mortality by 7 days p.i. ($P<0.05$, Kaplan-Meier test), with a median survival of 5 days (**Figure 4**). Median survival could not be determined in AL mice, as greater than 50% of young AL mice survived until 7 days p.i.

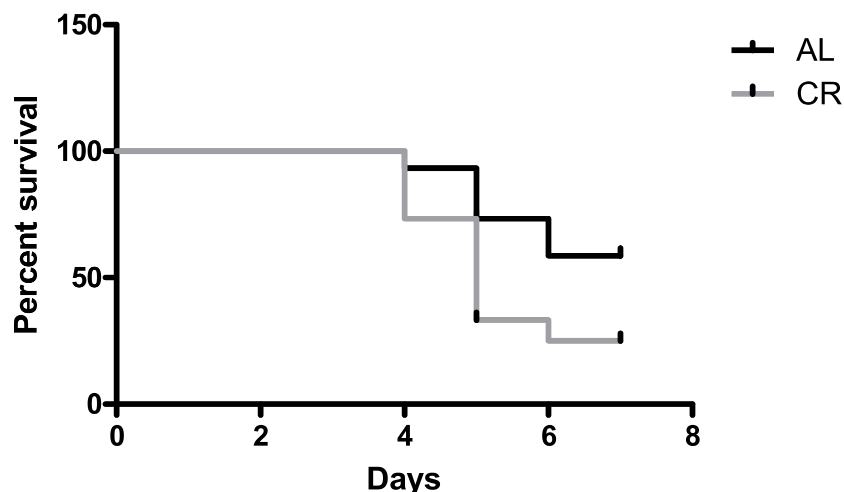


Figure 4. Decreased survival in young adult CR mice during influenza infection. Median survival of CR mice was 5 days, n=15 mice per group, $P<0.05$ at day 7.

Increased weight loss and anorexia in CR mice during influenza virus infection

Body weights were recorded daily throughout the course of infection, as weight loss is an important indicator of the severity of infection. Conversely, restoration of body weight is indicative of recovery from infection. As expected, AL mice weighed significantly more than CR mice at baseline (means ± SEM, 23.8g ± 0.2 vs. 17.7g ± 0.3, n = 15 mice per group, *t*-test, $P<0.0001$). Following infection, AL mice began to lose weight immediately, and lost more weight, as a percentage of baseline weight, than age-matched CR mice at 2 days p.i. ($P<0.05$) (**Figure 5**). In contrast to AL mice, CR mice maintained a constant body weight through 2 days p.i. However, by 4 days p.i., CR mice demonstrated increased weight loss, while AL mice began to recover body weight ($P<0.01$).

Weight loss is associated with the total or partial cessation of feeding, which is believed to be mediated, at least in part, by cytokines produced during the immune response to infection (Van Reeth 2000, Swiergiel 1997). Calorically-restricted mice were fed a controlled, 40%-restricted diet at baseline ($P<0.05$) (**Figure 6**). Following infection, both AL and CR mice exhibited a decrease in food intake through 2 days p.i. ($P<0.01$); however, the AL mice then began to recover, consistent with weight loss data. Interestingly, CR mice consumed 100% of their diet through the first day p.i. Food intake, in grams, was similar between AL and CR mice on days 1 through 3 p.i. However, unlike AL mice that began to recover intake at 4 days p.i., CR mice continued to consume approximately 60% less food than they did at baseline. As a result, CR mice consumed significantly less food than AL mice at 4 days p.i. ($P<0.05$).

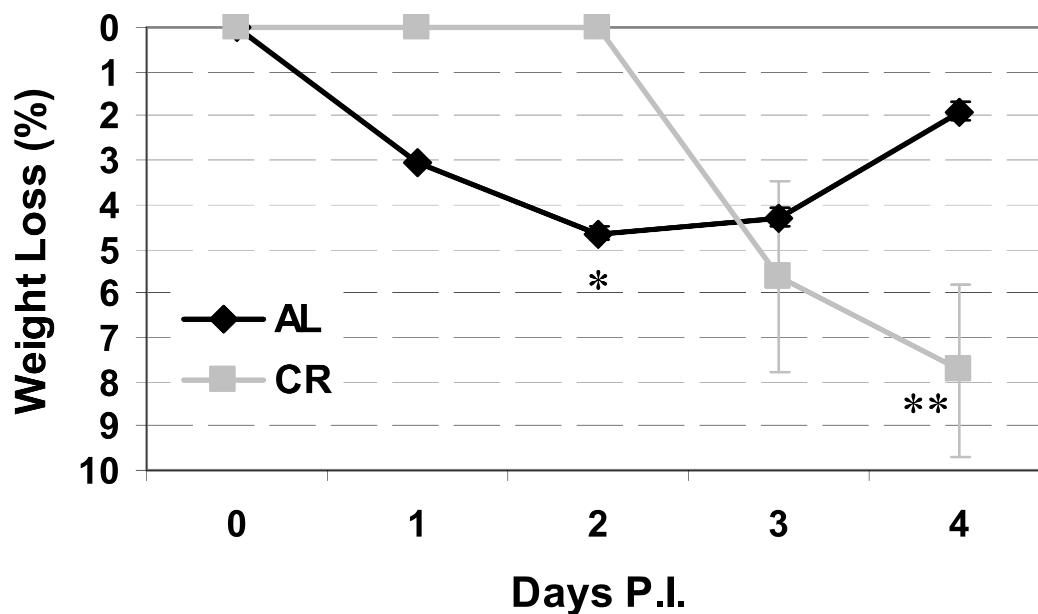


Figure 5. Increased weight loss in CR mice during influenza infection. AL mice exhibited an increased loss of body weight at 2 days p.i., but then began to recover. Young adult CR mice exhibited no weight loss through 2 days p.i. At 4 days p.i., CR mice demonstrated increased weight loss compared to AL controls, n=8 mice per group per day, *P<0.05, **P<0.01. Repeated a total of 3 times.

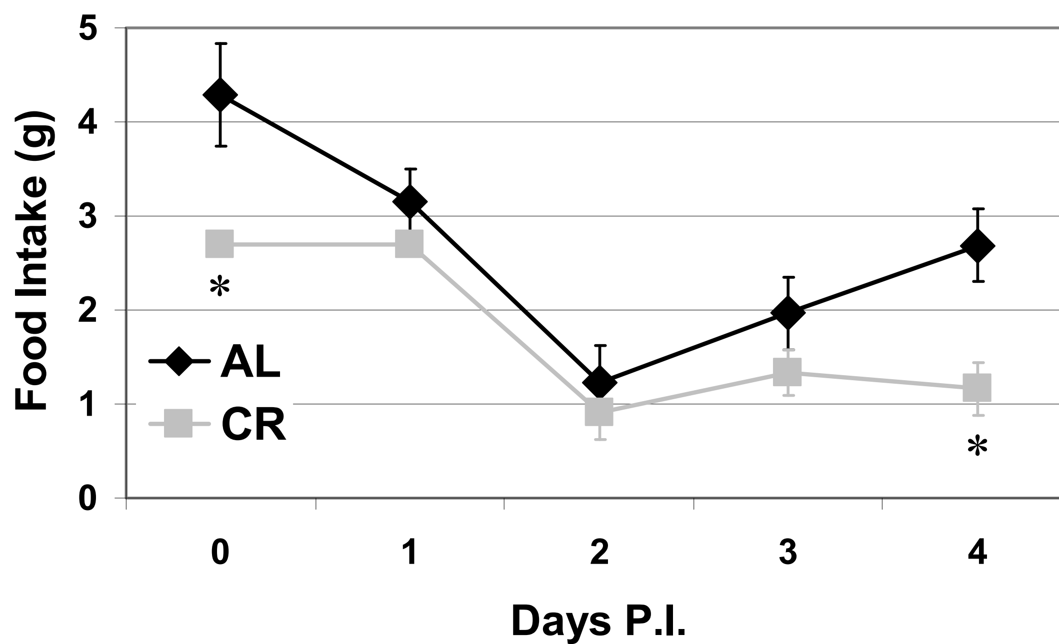


Figure 6. Decreased food intake in CR mice at baseline and during influenza infection. The CR mice were fed a 40% restricted diet at baseline. Following infection, young adult CR mice maintained 100% food intake through day 1 p.i. At 4 days p.i., CR mice consumed less food than AL controls, n=8 mice per group per day, **P*<0.05. Repeated a total of 3 times.

Increased lung virus in CR mice

To further assess the susceptibility of CR mice to influenza infection, lung virus burden was determined by two methods. First, lung virus titers were determined in lung homogenates from young adult AL and CR from days 0 through 4 p.i. using an MDCK cell infectivity assay. Virus was detected by the MDCK assay on day 1 p.i. in the lungs of CR mice, but not until 2 days p.i. in AL mice (**Table 1A**). Lung virus titers, as assessed by the MDCK method, did not differ from 2 through 4 days p.i.

Table 1A. Lung virus determined by MDCK ($TCID_{50}$/mL).

Days p.i.	AL	CR
0	ND	ND
1	ND	7.69 ± 0.2
2	6.50 ± 0.5	8.31 ± 0.2
3	8.34 ± 1.0	9.16 ± 0.2
4	6.75 ± 0.6	8.73 ± 0.3

ND, not detectable.

Table 1B. Lung virus determined by real-time qPCR (ng per lung).

Days p.i.	AL	CR
0	ND	ND
1	ND	663.8 ± 149.6
2	128.2 ± 30.5	*282.6 ± 14.0
3	691.8 ± 690.6	3094.4 ± 716.5
4	ND	608.8 ± 97.6

n=4 mice per group per day, *P<0.05 compared to AL on the same day; ND, not detectable. Repeated a total of 3 times.

A limitation of the MDCK infectivity assay is low sensitivity; therefore, a second method using RT-qPCR was employed to measure lung mRNA expression of M1 protein using a modification of a previously published method (Ward 2004). Since M1 protein is conserved among mouse-adapted influenza A viruses and is an abundant internal protein within the viral envelope, M1 expression is expected to correlate with virus levels in infected tissues. Further, we have validated this method by assessing intra- and inter-assay variation and directly comparing results obtained by RT-qPCR with those from the standard MDCK assay in the same animals (unpublished data, Nogusa & Gardner 2007). We have further found that weight loss significantly correlates with lung virus, as determined by M1 gene expression in lung (**Chapter 3.1**). In the current study, evaluation of M1 expression by RT-qPCR supported lung virus titers determined by MDCK assay. The expression of M1 mRNA was detected in the lungs of CR mice on day 1 p.i., but not until 2 days p.i. in AL mice (**Table 1B**). Gene expression of M1 was elevated in CR mice at 1 and 2 days p.i. compared to AL mice ($P<0.05$), and appeared to remain elevated through 4 days p.i., although this difference was not significant due to large variability in M1 expression in AL lungs on day 3. Finally, M1 mRNA expression was not detected in the lungs of AL mice at 4 days p.i., but remained elevated in CR mice. Combined data from the MDCK infectivity assay and M1 expression clearly suggest that lung virus is detected earlier and is increased in the lungs of young adult CR mice following influenza virus infection.

Increased lung pathology in CR mice

Virus replication during influenza infection occurs primarily in the respiratory epithelial cells, and morphologic changes, including destruction of lung tissue, are readily observed during the course of influenza infection (Bender 1995). Such changes are apparent even to the naked eye. This increased lung pathology is indicative of inflammation and contributes to increased mortality in influenza-infected mice (Smith 2007). In the current study, lung pathology was determined by a semi-quantitative assessment of epithelial erosion and cellular infiltration of lung tissue as indicated by H&E staining. Samples of lung tissue from AL and CR mice were compared at baseline and 4 days p.i. (**Figure 7**). As expected, lung pathology scores increased in AL ($P<0.05$) and CR mice ($P<0.001$) in response to influenza infection (**Figure 8**). However, CR mice exhibited increased lung pathology relative to AL mice both at baseline ($P<0.05$) and at 4 days p.i. ($P<0.001$). These data suggest that young adult CR mice exhibit increased inflammation in the lungs prior to and during influenza infection, as compared to AL mice. The increased lung pathology in young adult CR mice at baseline may also reflect morphological changes in lung associated with the onset of CR, as previously reported (Massaro 2004). The current study is the first to evaluate lung histology in 40% CR mice at an age of 6 months.

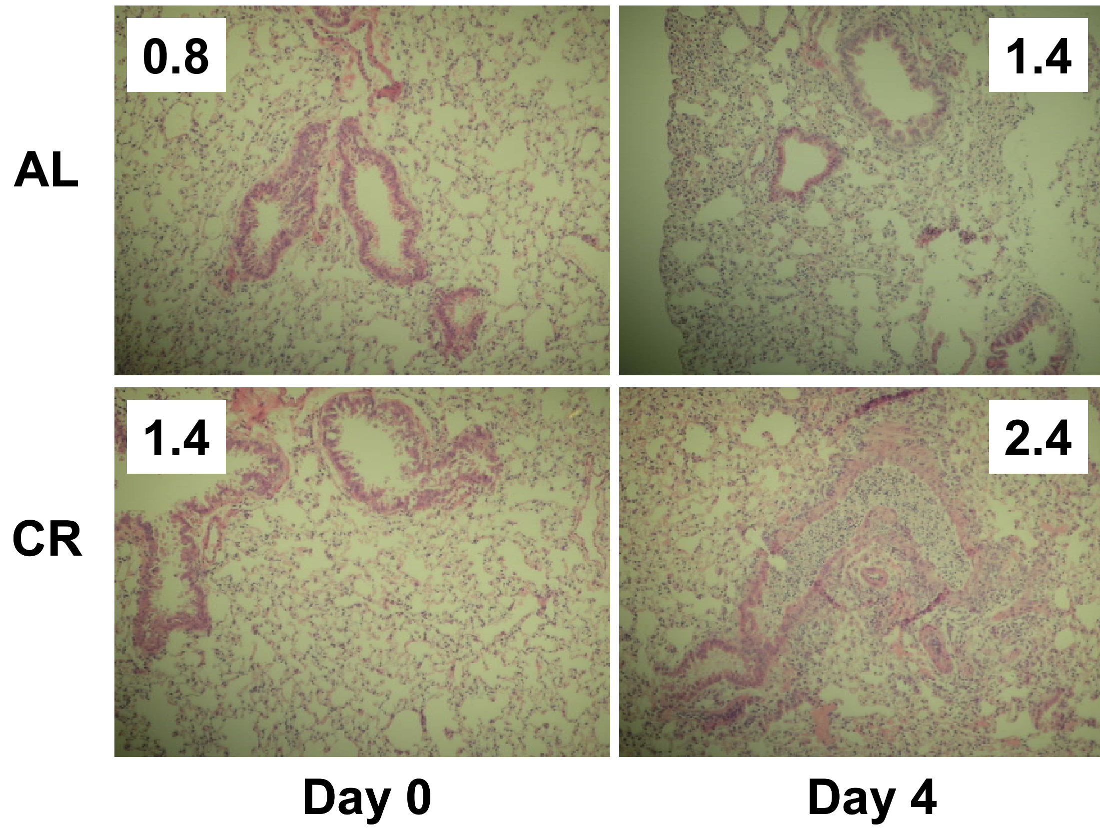


Figure 7. Representative H&E staining of lung tissues at baseline and 4 days following infection with influenza virus in AL and CR mice. Values indicate mean pathology scores. Repeated a total of 3 times.

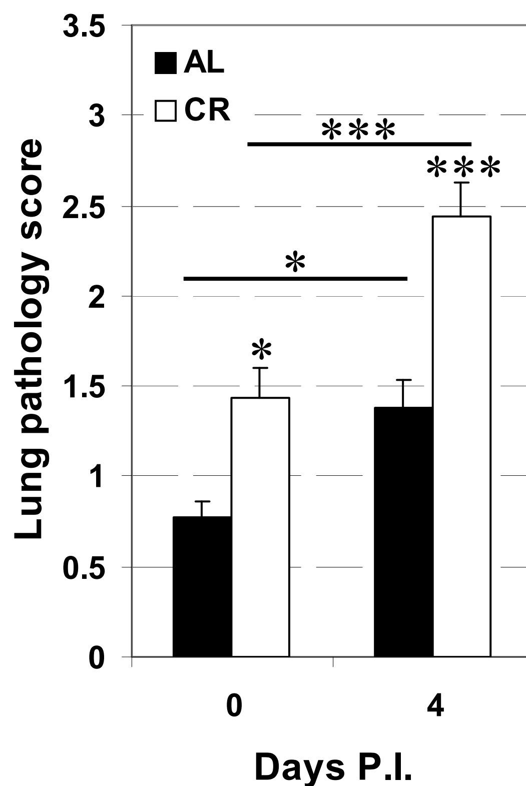


Figure 8. Increased lung pathology in CR mice at baseline and during influenza virus infection. Lung pathology scores increased from baseline to 4 days p.i. in both AL and CR mice. Further, CR mice exhibited increased pathology at both baseline and 4 days p.i. compared to AL controls, n=4 mice per group per day, *P<0.05, ***P<0.001. Repeated a total of 3 times.

Impaired influenza-induced NK cell cytotoxicity in CR mice

During infection, young adult CR mice exhibited increased mortality and weight loss, as well as elevated lung virus burden and pathology, compared to AL-fed controls. As previously demonstrated in aged CR mice, the increased susceptibility of young adult CR mice to influenza infection occurred early in the immune response, suggestive of an impairment in NK cell-mediated innate immunity (Gardner 2005).

To evaluate the ability of CR mice to mount an effective innate immune response to influenza virus infection, independent of the effects of advanced age, NK cell cytotoxicity was examined in young adult AL and CR mice. Cytotoxicity was concurrently assessed in lungs and spleens to determine if a change in the induction of NK cell cytotoxicity in the lung was also reflected in the spleen. Young AL mice demonstrated increased NK cell cytotoxicity during influenza virus infection in both the lung and spleen (**Figure 9**). In contrast, CR mice did not exhibit an increase in NK cell cytoxicity in either lung or spleen during infection. As a result, influenza-induced NK cell cytotoxicity was significantly elevated in young AL mice compared to CR mice at day 1 p.i. in lung ($P<0.01$) and at day 2 p.i. in spleen ($P<0.05$). NK cell cytotoxicity was not significantly different in CR mice compared to AL mice at baseline.

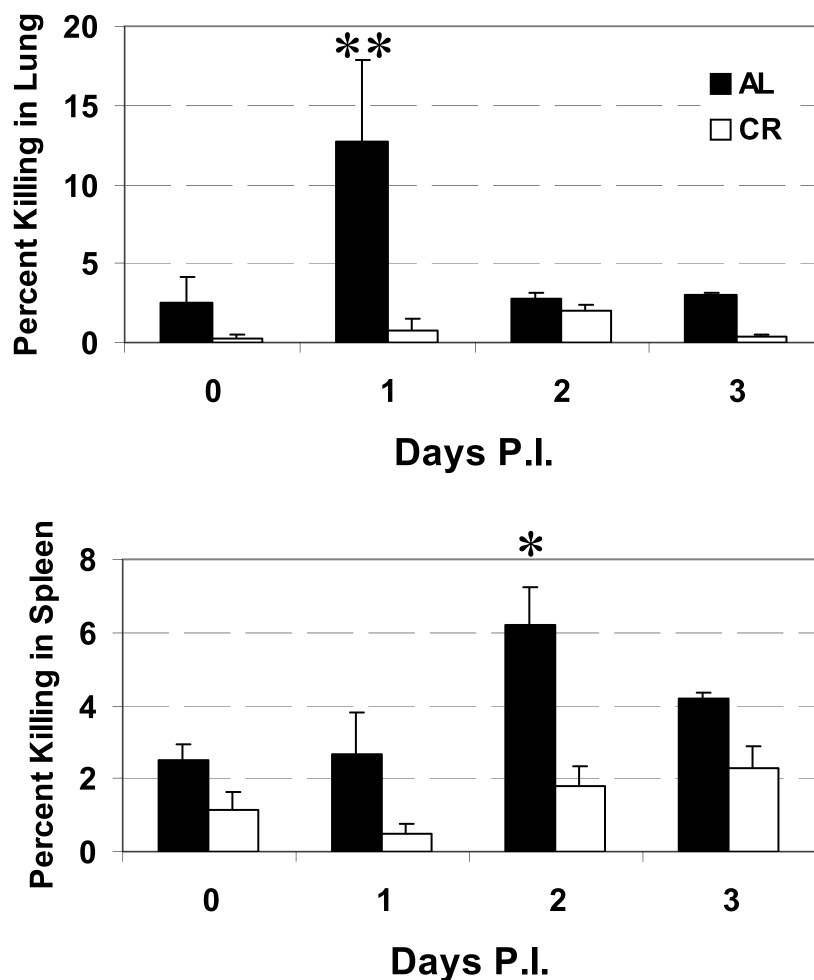


Figure 9. Decreased influenza-induced NK cell cytotoxicity in the lungs and spleens of CR mice. Influenza-induced NK cell cytotoxicity was increased in the lungs of AL mice at day 1 p.i. (top) and in the spleens of AL mice at day 2 p.i. (bottom). Young adult CR mice did not exhibit an increase in NK cell cytotoxicity in lung or spleen at any time point during influenza infection, n=4 mice per group per day, *$P<0.05$, **$P<0.01$. Repeated a total of 2 times.

Decreased total and NK1.1+ lymphocytes in the lungs and spleens of CR mice

It is important to assess changes in the lymphocyte population during infection as both a percentage of the total population and a total number per tissue, since a change in one may not be reflected by a change in the other. Therefore, the percentage and number of total lymphocytes, NK1.1+ lymphocytes, and CD8+ lymphocytes in the lungs and spleens of young adult AL and CR mice were determined by flow cytometry. The percentage ($P<0.001$) and number ($P<0.01$) of total lymphocytes in the lungs of CR mice were reduced compared to AL controls at baseline (**Table 2**). During infection, the percentage of total lymphocytes in the lungs of CR mice remained significantly less than AL controls at 2, 3, and 4 days p.i. ($P<0.001$). While the number of total pulmonary lymphocytes appeared less in CR mice than AL mice at all time points following infection, these differences were not significant. Similarly, the number of total lymphocytes in the spleens of CR mice was less than in the spleens of AL mice at baseline and throughout infection, significant only at 3 days p.i. (**Table 3**).

The assay to determine NK cell cytotoxicity utilizes a fixed ratio of effector cells from a mixed lymphocyte sample to YAC-1 target cells, such that a decrease in influenza-induced NK cell cytotoxicity can reflect either a decrease in activity or a relative decrease in the percentage or total number of NK cells in the sample. Therefore, it is important to consider NK cell cytotoxicity during an infection in the context of changes in the percentage and number of NK cells. Importantly, while there was no difference in the percentage of NK1.1+ lymphocytes in the lungs of CR mice compared to AL controls at baseline, there was a decrease in the percentage of NK1.1+ lymphocytes in the lungs of CR mice during infection, which reached significance at 3

days p.i. (*P*<0.05) (**Table 2**, **Figure 10**). There was also a decrease in the number of NK1.1+ cells in the lungs of CR mice compared to AL mice, which parallels the observed decrease in total lymphocytes. However, as observed in the evaluation of total lymphocyte number, the decrease in NK1.1+ cell number in the lungs of CR mice reached significance only at baseline (*P*<0.01). The percentage (*P*<0.05) and number (*P*<0.01) of NK1.1+ lymphocytes in the spleens of CR mice was less than in the spleens of AL mice at baseline (**Table 3**, **Figure 11**). During infection, the percentage and number of NK1.1+ cells in the spleens of CR mice were less than in AL, significant only in number on day 3 p.i. (*P*<0.05). The number of CD8+ lymphocytes in the spleens of CR mice was less than in AL mice at baseline (*P*<0.05), while the percentage of CD8+ cells increased in the spleens of CR mice, but not AL mice, during infection (*P*<0.01).

Overall, there was a decrease in total and NK1.1+ lymphocytes in the lungs and spleens of young adult CR mice compared to AL mice during influenza infection. These data suggest that the lack of influenza-induced NK cell cytotoxicity in CR mice may be explained, in part, by a relative decrease in the percentage and number of NK cells in the lungs of CR mice compared to AL controls.

Table 2. Percent and number of total, NK1.1+, and CD8+ lymphocytes in the lungs of AL and CR mice.

Days p.i.	Group	Lymphocytes[1]		NK1.1+[2]		CD8+	
		%	# (x10^4)	%	# (x10^4)	%	# (x10^4)
0	**AL**	62.0±2.7	38.6±10.9	11.5±1.6	4.6±1.8	8.9±0.8	3.8±0.6
	CR	***36.1±1.7	**4.3±2.0	10.0±1.3	**0.4±0.1	6.1±1.0	3.6±0.1
1	**AL**	48.3±3.3	23.7±5.5	15.4±1.1	3.8±1.1	6.0±0.7	2.1±0.3
	CR	39.7±1.2	19.6±2.8	6.5±0.5	1.3±0.2	8.6±1.5	3.1±1.2
2	**AL**	53.9±4.4	18.6±2.1	12.7±2.3	2.5±0.7	8.6±1.9	2.0±0.3
	CR	***35.6±2.1	8.4±2.4	5.8±0.7	0.4±0.1	6.4±0.4	1.0±0.1
3	**AL**	65.3±2.6	30.1±9.5	13.8±3.0	3.3±0.3	8.8±0.4	3.1±1.0
	CR	***30.7±1.5	10.0±0.6	*4.7±0.7	0.5±0.1	4.6±0.6	0.8±0.2
4	**AL**	62.0±1.9	11.0±2.7	16.7±3.0	1.7±0.4	8.5±0.4	1.4±0.2
	CR	***40.0±2.1	2.3±1.2	8.2±2.3	0.1±0.03	4.7[3]	0.3[3]

[1]Lymphocytes gated on total white blood cells. [2]NK1.1 and CD8 gated on lymphocytes. Values are means ± SEM, n=4 mice per group per day. Asterisks indicate differences between groups on that day, *P<0.05, **P<0.01, ***P<0.001.
[3]Insufficient data to determine SEM. Repeated a total of 2 times.

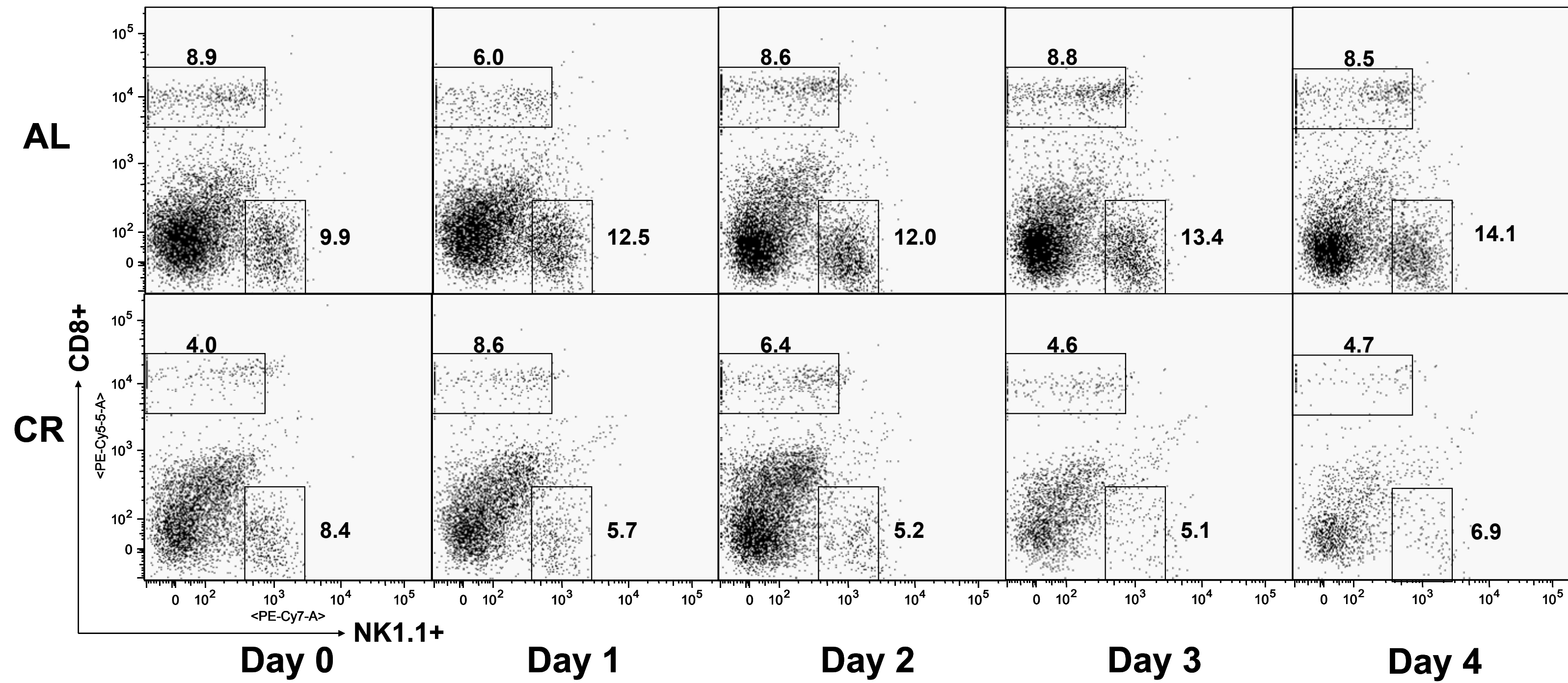


Figure 10. Representative flow cytometry data of the percentage of NK1.1+ and CD8+ lymphocytes in the lungs of AL and CR mice. Values indicate means ± SEM, n=4 mice per group per day. Repeated a total of 2 times.

Table 3. Percent and number of total, NK1.1+, and CD8+ lymphocytes in the spleens of AL and CR mice.

Days p.i.	Group	Lymphocytes		NK1.1+		CD8+	
		%	# ($x10^5$)	%	# ($x10^4$)	%	# ($x10^4$)
0	**AL**	91.3±0.1	20.0±6.0	3.4±0.5	6.1±1.3	5.5±0.9	10.6±2.9
	CR	90.6±1.1	5.4±1.7	*1.8±0.2	**0.9±0.2	6.3±0.6	*3.2±0.8
1	**AL**	91.6±0.5	19.1±7.5	2.5±0.5	4.7±1.9	2.1±0.7	4.6±2.4
	CR	91.8±0.4	9.3±0.7	1.4±0.1	1.3±0.1	3.5±0.5	3.2±0.5
2	**AL**	87.8±1.5	13.2±0.6	4.1±0.5	3.2±0.8	3.1±0.5	2.2±0.5
	CR	86.4±0.3	2.2±0.4	2.8±0.3	0.6±0.1	**7.5±1.2	1.6±0.4
3	**AL**	90.9±0.4	18.2±3.4	2.7±0.2	4.8±0.7	3.9±0.5	7.4±2.2
	CR	88.3±0.8	*2.0±0.2	1.5±0.2	*0.3±0.05	**8.3±0.6	1.7±0.2
4	**AL**	89.3±1.2	14.1±1.5	3.1±0.2	4.4±0.6	3.6±0.5	5.3±1.2
	CR	86.4±1.1	2.9±0.7	1.9±0.3	0.6±0.2	5.1±1.1	1.4±0.2

Values are means ± SEM, n=4 mice per group per day. Asterisks indicate differences between groups on that day, *P<0.05, **P<0.05. Repeated a total of 2 times.

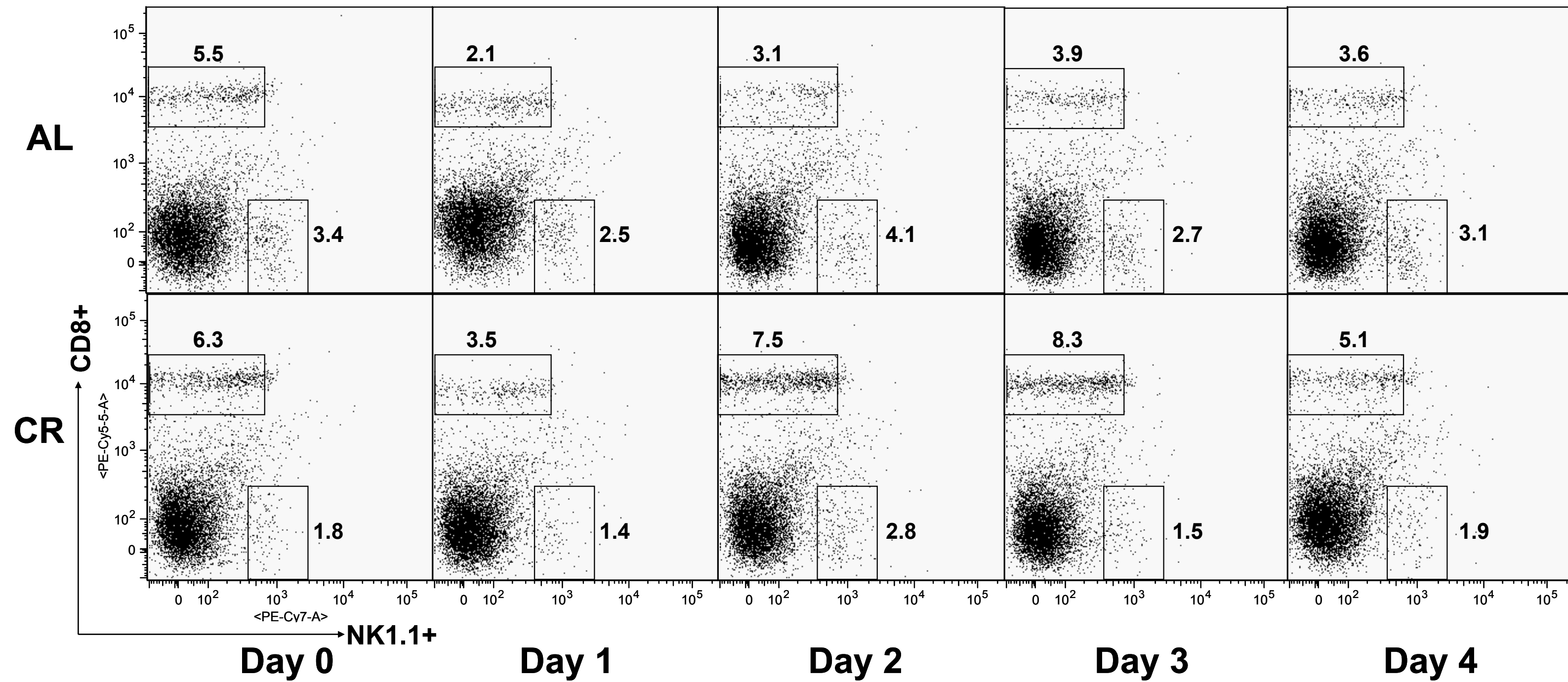


Figure 11. Representative flow cytometry data of the percentage of NK1.1+ and CD8+ lymphocytes in the spleens of AL and CR mice. Values indicate means ± SEM, n=4 mice per group per day. Repeated a total of 2 times.

Decreased perforin production by NK1.1+ cells in the lungs of CR mice

The cytotoxic effect of NK cells is mediated by the production and release of perforin and granzymes. The production of perforin and granzymes increases in response to virus infection and is associated with controlling virus replication, the production of IFN-γ, and host survival (van Dommelen 2006, Johnson 2003). Intracellular staining of perforin and granzyme B in NK1.1+ cells provides information on the cytotoxic potential of NK cells on a per cell basis. Therefore, the production of perforin and granzyme B by pulmonary NK cells was evaluated by intracellular staining in AL and CR mice in response to influenza virus infection. The intracellular production of perforin and granzyme B, as a fold increase in MFI, increased in AL mice in response to influenza virus infection, peaking at 2 days p.i. ($P<0.001$) (**Figure 12**). In CR mice, there was a similar increase in granzyme B-producing NK cells ($P<0.001$), but no change in perforin during the infection. As a result, perforin was decreased in NK cells of CR versus AL mice at 2 days p.i. ($P<0.01$). These data suggest a loss in NK cell cytotoxic function in CR mice on a per cell basis.

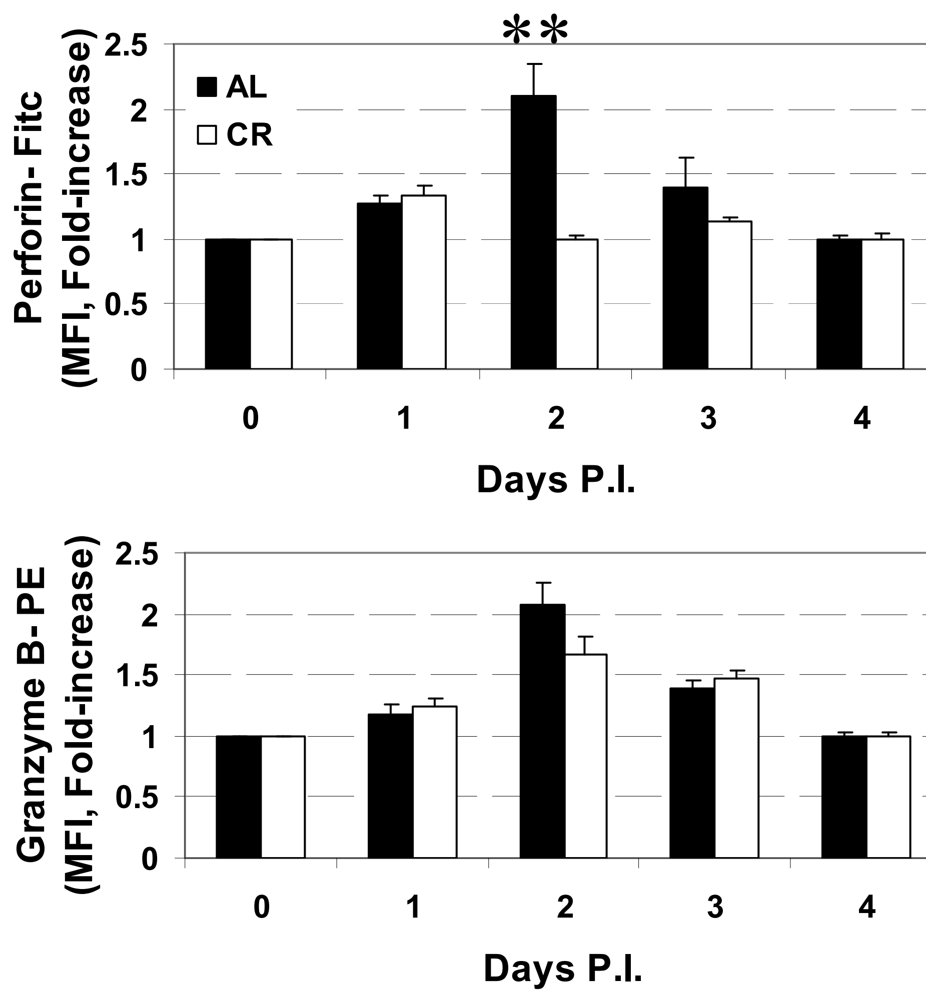


Figure 12. Intracellular perforin and granzyme B production by NK1.1+ lymphocytes in the lungs of AL and CR mice in response to influenza infection. The intracellular staining of NK1.1+ cells from CR mice demonstrated a decrease in the induction of perforin during infection, as compared to AL controls, n=4 mice per group per day, $^{**}P<0.01$. Repeated a total of 2 times.

Decreased IFN-α/β expression in the lungs of CR mice

The activation of NK cell effector functions, including the production of perforin and NK cell cytotoxicity, is mediated by cytokines, such as Type I IFN and IL-12 (Cousens 1997, Nguyen 2002, Liang 2003). Interferon-α/β expression was determined in the lungs of AL and CR mice at baseline and through 3 days p.i., and changes in IFN-α/β expression were reported as a fold increase over uninfected, normalized to the expression of GAPDH (**Figure 13**). There were no differences in IFN-α/β expression in the lungs of AL and CR mice at baseline. In contrast, however, the expression of both IFN-α ($P<0.01$) and IFN-β ($P<0.05$) were decreased in the lungs of CR mice compared to AL controls at 2 days p.i. IFN-α/β was then measured in the periphery by bioassay, and CR mice exhibited an increase in systemic antiviral activity ($P<0.01$) (**Figure 14**). These data suggest a local deficit in IFN-α/β expression in the lung and an increased systemic response to influenza infection in young adult CR mice.

Altered IL-12 production in the lungs of CR mice

Interleukin-12 production was quantitated by ELISA in the lungs of AL and CR mice at baseline and during influenza infection by ELISA. Calorically-restricted mice exhibited a decrease in IL-12 production compared to AL controls when evaluated at baseline ($P<0.001$, *t*-test) and at 2 days p.i. ($P<0.05$, *t*-test) (**Figure 15**). However, there was no difference in the production of IL-12 in the lungs of CR mice compared to AL mice at days 3 and 4 p.i. These data suggest a defect in the availability of the NK cell-activating cytokine IL-12 in the lungs of CR mice early during the innate immune response to influenza infection, jut prior to and following maximal NK cytotoxicity in AL mice.

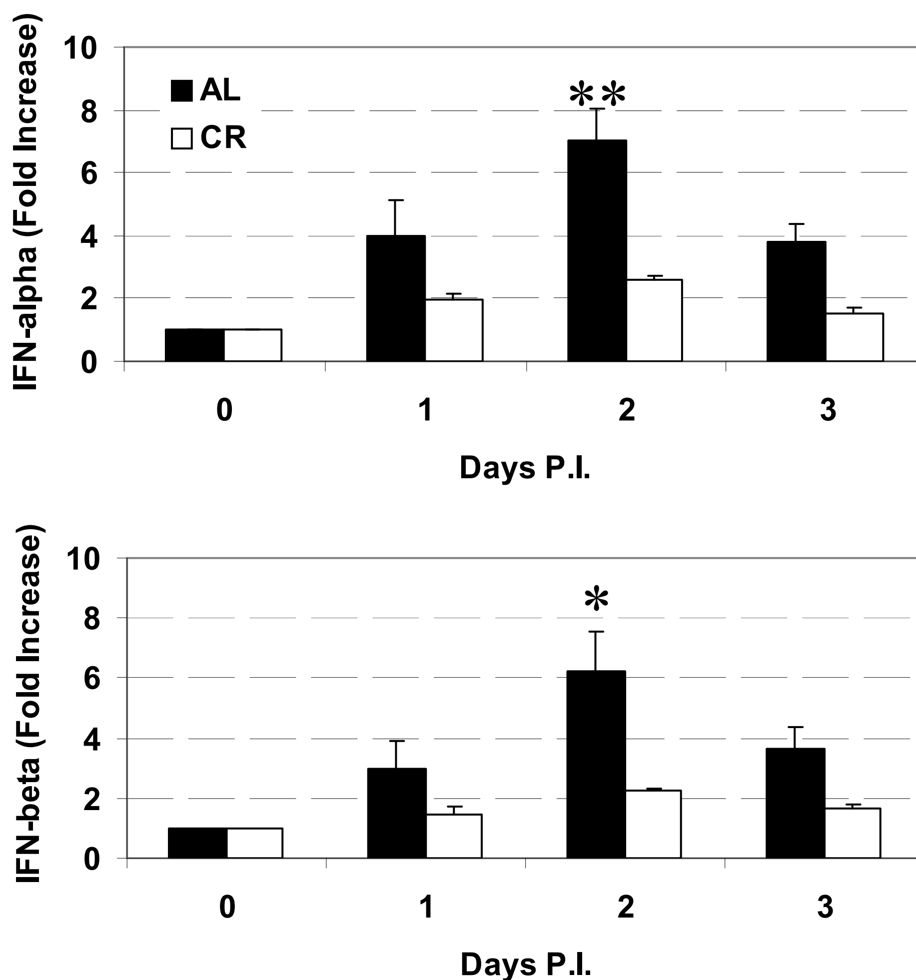


Figure 13. Decreased IFN-α/β expression in the lungs of CR mice during influenza infection. Young adult CR mice exhibited a decrease in the gene expression of IFN-α (top) and IFN-β (bottom) in lungs compared to AL controls in response to influenza infection, n=4 mice per group per day, *P<0.05, ***P*<0.01. Repeated a total of 2 times.

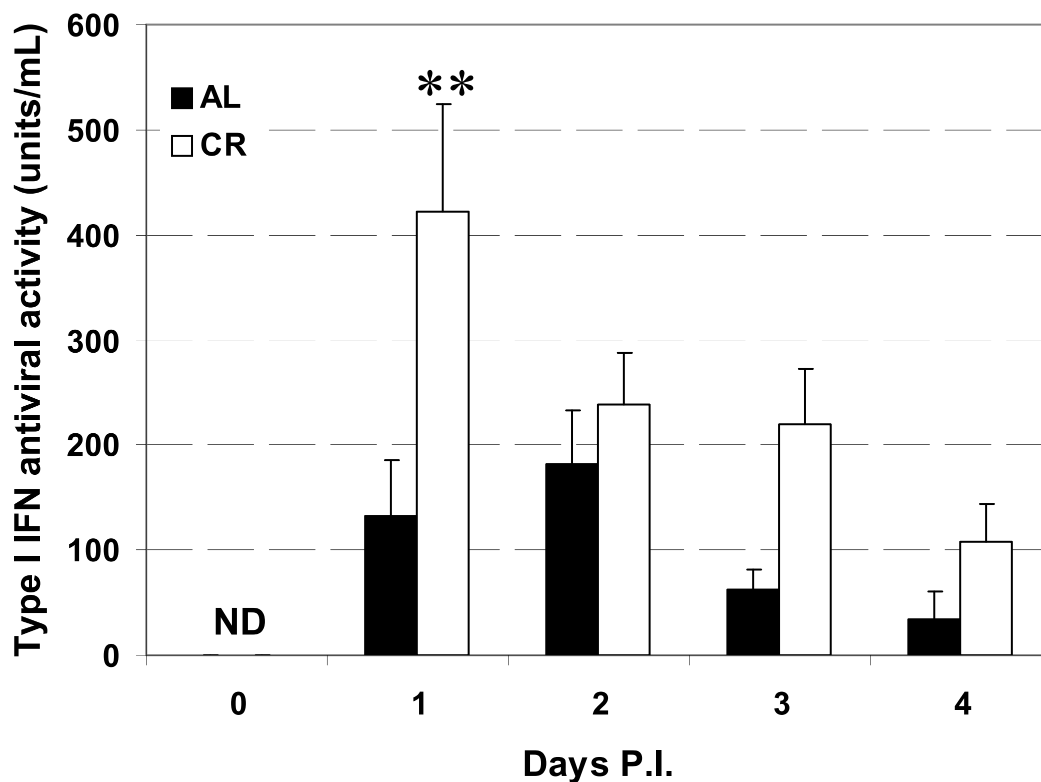


Figure 14. Increased systemic antiviral activity in CR mice during influenza infection. The antiviral activity of IFN-α/β was assessed in serum by bioassay. Young adult CR mice exhibited an increase in antiviral activity, n=8 mice per group per day, ***P*<0.01; ND, not detected. Repeated a total of 3 times.

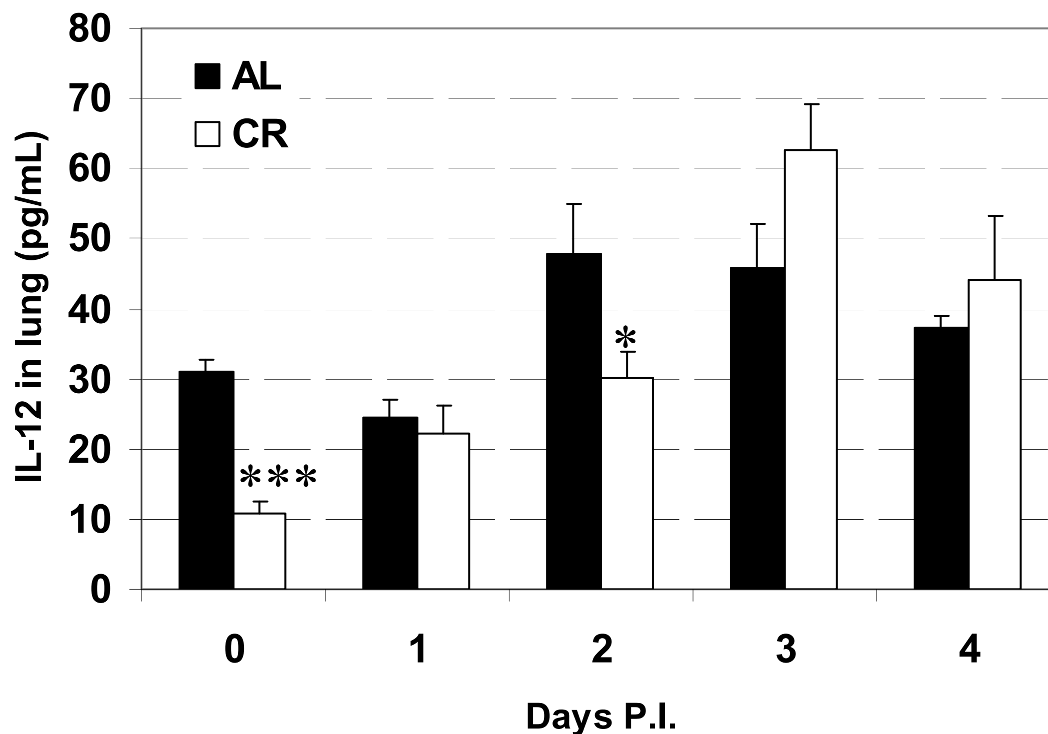


Figure 15. Altered IL-12 production in the lungs of CR mice during influenza infection. Young adult CR mice exhibited a decrease in the production of IL-12, as determined by ELISA, at baseline and at 2 days p.i., n=8 mice per group per day, *P<0.05 (*t*-test), ***P<0.001 (*t*-test). Repeated a total of 2 times.

Increased activation marker expression on NK1.1+ lymphocytes in the lungs and spleens of CR mice

NK cell activation is associated with the increased expression of surface markers, including CD25 and B220. The surface expression of CD25 (IL-2Rα) is associated with the proliferative response of activated NK cells (Son 2001); B220 (CD45R) is associated with NK cell cytotoxicity and is a marker of non-MHC restricted killing (Kassim 2006, Ballas 1990). Lymphocytes from the lungs of AL and CR mice were isolated at baseline through 4 days p.i., and the percentage, number, and mean fluorescence intensity (MFI) of NK1.1+ cells expressing activation markers were assessed by flow cytometry. MFI provides additional information regarding the number of surface antigens expressed per cell. The percentage of NK1.1+ lymphocytes expressing CD25 in lungs was increased in CR mice relative to AL controls on days 1 ($P<0.01$, *t*-test) and 4 p.i. ($P<0.001$, *t*-test) (**Figure 16**). The percentage of NK1.1+ lymphocytes expressing CD25 in spleens was increased in CR mice on days 3 and 4 p.i. ($P<0.05$, *t*-test). The percentage of NK1.1+ cells expressing B220 increased in CR mice in response to influenza infection ($P<0.001$) (**Figure 17**). The percentage of NK1.1+ cells expressing B220 was significantly increased in CR mice compared to AL controls at 3 days p.i. ($P<0.001$). Similarly, MFI increased in CR mice in response to influenza infection on days 2 ($P<0.001$) and 3 p.i. ($P<0.01$). The MFI of NK1.1+ cells expressing B220 was significantly increased in CR mice compared to AL controls on days 2 ($P<0.01$) and 3 p.i. ($P<0.05$). Despite the increased percentage of NK1.1 cells expressing activation markers in CR mice in response to influenza infection, there were no differences in the number of activated NK1.1+ cells in AL and CR mice at any time point.

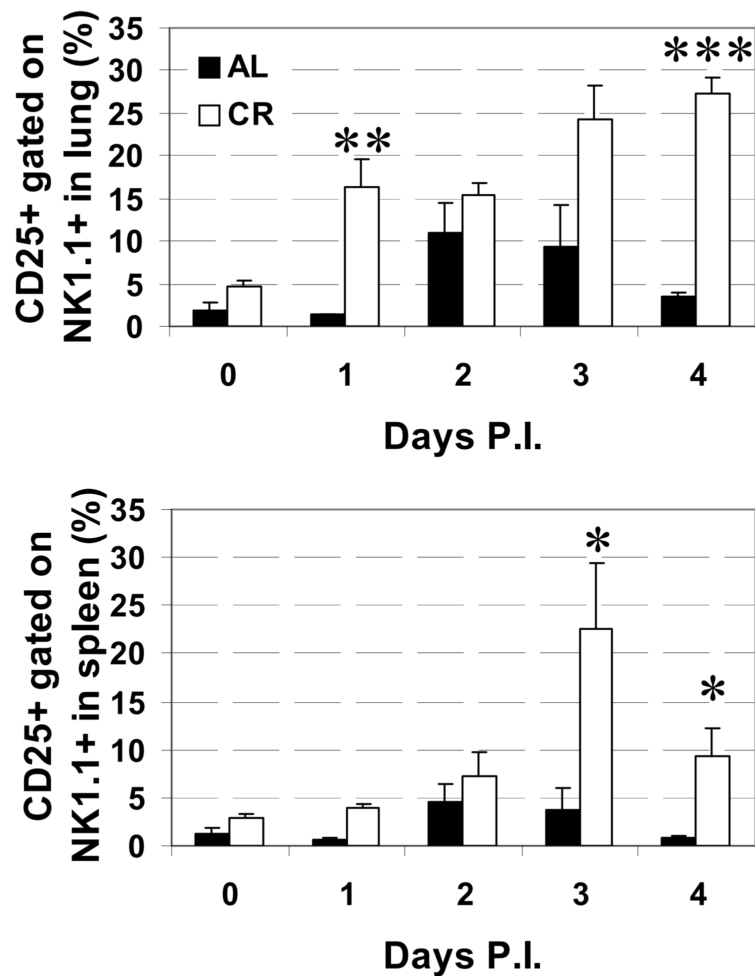


Figure 16. Increased expression of CD25 on NK1.1+CD8- lymphocytes in CR mice during influenza infection. Young adult CR mice demonstrated increased expression of CD25 on NK cells in the lung (top) and spleen (bottom) compared to AL controls in response to influenza infection, n=4 mice per group per day, *$P<0.05$, **$P<0.01$, ***$P<0.001$. Repeated a total of 2 times.

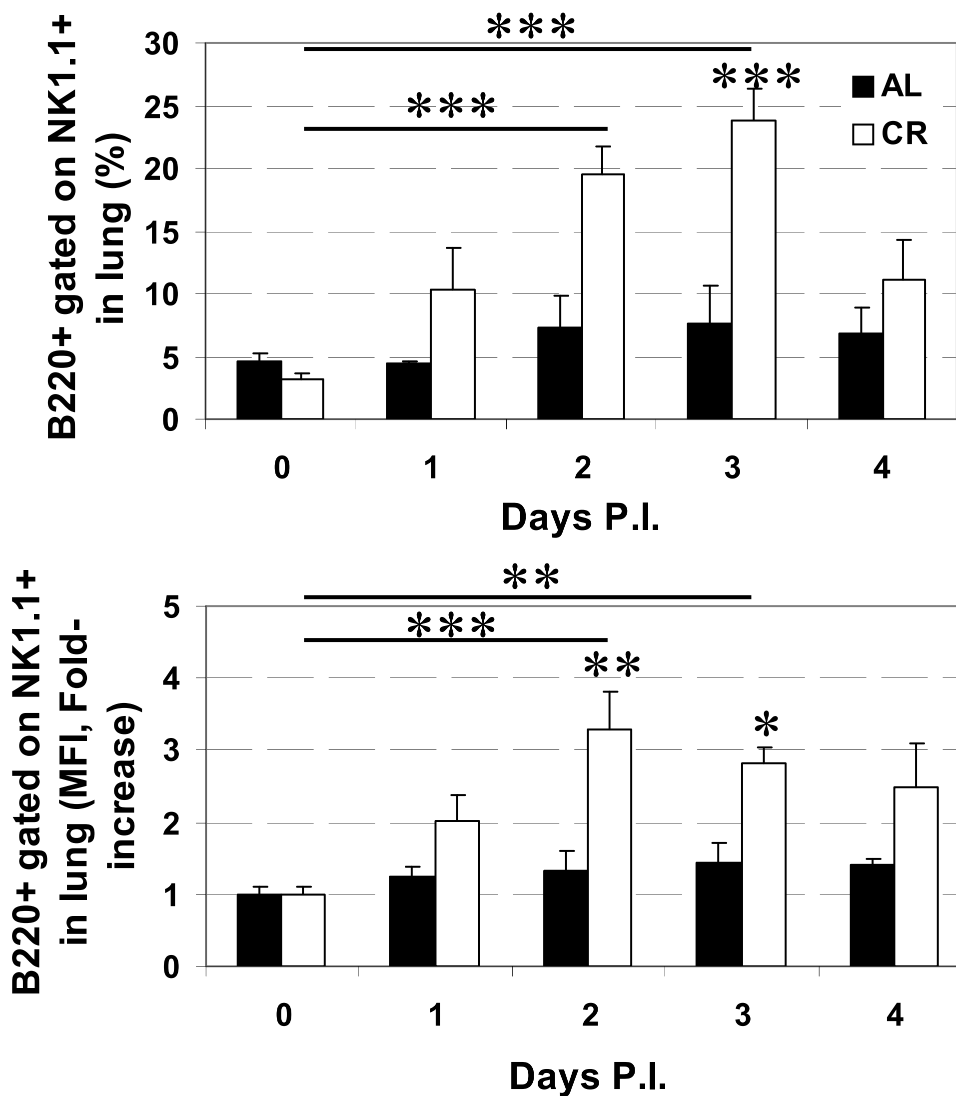


Figure 17. Increased expression of B220 on NK1.1+CD8- lymphocytes in the lungs of CR mice during influenza infection. Young adult CR mice demonstrated an increased expression of B220 on the surface of NK cells, as assessed by both percentage (top) and MFI (bottom), n=4 mice per group per day, *$P<0.05$, **$P<0.01$, ***$P<0.001$. Repeated a total of 2 times.

Increased percentage of NK1.1+ cells producing IFN-γ in the lungs of CR mice

Natural killer cells are the primary cell type responsible for producing IFN-γ during the innate immune response to infection, thus providing an important link to adaptive immunity (Biron 2001a). The percentage of NK1.1+ lymphocytes producing IFN-γ was assessed in the lungs and spleens of AL and CR mice by intracellular staining. We have consistently observed an approximate 10-fold increase in the percentage of NK1.1+ lymphocytes producing IFN-γ in the lungs of AL mice that corresponds with NK cell cytotoxicity in response to infection with this dose of influenza virus. The percent of NK1.1+ cells producing IFN-γ in the lungs of CR mice was significantly elevated compared to AL mice beginning at 2 days p.i. ($P<0.01$) and continuing through 4 days p.i. ($P<0.001$) (**Figure 18**). Further, CR mice exhibited a 20- to 25-fold increase in the percentage of NK cells producing IFN-γ over baseline at 3 and 4 days p.i. (**Figure 19**). In contrast, AL mice demonstrated a maximal 10-fold induction of IFN-γ production in the lungs of AL mice at day 1, consistent with the peak induction of NK cell cytotoxicity in these mice. Again, despite the increased percentage of IFN-γ-producing NK cells in the lungs of CR mice, there were no significant differences in cell number between the groups at any time point. Interferon-γ was then measured in lung homogenates from AL and CR mice. Interferon-γ was detected in the lungs of CR mice at all time points p.i., but was detected in only a limited number samples from AL mice on days 2 and 3 p.i. (**Table 4**). The percentage of NK1.1+ cells producing IFN-γ in spleens was always less than 3% and did not differ between AL and CR mice during infection.

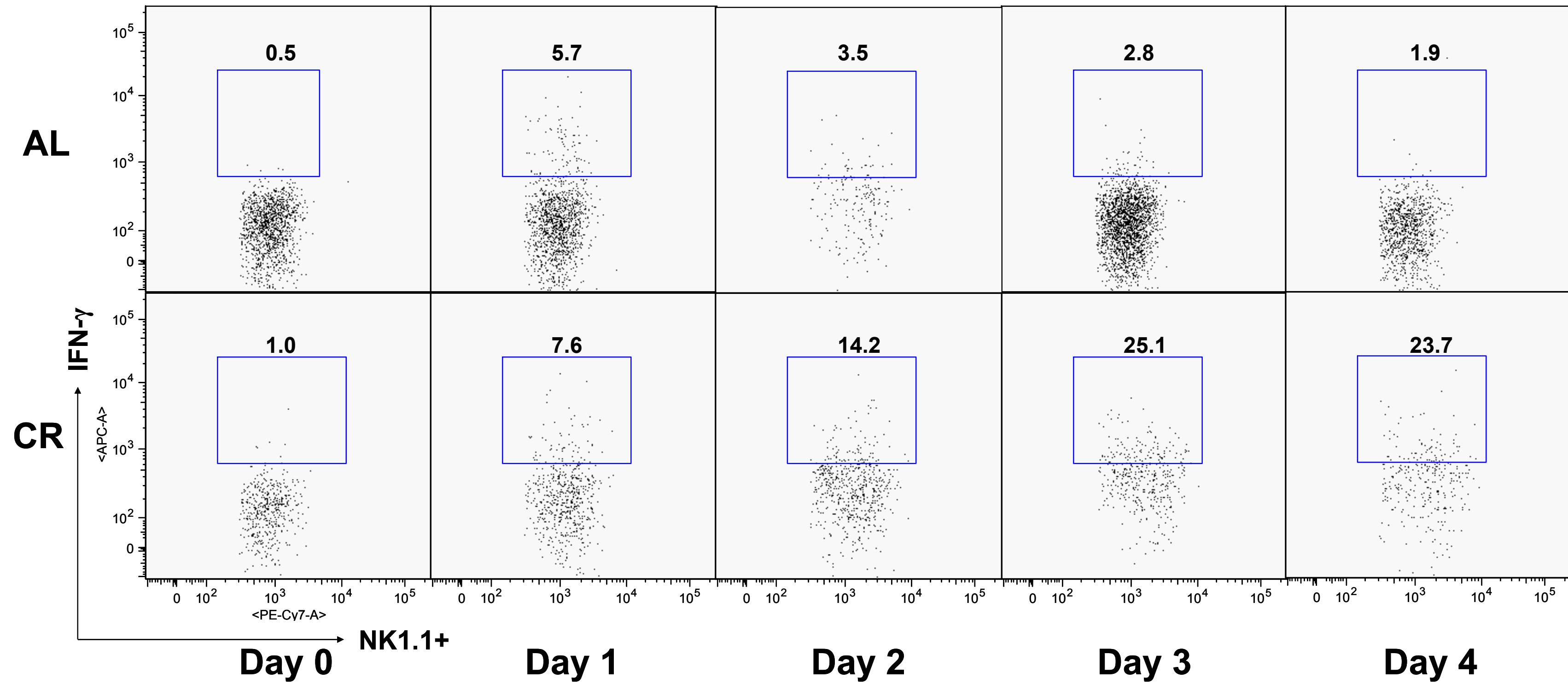


Figure 18. Representative flow cytometry data of the percentage of NK1.1+CD8- lymphocytes producing IFN-γ in the lungs of AL and CR mice during influenza infection. Values indicate means, n=4 mice per group per day. Repeated a total of 2 times.

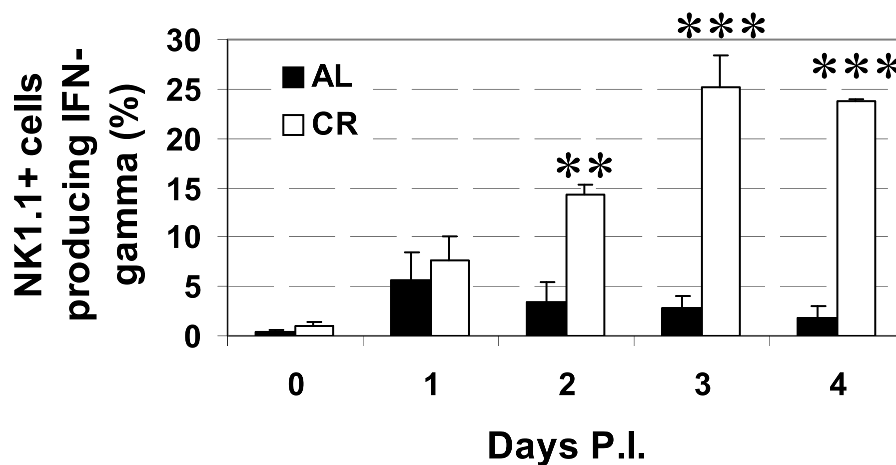


Figure 19. Increased percentage of IFN-γ-producing NK1.1+CD8- lymphocytes in the lungs of CR mice during influenza infection. Young adult CR mice exhibited a 20- to 25-fold increase in the percentage of NK cells producing IFN-γ in response to infection. The percentage of NK cells producing IFN-γ was increased in the lungs of CR mice compared to AL controls at 2 through 4 days p.i., n=4 mice per group per day, ***P*<0.01, ****P*<0.001. Repeated a total of 2 times.

Table 4. IFN-γ in the lung homogenates of AL and CR mice during influenza infection.

	IFN-γ (pg/mL)[1]	
Days p.i.	**YAL**	**YCR**
0	ND	ND
1	ND	75.3 ± 25.5
2	4.8[2]	59.2 ± 27.6
3	69.1[2]	212.6 ± 23.8
4	ND	100.8 ± 68.8

[1]Determined by ELISA

[2]Statistical analysis was not performed due to a limited number of mice with detectable IFN-γ in lung homogenates at these time points. Otherwise, n=4 per group per day. Repeated a total of 2 times.

Increased high side-scatter events in the lungs of CR mice

High side-scatter events reflect large or highly granulated cells, such as macrophages, dendritic cells, and neutrophils. Young adult CR mice exhibited an increase in the percentage of high side-scatter (SSC) events in the lung at baseline and during influenza infection compared to AL controls (day 2, $P<0.01$; days 0, 3, and 4, $P<0.001$) (**Table 5**). These data are in agreement with H&E staining that suggested an increase in cellular infiltration and lung pathology in CR mice. Taken together, these data suggest that young adult CR mice may exhibit an increase in the recruitment of inflammatory cells to the lungs during influenza infection.

Increased percentage of CD11b+ cells in the lungs of CR mice

The percentage of CD11b+ cells infiltrating the lungs of AL and CR mice were further assessed in response to infection. AL mice demonstrated a transient increase in CD11b+ cells in lung that followed a similar kinetics to the anorexia, weight loss, and recovery observed during influenza infection (**Figure 20**). In contrast, CR mice exhibited an increase in CD11b+ cells in lungs compared to AL mice at day 1 p.i. ($P<0.05$). Further, the percentage of CD11b+ in the lungs of CR mice remained elevated compared to baseline through 4 days p.i. ($P<0.05$), suggesting an early and prolonged inflammatory response to influenza infection.

Table 5. Percent and number of high side-scatter events, CD11b+, and CD11c+ cells in the lungs of AL and CR mice.

Days p.i.	Group	High SSC Events		CD11b+		CD11c+CD11b-	
		%	# (x10^4)	%	# (x10^4)	%	# (x10^4)
0	**AL**	30.0±2.3	20.2±6.7	12.4±1.1	2.5±0.9	12.1±2.4	2.2±0.7
	CR	***50.0±1.8	6.0±3.0	4.8±0.4	0.3±0.2	14.5±3.2	0.7±0.3
1	**AL**	42.3±2.7	20.2±3.2	17.5±2.6	3.6±1.0	8.9±0.9	1.8±0.3
	CR	46.2±0.5	22.6±2.7	15.5±4.6	3.9±1.5	5.8±1.5	1.2±0.2
2	**AL**	37.7±3.6	13.5±2.9	15.0±0.8	2.1±0.5	13.6±2.2	1.7±0.2
	CR	**50.8±2.6	11.6±3.2	19.0±3.5	2.2±0.7	5.1±0.4†	0.6±0.2
3	**AL**	27.6±2.1	11.8±2.3	18.1±2.5	2.3±0.8	10.5±0.5	1.2±0.2
	CR	***52.8±2.2	17.2±1.4	23.5±3.0†	4.2±0.8	3.1±0.2†	0.5±0.1
4	**AL**	29.3±1.3	5.1±1.3	20.9±5.0	1.2±0.6	14.8±2.2	0.7±0.2
	CR	***45.3±1.6	2.7±1.7	17.4±5.9	0.7±0.5	11.6±2.9	0.2±0.1

Values are means ± SEM, n=4 mice per group per day. Asterisks indicate differences between groups on that day, ***P*<0.01, ****P*<0.001. † indicates differences within the same group over time, *P*<0.05. Repeated a total of 2 times.

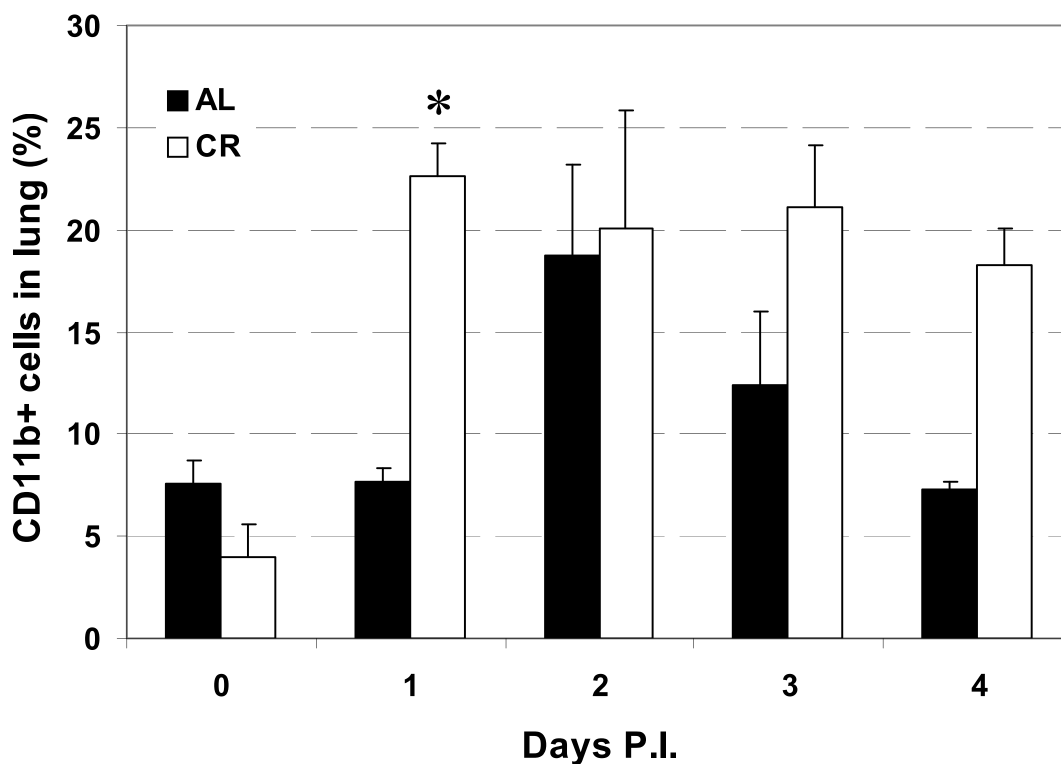


Figure 20. Increased percentage of CD11b+ cells in the lungs of CR mice during influenza infection. Young adult CR mice demonstrated an increase in CD11b+ cellular infiltration in lungs compared to AL controls at day 1 p.i. The percentage of CD11b+ cells in CR mice remained elevated over baseline through 4 days p.i. ($P<0.05$), n=4 mice per group per day, *$P<0.05$. Repeated a total of 2 times.

Increased systemic IL-6 production in CR mice

The production of cytokines IL-1β, IL-6, and TNF-α were examined by ELISA in both lung homogenates and plasma samples from baseline through 4 days p.i. Of these, only IL-6 in plasma could be detected (**Table 6**). Systemic IL-6 was detected in the plasma of AL mice on days 2 and 3 p.i., but was detected in CR mice at all days p.i., suggesting the possibility of prolonged systemic inflammation in young CR mice following influenza infection.

Increased percentage and number of CD11c+ cells expressing B220 in the lungs of CR mice

Plasmacytoid dendritic cells (pDCs), determined as CD11c+CD11b- cells expressing B220, are the major producers of IFN-α/β in response to virus infection, and thus, are important accessory cells in the activation of NK cell cytotoxicity (Garcia-Sastre 2006, Walzer 2005, McKenna 2005). The percentage and number of CD11c+CD11b- cells expressing B220 were increased in the lungs of CR mice compared to AL mice at 3 days p.i. ($P<0.05$) (**Table 7**). The expression of B220 on CD11c+ cells was also assessed as MFI and confirmed an increase over baseline ($P<0.001$), as well as an increase in the lungs of CR mice compared to AL controls ($P<0.01$) (**Figure 21**). Importantly, the kinetics of this increase in NK cell-activating pDCs followed both the inability to mount an effective NK cell cytotoxic response and the observed increase in lung virus burden in CR compared to AL mice. These kinetics were in agreement with the observed increase in NK cell activation and IFN-γ production in the lungs of CR mice late in the innate immune response to primary influenza infection.

Table 6. IL-6 in the plasma of AL and CR mice during influenza infection.

	IL-6 (pg/mL)[1]	
Days p.i.	**YAL**	**YCR**
0	ND	ND
1	ND	159.0 ± 45.4
2	175.1 ± 63.2	138.5 ± 58.8
3	61.6[2]	77.0 ± 31.0
4	ND	104.5 ± 52.3

[1]Determined by ELISA

[2]Statistical analysis was not performed due to a limited number of mice with detectable IL-6 in lung homogenates at this time point. Otherwise, n=4 per group per day. Repeated a total of 2 times.

Table 7. Percent and number CD11c+ cells expressing B220 in the lungs of AL and CR mice.

Days p.i.	Group	B220 on CD11c+ %	B220 on CD11c+ # ($x10^4$)
0	**AL**	5.4±0.7	1.0±0.3
	CR	6.5±0.5	0.3±0.1
1	**AL**	8.4±1.4	1.8±0.5
	CR	9.7±2.9	2.4±1.0
2	**AL**	9.0±3.7	1.5±0.9
	CR	18.4±1.4	2.2±0.7
3	**AL**	10.4±1.6	1.3±0.4
	CR	*25.2±2.0†	*4.4±0.7†
4	**AL**	16.0±3.7	0.7±0.2
	CR	17.2±4.9	0.6±0.5

Values are means ± SEM, n=4 mice per group per day. Asterisks indicate differences between groups on that day, *P<0.05. † indicates differences within the same group over time, P<0.05. Repeated a total of 2 times.

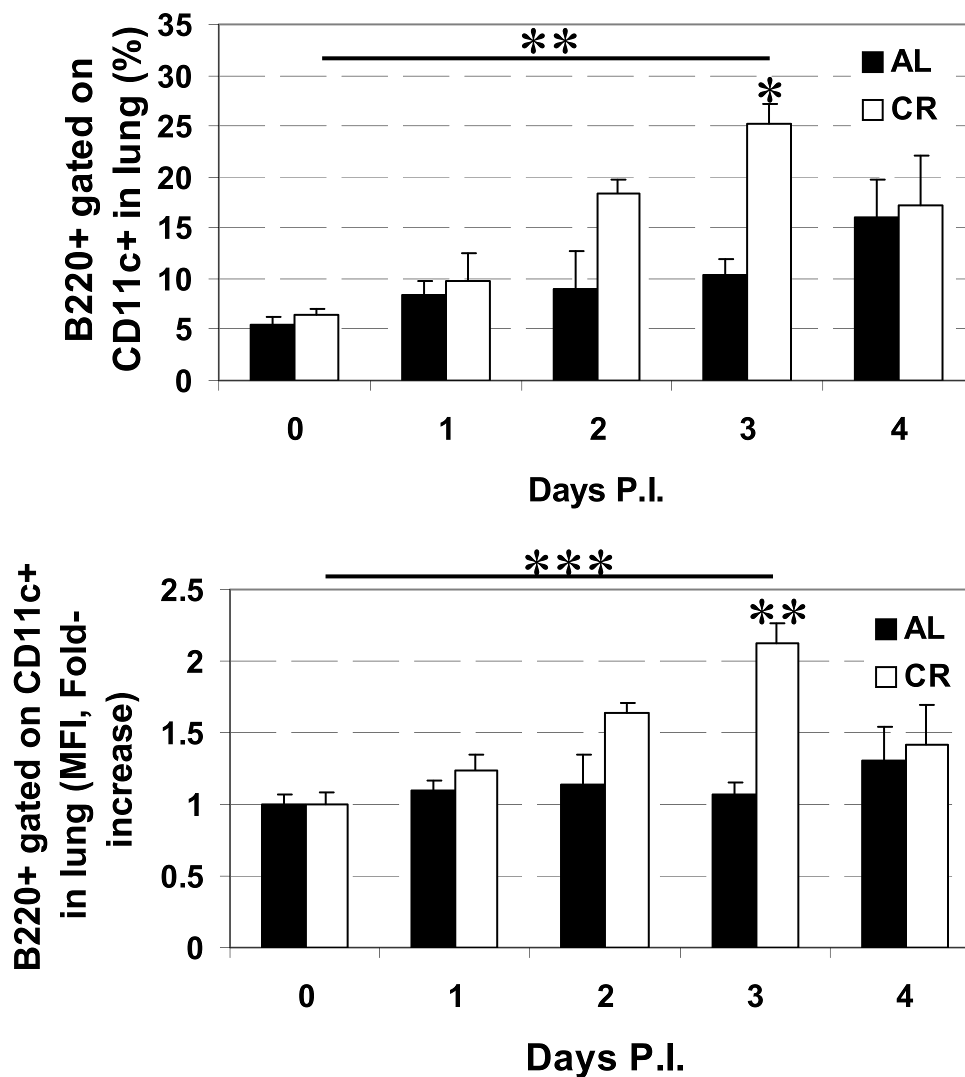


Figure 21. Increased percentage and MFI of CD11c+ cells expressing B220 in the lungs of CR mice during influenza infection. Young adult CR mice exhibited an increase in B220 expression on CD11c+ cells over baseline, assessed as both a percentage of high side-scatter cells (top) and a fold-increase in MFI (bottom), n=4 mice per group per day, **P*<0.05, ***P*<0.01, ****P*<0.001. Repeated a total of 2 times.

3.2.5 Discussion

The purpose of this study was to determine the effects of CR, independent of advanced age, on the susceptibility to primary influenza infection. Young adult CR mice exhibited an increase in mortality, weight loss, lung virus titers, and lung pathology in response to influenza infection that was associated with an inability to mount an effective NK cell-mediated innate immune response to influenza infection. These data clearly demonstrate an age-independent increase in the severity of influenza infection in young adult CR mice, as compared to young adult AL-fed controls.

The anorectic response to infection is believed to be mediated, at least in part, by the inflammatory cytokine and chemokine milieu (Van Reeth 2000, Swiergiel 1997). Young adult CR mice demonstrated increased weight loss in response to influenza infection, which was consistent with increased lung pathology and CD11b+ cellular infiltration in lung. The AL mice ceased eating immediately, lost more weight than CR mice through 2 days p.i., and then began to recover. Interestingly, CR mice maintained 100% of their baseline food intake at 1 day p.i. and 100% of their baseline body weight through 2 days p.i. This delay in anorexia and associated weight loss in CR mice might be explained by a recent report that CR mice maintain hunger signals throughout life, despite long-term energy balance (Speakman 2007). In these studies, re-feeding an AL diet to mice previously fed a chronic CR diet resulted in a transient hyperphagic response, followed by normal AL food intake and the re-establishment of energy balance at an increased body weight. This suggests that neuroendocrine signaling in response to long-term CR might allow CR mice to maintain food intake, despite infection, for a period of time.

In our previous study, aged CR mice appeared to exhibit a decrease in influenza-induced NK cell cytotoxicity in lung. Additional information on the effects of CR on NK cell cytotoxicity is limited, but an early study demonstrated a decrease in basal NK cell cytotoxicity and an increase in PolyI:C-stimulated NK cell cytotoxicity in splenocytes from aged CR mice compared to aged AL mice (Weindruch 1983). The effects of CR on NK cell cytotoxicity, however, have not been studied in young mice or in response to infection. Our data are the first to demonstrate a defect in influenza-induced NK cell cytotoxicity in young adult CR mice that is associated with an increased susceptibility to infection.

While findings in non-human primates have been contradictory to date, studies in aged CR rodents have shown a clear decrease in the total number of lymphocytes in spleens, but no change in total circulating peripheral blood mononuclear cells (Nikolich-Zugich 2005). Total lymphocytes in young adult CR mice have not been examined compared to young adult AL mice, nor have differences in the pulmonary lymphocyte compartment been considered in response to infection. We observed a consistent decrease in total lymphocytes in the lungs and spleens of young adult CR mice, both at baseline and in response to infection. There was no difference in the percentage of NK cells in the lungs of AL and CR mice at baseline; however, as a result of the decrease in total lymphocytes, the absolute number of NK cells in the lungs of CR mice was reduced compared to AL at baseline. Further, both the percentage and number of NK cells in the lungs of CR mice were decreased compared to AL mice during infection. A decrease in the percentage of NK cells in the mixed lymphocyte population can result in a decrease in NK cell cytotoxicity as determined by the ^{51}Cr-release assay. Therefore, it was important

to consider other aspects of NK cell activation and determine NK cell function on a per cell basis.

Natural killer cells induce cytotolysis by the production and release of perforin and granzymes (van Dommelen 2006). In response to primary influenza infection, NK cells from AL mice exhibited a 2-fold increase in both perforin and granzyme B. Importantly, CR mice demonstrated an increase in granzyme B, but no induction in the production of perforin. The essential role of perforin in the innate immune response to influenza has been demonstrated, as mice deficient in the perforin gene demonstrate no NK cell-mediated killing and increased mortality and lung virus titers in response to influenza infection (Liu 2003). Therefore, our data suggest that the loss of influenza-induced NK cytotoxicity in CR mice may be due to both a decrease in total NK cells in lung and an inability to induce the production of perforin in response to infection.

The endogenous production of IFN-α/β is critically important to host defense to viruses, as IFN-α/β activates NK cells, induces an antiviral state, inhibits virus replication, and increases MHC class I expression and antigen presentation in all cells (Garcia-Sastre 2006, Nguyen 2002, Samuel 1991). Importantly, the induction of perforin and NK cell-mediated cytolysis by Type I IFN has been demonstrated (Liang 2003). Therefore, IFN-α/β gene expression was assessed by RT-qPCR in the lungs of AL and CR mice during primary influenza infection. The expression of IFN-α/β in the lungs of young CR mice was lower than AL throughout the time examined (1-3 days p.i.) and was significantly decreased at 2 days p.i. These data have clear implications for the ability of CR mice to mount an antiviral response to influenza virus at the site of infection, the lung, as well as to induce perforin production and NK cell cytotoxicity. In contrast,

however, there was an increase in systemic Type I antiviral activity as assessed by bioassay in the serum of CR compared to AL mice, suggesting that the effects of CR on the antiviral response to influenza infection in young adult mice may differ by tissue. Further study is required to determine if virus might leave the lung in CR mice, resulting in systemic antiviral activity.

The cytokine IL-12 (NK cell stimulating factor) is produced by macrophages and dendritic cells in response to virus infections and mediates early NK cell responses (Trinchieri 2003, Biron 2001a, Biron 2001b, Julkenen 2001). Kinetic studies in mice infected with MCMV have shown that, in addition to IFN-α/β, the endogenous production of IL-12 acts to induce NK cell activity and contributes to peak IFN-γ production by NK cells, thus providing an important link to adaptive immunity (Nguyen 2002, Cousens 1997, Pien 2000, Orange 1996). The *in vitro* production of IL-12 by DCs from young (8 wk) CR mice was recently reported to be decreased compared to IL-12 production by DCs from AL mice (Niiya 2007). Our *in vivo* data suggest a defect in the availability of IL-12 in the lungs of CR mice early in the innate immune response to influenza infection, at the time when maximal NK cytotoxicity was induced in AL mice. However, later in the innate immune response, at days 3 and 4 p.i., there was a trend towards increased IL-12 production in the lungs of CR mice. This late increase in IL-12 production may represent a compensatory mechanism in which CR mice attempt to increase NK cell activity and the production of IFN-γ in an effort to stimulate adaptive immunity after innate immunity has failed to control the influenza virus.

Consistent with this hypothesis was an increase in NK cell activation, as determined by increased CD25 and B220 surface expression, and an increase in the

intracellular production of IFN-γ. The overall increase in these activities occurred late in the innate immune response to infection, however, after the observation of maximal NK cell cytotoxicity in young AL mice.

Given the differential effects of CR on NK cell activation and cytotoxicity, as well as observed alterations in the availability of NK cell-activating cytokines, it is important to consider the contribution of accessory and inflammatory cells to the innate immune response to influenza infection in AL and CR mice. The observed increase in cellular infiltration of CD11b+ cells in the lungs of young adult CR mice in response to influenza infection, along with the increase in pro-inflammatory cytokines, suggest that CR mice might mount a hyper-inflammatory response as a secondary effort to control influenza infection, which although indicative of an increased immune response in young adult CR mice, could also contribute to the observed increase in lung pathology and mortality.

The cellular infiltrate in the lungs of CR mice also included an increased percentage and number of CD11c+CD11b-B220+ pDCs, which are largely responsible for the production of NK cell-activating cytokines in response to multiple virus infections (Walzer 2005, Garcia-Sastre 2006, McKenna 2005). The kinetics of this increase in pDCs was in accord with the observed increase in NK cell activation, as assessed by CD25 and B220 surface expression on NK cells, as well as increased intracellular NK cell production of IFN-γ. However, these events may have been insufficient or occurred too late to alter the course of the infection.

3.3 Supplementation with active hexose correlated compound increases the innate immune response of young mice to primary influenza infection

(Published as Ritz 2006b)

Barry W. Ritz, Shoko Nogusa, Elizabeth A. Ackerman, and Elizabeth M. Gardner

Department of Bioscience and Biotechnology, 3141 Chestnut Street, Drexel University, Philadelphia, PA 19104

Abbreviations used: AHCC, Active Hexose Correlated Compound; CD, cluster of differentiation; HAU, hemagglutination units; H & E, hematoxylin-eosin Y; IFN, interferon; IHC, immunohistochemistry; IL, interleukin; i.n., intranasal; i.p., intraperitoneal; MDCK, Madin-Darby canine kidney; NK, natural killer; TCID, tissue culture infective dose; TNF, tumor necrosis factor.

This work was funded in part by a non-restrictive grant from Amino Up Chemical Co., Sapporo, Japan, manufacturer of the agent that was studies.

No supplementary data online. The authors report no conflicts of interest.

Running Title: Innate Immunity During Influenza Infection

3.3.1 Abstract

The emergence of H5N1 avian influenza and the threat of new or adapted viruses in bioterrorism have created an urgent interest in identifying agents to enhance the immune response to primary virus infection. Active hexose correlated compound (AHCC) is a natural mushroom extract reported to increase natural killer (NK) cell activity, survival, and bacterial clearance in young mice. However, the effects of AHCC on the response to viral infections have not been studied. In the current study, young C57BL/6 mice were supplemented with 1g AHCC/(kg body weight · d) for 1 wk prior to and throughout infection with influenza A (H1N1, PR8). Supplementation increased survival, decreased the severity of infection, and shortened recovery time following intranasal infection with FLU, as determined by the recovery of body weight and epithelial integrity in lung. AHCC increased NK activity in the lungs at d 1 ($P<0.05$) and d 4 ($P<0.01$) and in the spleen at d 2 post-infection ($P<0.01$). Supplementation increased the percentage ($P<0.05$) and number ($P<0.01$) of NK1.1+ cells in the lung and reduced the infiltration of lymphocytes and macrophages compared to controls ($P<0.01$). These data suggest that AHCC supplementation boosts NK activity, improves survival, and reduces the severity of influenza infection in young mice. Bolstering innate immunity with dietary bioactives may be one avenue for improving the immune response to primary flu infection.

3.3.2 Introduction

Influenza virus is a public health concern in the United States, causing disease among all age groups. Although children demonstrate the highest rates of infection, rates of serious illness and death are highest among persons aged $\geq$65 years, young children aged <2 years, and persons of any age with medical conditions putting them at increased risk for complications from influenza. Among children aged 0-4 years, hospitalization rates have ranged from approximately 1 in 1000 for children without high-risk medical conditions to 1 in 200 for those with high-risk medical conditions (ACIP 2005). Within the 0-4 year age group, hospitalization rates are highest among children aged 0-1 years and are comparable to rates reported among persons aged $\geq$65 years (ACIP 2005). Thus, in 2004-05 the Advisory Committee on Immunization Practices (ACIP) recommended that all children aged 6-23 months receive yearly influenza vaccinations (ACIP 2005).

The highest rates of infection with influenza virus occur in young children, in part, because of a lack of prior immunity from previous exposure to the virus. Immature immune systems rely heavily on innate defenses. Natural killer (NK) cells require neither prior exposure to virus nor antigen presentation in order to target and kill virus infected cells, and thus, provide one of the first lines of defense against many different viral infections, including influenza. NK cell-mediated killing controls viral replication until the virus is cleared by the adaptive immune response. However, in some cases a sufficient NK cell response may eliminate an infection completely (Goldsby 2003). The roles of NK cells in controlling influenza infection at the site of infection, i.e. the lung, and in activating adaptive, antigen-specific immunity in response to primary influenza infection have not been fully characterized (Biron 2001a, Solana 2000, Neff-La Ford

2003). We (Plett 2000, Gardner 2005) and others (Hunzeker 2004, Tamura 2004) have demonstrated an increase in NK activity in the lungs of young mice following influenza infection. Young mice subjected to restraint stress and infected with intranasal (i.n.) influenza virus demonstrated suppressed NK cell activation and function that was followed by enhanced viral replication (Hunzeker 2004). Depletion of pulmonary NK cells has been shown to increase the mortality of mice infected with influenza and delay the initiation of a virus-specific CD8+ T cell response (Neff-La Ford 2003). In our previous studies, a reduction in NK response to influenza infection in aged calorie-restricted mice was associated with an increase in viral titer in lung and early mortality at 4 days P.I., before the initiation of a CTL response could be generated (Gardner 2005). Although basal NK activity does not differ between young and aged mice, there is an age-associated decline in cytokine-inducible NK activity that is associated with a delay in viral clearance and a decreased and delayed adaptive response (Biron 2001a, Neff-La Ford 2003, Plett 2000). A decrease in inducible NK activity has also been observed in aging humans (Solana 2000). These data clearly indicate that NK cells are important in maintaining both the innate and adaptive immune responses and in controlling virus burden during primary influenza infection. Efforts to enhance the activation of NK cells involved in innate immunity, then, would also be expected to lead to the subsequent enhancement of adaptive immune responses. As a result, inducible NK activity is a potential therapeutic target of current interest (Tamura 2004).

In the current study, we examined the effect of a dietary supplement known as active hexose correlated compound (AHCC) on the influenza-induced NK cell response during primary influenza infection in young mice. This compound is an enzyme-fermented

extract of the mycelia of *Basidiomycetes* mushrooms and is marketed in the U.S. as a dietary supplement, or nutraceutical, containing a mixture of polysaccharides, amino acids, lipids, and minerals. The predominant components of AHCC are oligosaccharides, totaling approximately 74% of the mixture. Of these, nearly 20% are partially acetyated α-1,4-glucans with an average molecular weight of 5000. These oligosaccharides are believed to account for the biological activities of AHCC. Supplementation with AHCC has shown a generalized positive effect on the immune systems of both rodents (Aviles 2004, Matsushita 1998, Burikhanov 2000, Wang 2001, Gao 2005) and humans (Matsui 2002, Ghoneum 1995), as well as antioxidant effects (Ye 2003, Wang 2001), and is well-tolerated by both rodents and humans, with no reported adverse effects. Studies to date have suggested that AHCC may increase NK activity in humans (Ghoneum 1995) and rodents (Matsushita 1998, Matsui 2002) with malignancies. In response to infection, AHCC supplementation increased percent survival, mean time until death, and bacterial clearance (*Klebsiella pneumoniae*) in young mice stressed by 15-20% head-down tilt (Aviles 2004). However, no studies have previously examined the effect of AHCC supplementation on the immune response to influenza infection or viral clearance.

3.3.3 Materials and Methods

Animals. The protocol was approved by the Drexel University Institutional Animal Care and Use Committee. Specific pathogen-free young (6-8 wk old) male C57BL/6 mice were obtained from Jackson Laboratories. Mice were housed in microisolator cages in a barrier room of the AAALAC-accredited animal facility at Drexel University and

acclimated for at least 1 wk before the initiation of experiments. Mice were monitored and weighed daily.

Supplementation with AHCC. AHCC (Amino Up Chemical Company, Sapporo, Japan) was administered orally by pipette at a concentration 1g AHCC/(kg of body weight · d) in 20μL distilled water. Control mice received 20μL distilled water per d. Mice were supplemented for 7 d prior to and throughout the course of infection with influenza. This dose of AHCC has been used previously and does not produce toxic effects in young mice (Aviles 2004).

Virus. Influenza A/Puerto Rico/8/34 (PR8, H1N1; a kind gift from Dr. Walter Gerhardt, University of Pennsylvania) was propagated in specific pathogen-free eggs (B & E Eggs), and cell-free supernatants were stored at -70°C for subsequent use. At baseline (d 0), mice were anesthetized by intraperitoneal (i.p.) injection with Avertin (2,2,2-tribromoethanol, Sigma) and were infected intranasally (i.n.) with 100 hemagglutination units (HAU) of PR8.

Isolation of mononuclear cells from spleens and lungs. The procedure for the isolation of mononuclear cells from spleens and lungs has been described in detail previously (Po 2002). Briefly, mice were euthanized by CO_2 asphyxiation, and spleens and lungs were aseptically removed. Spleens were homogenized by dounce and resuspended in RPMI-1640 (Mediatech). Lungs were minced with dissecting scissors and incubated at 37°C for 1.5 h in a cocktail containing 3mg/ml Collagenase A and 80 Kuntz units of DNAse I/ml (Roche) with 5% fetal bovine serum [(FBS) Mediatech] in Iscove's Modified Dulbecco's Medium [(IMDM) Mediatech]. The digested lung samples were passed through a 40μm nylon mesh (Fisher) and centrifuged. Lung homogenates were centrifuged, and

supernatants were aliquoted and stored at -70°C for subsequent analysis of virus titers. The pellets were resuspended and washed twice with 5% FBS in IMDM. The cell suspensions from spleens and lungs were layered on Histopaque-1083 (Sigma) and subjected to density gradient centrifugation. Cells from each tissue were resuspended to the appropriate concentration for use in subsequent assays.

Lung virus titers. Supernatants from lung homogenates were serially diluted and used to infect Madin-Darby canine kidney (MDCK) cells. After incubation at 37°C for 24 h, 0.02% TPCK-treated trypsin (Sigma) was added, followed by an additional 48 h incubation. Chicken red blood cells (B & E Eggs) were resuspended at 0.05% in PBS and added to the cultures. Virus titers were then determined based on the presence or absence of hemagglutination, as previously described (Po 2002), and reported as the 50% tissue culture infectious dose ($TCID_{50}$).

NK cell activity in lungs. A standard 4 h ^{51}Cr-release assay with YAC-1 target cells was utilized to assess NK activity as previously described (Plett 2000). Briefly, $1x10^6$ YAC-1 cells were incubated with 200 µCi $Na^{51}CrO_4$ (PerkinElmer) for 2 h at 37°C. During this incubation, cells were mixed every 20 min to ensure maximal uptake of $Na^{51}CrO_4$. The cells were then washed twice with RPMI-1640, resuspended in RPMI-1640 complete medium containing 10% FBS and rotated for 1 h at room temperature. After the final wash, YAC-1 cells were resuspended at $1x10^5$ cells/ml in complete medium and plated in round bottom 96-well microtiter plates (VWR). Cell preparations were then added to wells at an effector to target (E:T) ratio of 50:1. All samples were assayed in triplicate. Target cells were incubated in medium alone to assess spontaneous release or with 5% Triton X-100 (Sigma) to quantitate maximum release. After a 4 h

incubation at 37°C, supernatants were harvested onto UniFilter microplates (PerkinElmer), and radioactivity in supernatants was quantitated using a γ-counter (Packard TopCount). Percent cytotoxicity was calculated as follows: % Cytotoxicity = (Experimental CPM – Spontaneous CPM) / (Maximum CPM- Spontaneous CPM) x 100. Spontaneous release was always < 5% of maximal release.

Immunophenotyping. Following multiple washes, $5x10^5$ mononuclear cells from spleen or lung were resuspended in PBS containing fluorochrome-conjugated antibodies (eBioscience) to CD4 (Pe-Cy7), CD8 (APC), NK1.1 (PE), and CD11b (Fitc) and incubated on ice in the dark for 30 min. Cells were then washed three times in HBSS (Mediatech) containing 1% FBS, resuspended in PBS containing 1% paraformaldehyde (Sigma), and stored at 4°C until analysis. Samples were acquired on a FACSCanto flow cytometer (Becton Dickinson) and analyzed using FACSDiva software.

Tissue staining. Formalin-fixed lung tissue was paraffin embedded, sliced, and mounted onto glass slides. Slides were baked at 65°C for 30 min and deparrafinized by xylene wash. Rehydration of tissue was carried out through a graded alcohol series (100%, 95%, and 80%). Slides were then washed and either stained for histology using the hematoxylin-eosin (H & E) method or macrophages were stained by immunohistochemistry (IHC) using a Vector kit (Vector Labs), following the manufacturer's instructions. H & E slides were stained with hematoxylin (Harleco) for 8 min. Slides were rinsed in tap water, dipped in acid alcohol, and rinsed again. Slides were then dipped in ammonia water and rinsed in tap water for 4 min. Following multiple dips in 95% alcohol, slides were counterstained in eosin Y (1% alcoholic, Harleco). For IHC staining, antigen retrieval was achieved using trypsin digestion at

37°C for 30 min. Endogenous peroxidase activity was quenched by addition of 3% hydrogen peroxide for 10 min and washed in 1X PBS. Non-specific binding was blocked using normal rabbit serum (Vector) for 30 min. Macrophages were then stained using F4/80 rat anti-mouse primary antibody (eBioscience) for 30 min and washed in 1X PBS. An isotype control slide was also stained with mouse IgG2a (DakoCytomation). A secondary rabbit anti-rat biotinylated antibody was diluted in 1X PBS (containing 1.5% blocking serum) and then added to slides (Vector), followed by a wash in 1X PBS. Next, avidin-biotin complex [(ABC) Vector] reagent was added to slides and incubated for 30 min. Slides were then washed in 1X PBS, incubated with diaminobenzidine [(DAB) Pierce] solution diluted 1:10, and washed in tap water. Slides were counterstained in hematoxylin (Harleco) for 10 min and rinsed in running tap water for 5 min. All slides were then dehydrated in a graded alcohol series, washed in xylene, and mounted with coverslips.

Statistics. All statistics were performed using GraphPad InStat 3 software. Survival data were analyzed by Kaplan-Meier test, whereas comparisons between and within groups were analyzed using 1-way ANOVA with Tukey-Kramer multiple comparisons. Mann-Whitney *U*-tests were used when data were not normally distributed. Statistical significance was accepted at $P<0.05$.

3.3.4 Results

Active Hexose-Correlated Compound (AHCC) increased survival and maintained weight of young mice after primary influenza infection. Mice supplemented with AHCC exhibited 95% survival compared to 75% survival in control mice through 10 d post-infection (**Figure 1**). Importantly, there were no differences in the mean body weights of control or AHCC supplemented mice at d -7 (25.6g and 25.0g, respectively) or at d 0 (26.0g each). However, AHCC-supplemented mice lost significantly less weight ($P<0.001$) and also recovered weight more quickly than control mice following infection (**Figure 2**). AHCC-supplemented mice exhibited a maximal weight loss of 1.8g (7% of initial body weight) at d 4, while control mice exhibited a maximal loss of 5.9g (23%) at 6-7 d post-infection.

Lung virus titers were reduced in mice supplemented with AHCC. Virus was undetectable at baseline, and values post-infection were adjusted for background. Viral load in the lungs of control mice was significantly higher in AHCC-supplemented mice at 5, 7, and 10 d post-infection (**Table 1**, $P<0.05$).

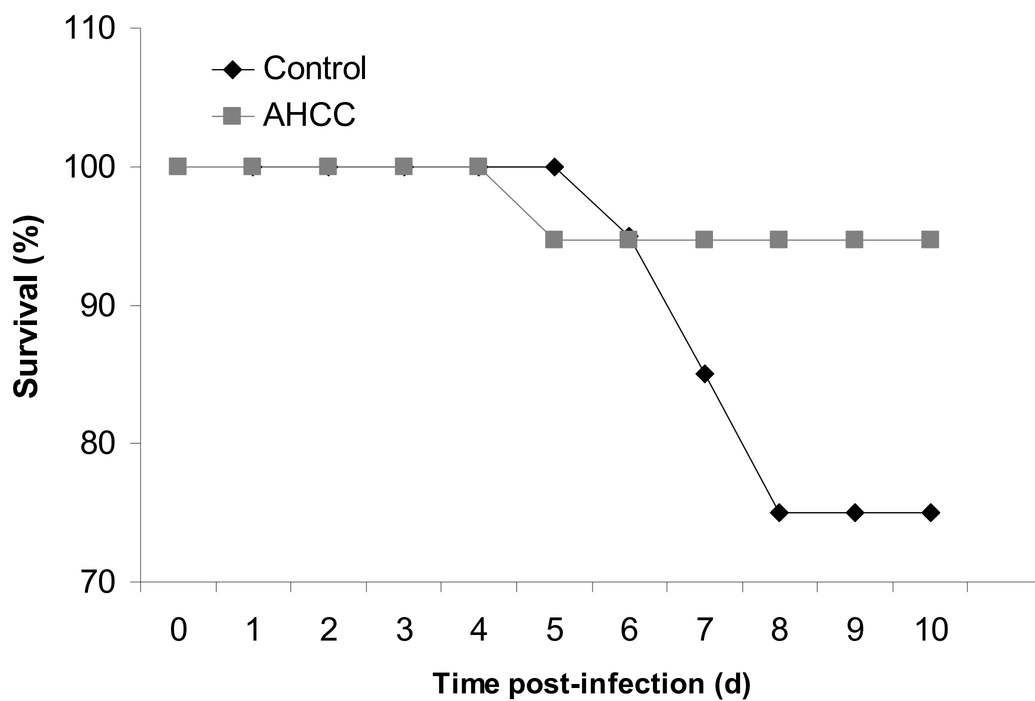


Figure 1. Percent survival of control and AHCC-supplemented young mice (6-8 wk) from infection with 100 HAU influenza A/PR8 through 10 d post-infection, n = 20 mice per group.

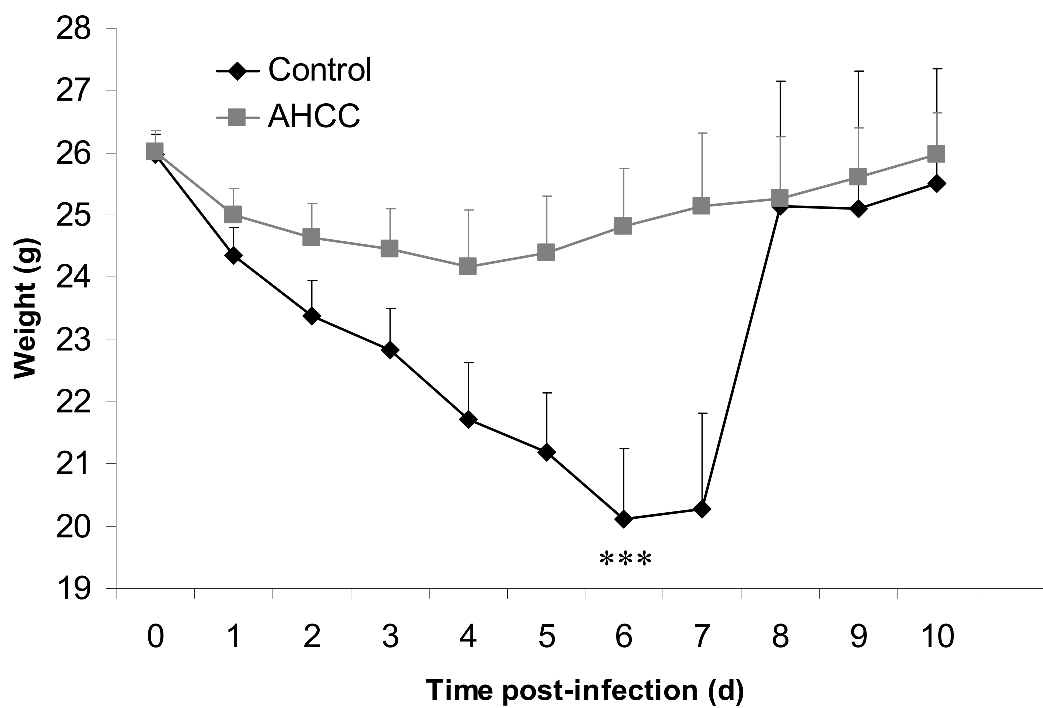


Figure 2. Weight loss in g in control and AHCC-supplemented young mice following infection with 100 HAU influenza A/PR8 through 10 d post-infection. Values are means ± SEM, n = 20 mice per group. ***Different from AHCC, *P*<0.001. Repeated a total of 2 times.

Table 1. Lung virus titers in control and AHCC-treated mice following influenza infection[1]

	d 3	d 5	d 7	d 10
		$TCID_{50}$, log_{10}		
Control	1.83 ± 0.3	4.25 ± 0.5***	4.50 ± 0***	2.50 ± 0.6*
AHCC	2.50 ± 0	1.50 ± 0.6	0.17 ± 0.3	0.5 ± 0

[1]Values are means ± SEM, n=3 mice per group per d. Asterisks indicate different from AHCC at that time: *$P<0.05$, ***$P<0.001$. Repeated a total of 2 times.

Supplementation with AHCC improved lung epithelial integrity following influenza infection. At d 10, lungs from control mice exhibited erosion of the epithelium, while recovery was apparent in lungs from AHCC-supplemented mice (**Figure 3**). Lung tissue from both control and supplemented mice showed cellular infiltration at d 10.

Mice supplemented with AHCC demonstrated enhanced NK cell activity in the lung and spleen and increased NK cell percentage and number in lungs during influenza infection. The kinetics of NK activity in response to influenza infection was altered in the lungs of AHCC-supplemented mice compared to controls (**Table 2**). While both groups demonstrated a peak in NK activity at d 3 post-infection ($P<0.001$), supplemented mice exhibited a significant increase in NK cytotoxicity at d 1 ($P<0.05$) and maintained a significantly elevated activity at d 4 compared to controls ($P<0.01$). Similarly, NK activity was increased in the spleens of supplemented mice at d 2 (**Table 3**, $P<0.01$). Alterations in NK activity were associated with an increase in both the percentage ($P<0.05$) and absolute number ($P<0.01$) of NK1.1+ lymphocytes at d 2 in the lungs of AHCC-supplemented mice compared to controls (**Table 4**). No changes in the percent or number of NK cells were apparent in the spleens of mice from either group throughout the course of infection (data not shown).

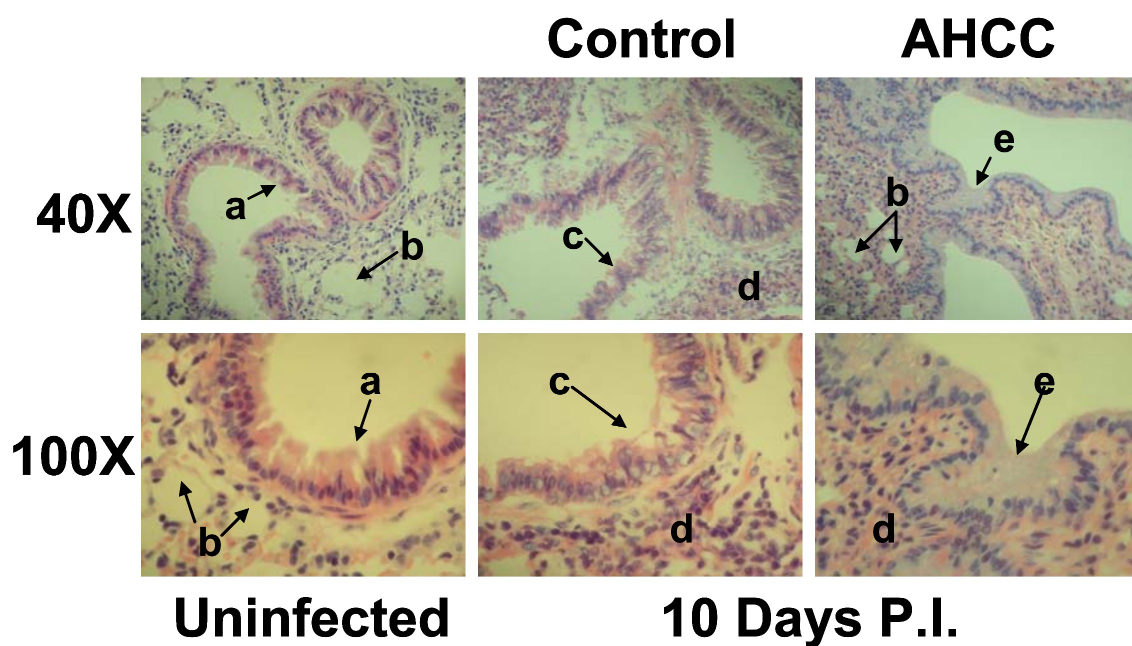


Figure 3. Representative H & E staining of lung tissue from control and AHCC-supplemented young mice at baseline and 10 d post-infection with 100 HAU influenza A/PR8. Healthy columnar epithelial cells (a) and clear alveoli (b) are illustrated in uninfected tissue in contrast to eroded epithelium (c) and cellular infiltration (d) in control mice at d 10. Lung tissue from AHCC-supplemented mice demonstrated cellular infiltration and a recovery of epithelial integrity (e) at d 10.

Table 2. NK cell activity in control and AHCC-treated mice lungs following influenza infection[1]

	d 0	**d 1**	**d 2**	**d 3**	**d 4**
			% cytotoxicity		
Control	3.1 ± 1.1[ab]	1.8 ± 0.3[a]	10.1 ± 1.3[b]	20.2 ± 0.7[c]	4.8 ± 2.5[ab]
AHCC	2.4 ± 1.4[a]	6.9 ± 0.1[a]**	12.1 ± 0.9[a]	21.2 ± 1.0[b]	8.8 ± 1.0[a]**

[1]Values are means ± SEM, n=3 mice per group per d. Means for a group without a common letter differ, *P*<0.05. Asterisks indicate different from control at that time: ***P*<0.01. Repeated a total of 2 times.

Table 3. NK cell activity in control and AHCC-treated mice spleens following influenza infection[1]

	d 0	d 1	d 2	d 3
		% cytotoxicity		
Control	5.6 ± 1.0	14.2 ± 3.2	10.4 ± 1.6	6.3 ± 1.2
AHCC	5.3 ± 0.7[a]	13.6 ± 2.5[ab]	20.4 ± 3.6[b]**	6.7 ± 0.5[a]

[1]Values are means ± SEM, n=3 mice per group per d. Means for a group without a common letter differ, $P<0.05$. Asterisks indicate different from control at that time: **$P<0.01$. Repeated a total of 2 times.

Table 4. Percent and number of NK cells in control and AHCC-treated mice lungs following influenza infection[1]

	d 0	d 1	d 2	d 3
		%		
Control	13.6 ± 0.9[1]	17.5 ± 1.4	18.2 ± 1.7	13.8 ± 0.8
AHCC	11.2 ± 5.1	18.0 ± 2.8	29.2 ± 5.6*	18.9 ± 0.7
		n x 10^{-5}		
Control	2.7 ± 0.6[2]	3.9 ± 0.5	3.6 ± 0.3	4.5 ± 0.7
AHCC	2.2 ± 0.4	3.7 ± 1.6	6.5 ± 0.03**	3.3 ± 0.6

[1]Values are means ± SEM, n=3 mice per group per d. Asterisks indicate different from control at that time: *P<0.05, **P<0.01. Repeated a total of 2 times.

Mice supplemented with AHCC exhibited reduced lymphocyte and macrophage infiltration in lung during influenza infection. The number of total lymphocytes (*P*<0.01), as well as the number of CD4+ and CD8+ T cells (*P*<0.05), was significantly elevated in the lungs of control mice at d 7 post-infection (**Table 5**). Control mice also demonstrated a significant increase in the number of macrophages infiltrating the lung at d 7, determined as CD11b+ cells (**Table 6**, *P*<0.05). Macrophage infiltration in lung was confirmed by IHC staining against F4/80 primary antibody (**Figure 4**). The peak in lymphocyte and macrophage infiltration at 7 d post-infection corresponded with the height in the severity of infection in control mice, as indicated by maximal weight loss.

Table 5. Number of lymphocytes and T cells in control and AHCC-treated mice lungs following influenza infection[1]

	d 0	d 3	d 5	d 7	d 10
			n x 10^{-5}		
Total lymphocytes					
Control	12.7 ± 1.0[a]	10.9 ± 2.6[a]	137 ± 44[a]	274 ± 43[b]	100 ± 35[a]
AHCC	8.5 ± 0.8	8.3 ± 1.2	123 ± 30	123 ± 67	62 ± 11
CD4+					
Control	1.5 ± 0.2[a]	1.9 ± 0.4[a]	19.1 ± 8.7[a]	49.2 ± 8.8[b]	21.2 ± 8.4[a]
AHCC	0.9 ± 0.1	1.5 ± 0.3	15.9 ± 4.1	27.1 ± 12	11.6 ± 3.3
CD8+					
Control	0.8 ± 0.1[a]	0.8 ± 0.2[a]	23.7 ± 11[a]	66.9 ± 20[b]	19.9 ± 13[a]
AHCC	0.6 ± 0.1	0.8 ± 0.3	13.7 ± 4.1	20.9 ± 13	7.3 ± 2.8

[1]Values are means ± SEM, n=3 mice per group per d. Means for a group with superscripts without a common letter differ, $P<0.05$. Repeated a total of 2 times.

Table 6. Number of CD11b+ macrophages in control and AHCC-treated mice lungs following influenza infection[1]

	d 0	d 3	d 5	d 7	d 10
			n x 10^{-5}		
Control	0.5 ± 0.02[a]	1.5 ± 0.8[a]	43.5 ± 20.3[a]	166.2 ± 17.8[b]**	4.9 ± 2.8[a]
AHCC	0.4 ± 0.01	1.7 ± 1.2	63.7 ± 29.6	50.9 ± 45.6	2.0 ± 0.4

[1]Values are means ± SEM, n=3 mice per group per d. Means for a group with superscripts without a common letter differ, $P<0.05$. Asterisks indicate different from AHCC at that time: **$P<0.01$. Repeated a total of 2 times.

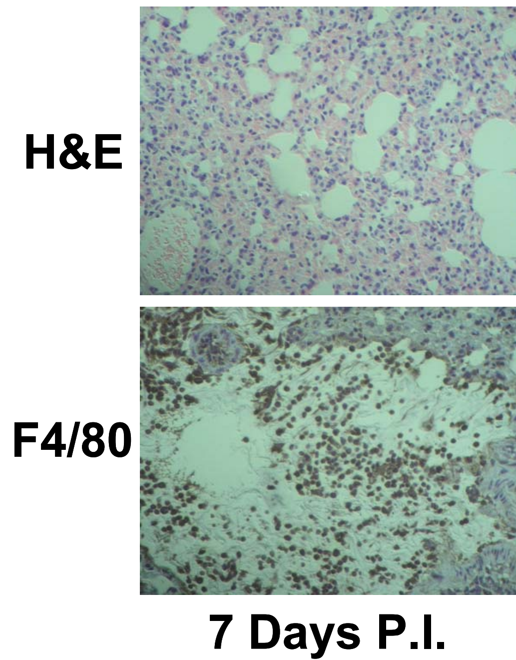


Figure 4. Representative IHC staining of macrophages in lung tissue from young control mice at baseline and 7 d post-infection with 100 HAU influenza A/PR8. Cellular infiltration identified by H & E staining (top) stained positive for the macrophage marker F4/80 (bottom).

3.3.5 Discussion

Young mice supplemented with AHCC daily before and during the course of influenza infection exhibited increased survival, enhanced NK activity in lung and spleen, and rapid virus clearance from the lung relative to young mice given a vehicle control. The importance of NK cells in controlling primary influenza infection in the lung prior to the initiation of a virus-specific immune response has been suggested (Biron 2001a, Solana 2000, Neff-La Ford 2003, Bender 1995). Upon activation, NK cells respond rapidly, peaking within the first few days of infection. By targeting and eliminating virus-infected cells, NK cell-mediated cytolysis acts to eliminate the source of the replicating virus (Goldsby 2003). Activated NK cells also produce IFN-γ, which further increases the cytotoxicity of NK cells and activates antigen-specific CD8+ cytotoxic T lymphocytes (CTLs) (Nguyen 2002, Bender 1991, Moskophidis 1998). Our data indicated that influenza-induced NK activity in lung was enhanced by supplementation with AHCC, which was associated with the initiation of viral clearance and a significant decrease in lung virus titer compared to controls as early as 5 d post-infection. Although we did not evaluate the production of antigen-specific CTLs, our previous kinetic studies demonstrated a peak in CTL activity and viral load at 7 d post-infection that was not followed by viral clearance until d 10 (Po 2002). The adaptive immune response requires time to evoke antigen presentation, such as by macrophages and dendritic cells, and T cell proliferation, including an increase in both CD4+ and CD8+ T lymphocytes. In the current study, lymphocyte and macrophage infiltration in lung following infection peaked at d 7 in control mice, as expected, but was less pronounced in AHCC-supplemented mice. These observations suggest the possibility that the more robust NK response was

associated with a decreased reliance on the adaptive immune response for viral clearance. Future studies will aim to evaluate the effect of AHCC supplementation on adaptive immunity. Taken together, however, the current data suggest that an enhanced NK cell response in AHCC-supplemented mice may have contributed to a decreased susceptibility to influenza infection.

Although the exact mechanism by which AHCC boosts NK activity remains under investigation, we speculate that α-1,4-glucans are recognized by C-type lectins, such as Dectin-1 on NK cells, thus initiating innate immunity. C-type lectins are also expressed on other cell types, including macrophages, dendritic cells, and γδ-T cells, that may further influence NK cells and the innate immune response through the production of cytokines. IFN-α/β, for example, is a cytokine produced during infection that induces an antiviral state in uninfected cells, thus limiting virus replication (Nguyen 2002). Previous kinetic studies of cytokine production in bronchoalveolar fluid and lung homogenates after influenza infection in mice have shown early production of IFN-α/β before the initiation of an influenza-specific adaptive immune response in lung (Van Reeth 2000). Along with Type I IFN, IL-12 and IL-18 are also produced early in the innate immune response and act synergistically to activate NK cells to produce IFN-γ (Nguyen 2002, Julkunen 2001, Brydon 2005, Liu 2004). Previous reports suggest that AHCC influences the production of a variety of cytokines (Aviles 2004), including enhanced IL-12 (NK stimulatory factor) production by macrophages (Yagita 2002) and IFN-γ production by antigen-specific CD8+ T cells (Gao 2005). Therefore, it is possible that the enhanced NK cytotoxicity in AHCC-supplemented mice was due to alterations in endogenous cytokine production, improving the ability of NK cells to become activated during primary

infection. In addition to producing IL-12, macrophages are also potent producers of inflammatory cytokines in response to influenza infection, including TNF-α, IL-1β, and IL-6. While these cytokines play an essential role in viral clearance, they are also associated with inflammation, tissue damage, and symptoms of disease (Tamura 2004, Van Reeth 2000, Julkunen 2001, Brydon 2005). In the current study, AHCC-supplemented mice exhibited both a reduction in macrophage infiltration and improved epithelial integrity in lung following infection compared to controls. As such, further studies are required to address the potential influence of AHCC on cytokines involved in both NK activation and the inflammatory response to influenza infection.

Finally, both the young and the elderly are at an increased risk for morbidity and mortality associated with influenza infection. AHCC has previously been reported to increase the number of NK cells in aged mice (Ghoneum 1992), and future studies should determine if AHCC supplementation may abrogate the age-associated decline in inducible NK activity. Additionally, mice and humans demonstrate multiple age-associated impairments in immunity (Murasko 2003, Murasko 2005), including a reduced CTL response to influenza infection (Po 2002, Bender 1991) and a loss of antibody production in response to influenza vaccination (Gardner 2001). Given the ability of AHCC supplementation to enhance influenza-induced NK activity in young mice and the clear connection between the NK cell-mediated innate immune response to influenza infection and the activation of adaptive immunity, future investigations should consider the possibility that AHCC may mitigate certain aspects of immunosenescence in response to influenza. In summary, if our data can be extended to the human circumstance, we suggest that supplementation with AHCC, a natural bioactive dietary supplement, may

provide a feasible approach to improving the immune response to viral infections, such as influenza.

Acknowledgments

Jessica Battisto, B.S., provided abundant assistance with flow cytometry, and Veronica Holmes, B.S., provided instruction on tissue staining.

CHAPTER 4: DISCUSSION

4.1 ***Introduction.*** Seasonal influenza and related secondary pneumonias remain a leading cause of infectious disease morbidity and mortality among the elderly, despite vaccination. While the age-associated decline in the immune response to influenza vaccination has been well studied, much less is known about the primary immune response to influenza infection, and in particular, the role of innate immunity in this process. The study of primary influenza infection is especially relevant in this era of threats from highly-virulent H5N1 avian influenza and bioterrorism, and these circumstances further emphasize the need for a comprehensive understanding of the early, innate immune response to influenza infection in both the young and the old. It is also unknown whether vaccines can be produced quickly enough to respond to such threats or if traditional antiviral therapies will be effective. Therefore, additional parameters that may influence immunity, such as nutritional status, must be considered in the context of the innate immune response to primary influenza infection. In this thesis, a series of studies was conducted to investigate the influence of age and nutrition on the innate immune response to primary influenza infection in a mouse model. Notable outcomes that will now be discussed in detail confirm that NK cells play an important role in controlling primary influenza infection in both young and aged mice and that NK cells respond, both positively and negatively, to nutritional interventions. Future research strategies are discussed, including a detailed plan for characterizing the relationship between immunity and metabolism during primary influenza infection.

4.2 *Natural killer cells control influenza infection in young and aged mice.* Previous studies have indicated that recovery from influenza infection is dependent on cytotoxic T lymphocyte (CTL) activity and that aging is associated with impaired CD8+ T cell function and prolonged virus shedding (Po 2002). Additional observations, including a decrease in cytokine-inducible NK cell activity in both aged mice (Plett 2000) and humans (Kutza 1996), suggested that an age-associated defect in the NK cell response to influenza virus might contribute to the increased susceptibility to infection. While the importance of NK cells in limiting virus replication before the initiation of a virus-specific adaptive immune response has been demonstrated in multiple virus infections, including HSV and MCMV (Andoniou 2006, Biron 2001a), the role of NK cells in controlling influenza infection has received little attention and has not been studied in the context of aging (Bender 1995). Therefore, it was important to evaluate NK cell-mediated immunity in response to influenza infection in young and aged mice and to characterize the relationship between the influenza-induced NK cell cytotoxic response and lung virus titer following intranasal infection.

Young (6-8 wk) and aged (22 mo) C57BL/6 male mice were infected with influenza A virus (H1N1, PR8), and NK cell cytotoxicity, lung virus accumulation, and weight loss were monitored during the early response to infection. Aged mice demonstrated a decrease in NK cell activity in response to primary influenza infection, as compared to young mice (Figure 3.1.1). Also in aged mice, increased lung virus by the first day post-infection (p.i.) (Table 3.1.3) indicated an early increase in the severity of influenza infection. Therefore, in conjunction with our previous studies, we have concluded that both a loss of NK cell cytotoxicity and an impaired CTL response

contribute to a reduced ability to control and clear influenza virus from the lungs of aged mice (**Figure 1**).

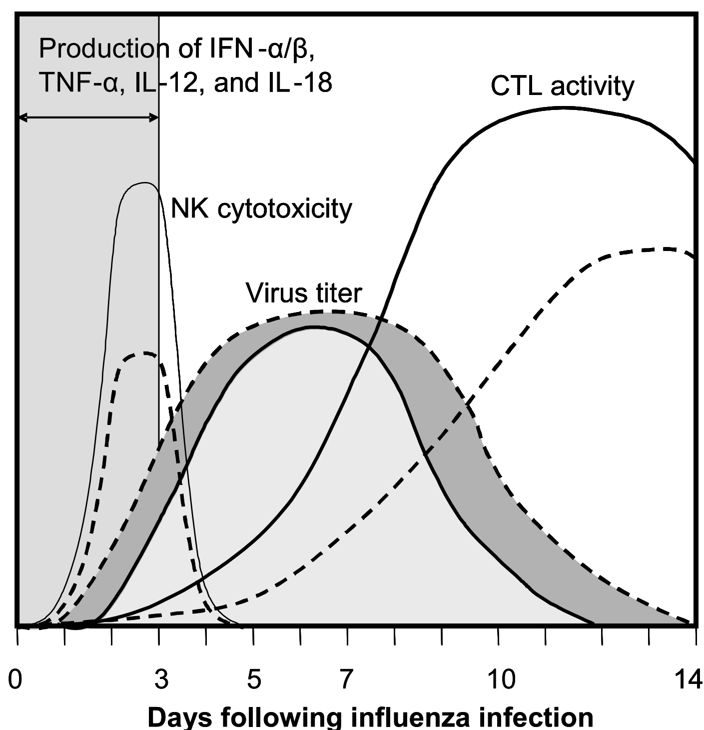


Figure 1. Natural killer (NK) cells and the immune response to influenza infection in young and aged mice. A decrease in influenza-induced NK cell cytotoxicity in aged mice (dashed line) precedes an early and prolonged virus titer in lung and a reduced and delayed cytotoxic T lymphocyte (CTL) response, as compared to young mice (solid line).

While these data demonstrate a loss of NK cell activity and an increase in lung virus in aged mice, they do not imply causality. Therefore, it was important to determine if a decrease in influenza-induced NK cell activity was directly associated with an increase in lung virus and severity of infection.

Since a defect in CTL activity was previously shown to contribute to the age-associated decline in the immune response to influenza, it was necessary to manipulate NK cell activity in young mice in order to clearly delineate the role of NK cells in controlling lung virus. Therefore, NK cells were depleted from young, immunocompetent mice using an anti-NK1.1 antibody, and the severity of infection was monitored as weight loss and lung virus burden. The depletion of NK cells from young mice resulted in an elimination of NK cell cytotoxicity, as expected (Figure 3.1.3), as well as increased weight loss (Figure 3.1.4) and lung virus titers (Tables 3.1.2 and 3.1.3), compared to controls. To our knowledge, these data are the first to provide conclusive evidence that NK cells control influenza virus at the site of infection, i.e., the lungs, and limit severity early in the course of infection.

Important and novel observations were also made in the parallel evaluation of young, aged, and young NK cell-depleted mice. A reduction in NK cell number and function (aged mice) and the absence of NK cell number and function (young NK cell-depleted mice) resulted in a dose-dependent increase in weight loss. Across all three groups, weight loss was significantly correlated with lung virus burden (P=0.004), determined as M1 gene expression by RT-qPCR (Figure 3.1.4). This correlation was strengthened by the elimination of data points from aged mice. Although it is well known that influenza infection results in weight loss, the association between body weight and lung virus has not been explored in detail. We propose that the ability to lose weight may be more than just an artifact of infection; instead, weight loss may be an important component in the immune response to influenza infection, as energy is

mobilized to fight the infection. This concept is further explored in the discussion of CR and the immune response to influenza infection.

***Future studies*.** The importance of NK cells in controlling lung virus and weight loss early in the course of influenza infection and the observed defect in influenza-induced NK cell cytotoxicity in aged mice, suggest that an age-associated decline in NK cell-mediated innate immunity contributes to the increased susceptibility of aged mice to influenza infection. This discovery underscores the need for additional studies to determine the mechanism for the observed decrease in influenza-induced NK cell activity in aged lungs.

The age-associated decrease in NK cell activity could result from an intrinsic defect in the NK cell itself, an extrinsic defect in the NK cell-activating environment, or a combination of both. The use of an adoptive transfer model (**Figure 2**), in which NK cells from a young mouse are transferred into an aged mouse and NK cells from an aged mouse are transferred into a young mouse, assessed in parallel with young and aged mice not receiving external NK cells (controls), would allow us to begin to delineate the underlying mechanism for the loss of NK cell-mediated killing in aged mice.

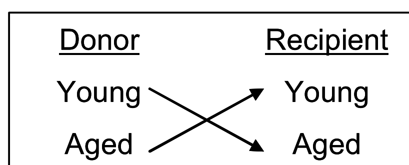


Figure 2. Adoptive transfer model.

Such a study would require preliminary experiments to determine the stability and yield of NK cells once removed from the donor mouse. If feasible and if the results of

adoptive transfer studies suggest a defect in the NK cell itself, meaning that the transfer of NK cells from aged mice to a young environment does not restore function, it would then be necessary to identify potential mechanisms for an intrinsic defect. An age-associated loss in NK cell function due to increased NK cell apoptosis or a defect in NK cell receptor expression in aging has previously been proposed (Plett 2000). Signaling events that mediate NK cell effector functions following receptor-ligand binding on the surface of NK cells could also be impaired in aging. Differences in signaling events, such as those mediated by the JAK/STAT pathway, have not been studied in aged mice and warrant further attention.

If the NK cell-activating environment is altered during the influenza-induced NK cell response of aged mice, such that NK cells from young mice exhibit decreased function when transferred to aged mice, it would be important to assess NK cell-activating cytokines and the accessory cell types that produce them in the lungs of young and aged mice. Cytokines that are important for NK cell survival, activation, and proliferation, include IFN-α/β, IL-12, IL-15, and IL-18 (Biron 2001a, Nguyen 2002, Cousens 1997). Protocols have been established to evaluate IFN-α/β expression in lungs by RT-qPCR, and it was previously shown that altered IFN-α/β response may be partly responsible for reduced inducible NK activity in aged mice (Plett 2000). Additional NK cell-activating cytokines could also be evaluated by PCR, and the appropriate sequences for this work are provided in **Appendix II**.

Natural killer cell-activating cytokines are produced by accessory cells, including dendritic cells (DCs), which make crucial contributions affecting the overall magnitude of NK cell activation. Specifically, *in vitro* evidence suggests that DCs are essential for

the induction of IFN-γ production, cytotoxicity, and proliferation in resting NK cells (Walzer 2005). Therefore, it is likely that DCs play a role in NK cell activation in the lungs of young and aged mice during primary influenza infection, although this has not been studied. Additionally, DC and NK cell activation is mediated, in part, via toll-like receptor (TLR) signaling, which is reduced in aging (Renshaw 2002, van Duin 2007). Alterations in TLRs, specifically TLRs 3 and 7, would be expected to influence the innate immune response to influenza infection, and should be considered as a potential mechanism in the age-associated decline in innate immunity.

4.3 ***Caloric restriction increases severity of influenza infection.*** Caloric restriction, without malnutrition, extends lifespan and postpones age-related changes in immunity. However, our laboratory observed an anomaly in which aged (22 mo) CR mice exhibited increased mortality, impaired virus clearance, and reduced NK cell cytotoxicity during influenza infection compared to aged *ad-libitum* (AL) controls (Gardner 2005). To determine if CR alone, independent of advanced age, results in an increased susceptibility to influenza infection, young adult (6 mo) CR and AL C57BL/6 male mice were infected with 10^4 $TCID_{50}$/ 100 HAU of influenza A virus (H1N1, PR8). Young adult CR mice exhibited increased mortality (Figure 3.2.4), weight loss (Figure 3.2.5), lung virus burden (Table 3.2.1), and lung pathology (Figure 3.2.8) compared to age-matched *ad-libitum* (AL)-fed controls, firmly establishing ***an age-independent and detrimental effect of CR on the immune response to primary influenza infection***.

The data presented in this thesis clearly demonstrate that young adult CR mice failed to induce NK cell cytotoxicity in response to influenza infection (Figure 3.2.9), in

agreement with our observation in aged CR mice. The loss of NK cell cytotoxicity in CR mice was accompanied by an early increase in lung virus at 1 to 2 days p.i. (Table 3.2.1). Since the first study in this thesis established the critical importance of NK cells in controlling influenza virus in lung regardless of age, these data suggest that the increased susceptibility of CR mice to influenza infection is related to an impaired NK cell-mediated innate immune response to primary influenza infection. Therefore, it was important to further characterize innate immunity in young adult CR mice, including an evaluation of NK cell percentage and number, NK cell-activating cytokines, NK cell activation, additional NK cell effector functions, and the participation of accessory and inflammatory cell types in response to influenza infection (**Figure 3**).

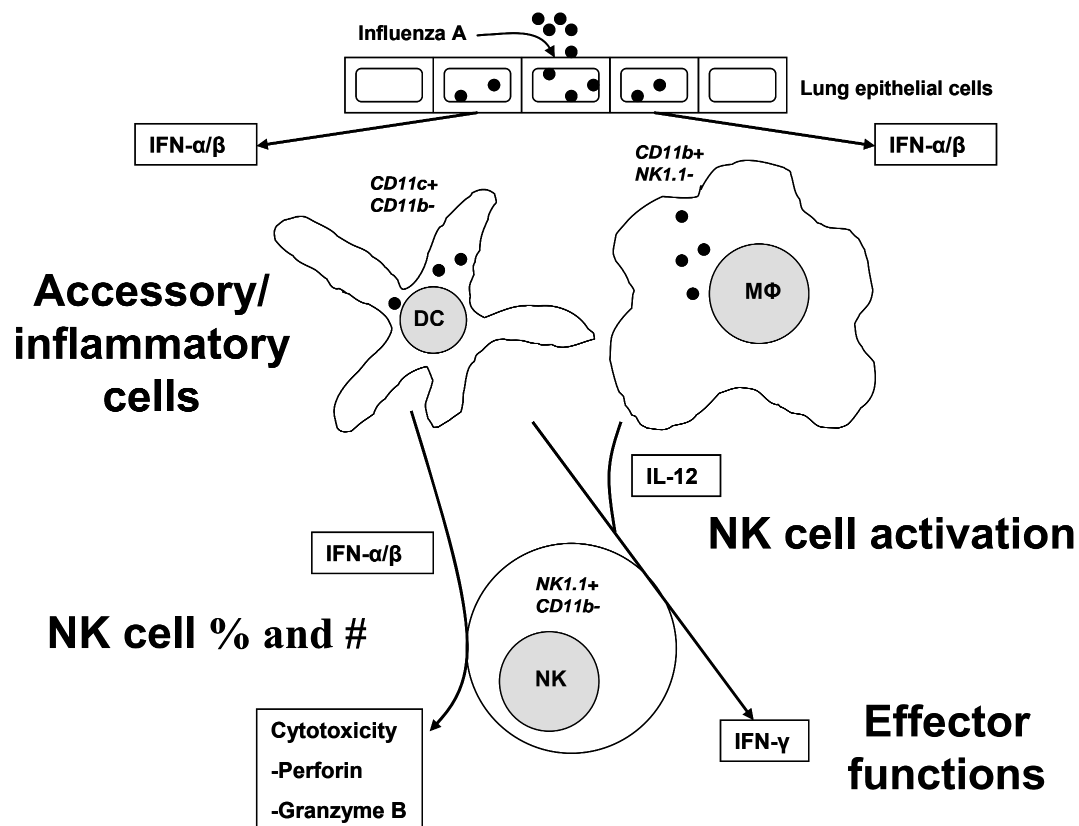


Figure 3. The study of innate immunity includes an evaluation of NK cell percentage and number, NK cell activation, NK cell effector functions, and cytokine production by accessory and inflammatory cells.

We observed a decrease in total and NK1.1+ lymphocytes in the lungs of CR mice, as compared to AL controls, both at baseline and in response to influenza infection (**Table 1**). A decrease in the percentage of NK cells in the mixed lymphocyte population can contribute to a decrease in NK cell cytotoxicity. Therefore, it was important to consider other aspects of NK cell activation and to determine NK cell effector functions on a per cell basis.

Table 1. Summary of flow cytometry data from the lungs of CR mice compared to AL at baseline and 3 days p.i.: total and NK1.1+ lymphocytes.

	Total lymphocytes		**NK1.1+**	
	%	**#**	**%**	**#**
Baseline	↓***	↓**	=	↓**
3 days p.i.	↓***	↓	↓*	↓

*P<0.05, **P<0.01, ***P<0.001

Kinetic studies in mice infected with MCMV demonstrate that the endogenous production of cytokines IFN-α/β and IL-12 in lung acts to activate NK cell effector functions, including cytotoxicity and the production of IFN-γ, thus providing an important link to CTL-mediated adaptive immunity (Garcia-Sastre 2006, Biron 2001b, Nguyen 2002). In response to influenza virus, the expression of IFN-α/β (Figure 3.2.13) and the production of IL-12 (Figure 3.2.15) were reduced at 2 days p.i. in the lungs of CR mice compared to AL-fed controls (summarized in **Figure 4**). These data have clear

implications for the ability of CR mice to mount an antiviral response to influenza infection in the lungs, as well as to induce NK cell cytotoxicity.

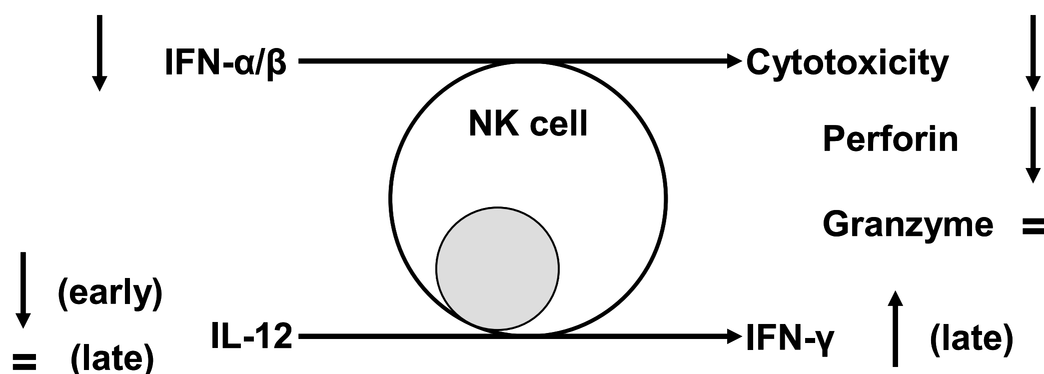


Figure 4. Natural killer cell-activating cytokines and effector functions in the lungs of young adult CR mice in response to primary influenza infection. Initially, CR mice demonstrated a decrease in IFN-α/β expression and IL-12 protein levels in lungs, as well as a decrease in perforin and NK cell cytotoxicity compared to AL controls in response to infection. However, IL-12 production normalized and IFN-γ increased in the lungs of CR mice later during the immune response, after maximal NK cell cytotoxicity in the lungs of AL mice.

Upon cytokine activation, NK cells induce cytotoxicity via the production and release of perforin, a cell membrane disruptor, and granzymes, a series of serine proteases (van Dommelen 2006). An increase in perforin was observed in pulmonary NK cells of AL mice, but not of CR mice, in response to influenza infection (Figure 3.2.12). In studies of perforin gene knockout mice, the inability to induce a perforin response to infection resulted in no NK cell cytotoxicity and an elevated susceptibility to influenza infection (Liu 2003). Therefore, it is reasonable to propose that the observed decrease in

IFN-α/β precedes reduced NK cell cytotoxicity in the lungs of young adult CR mice. Indeed, IFN-α/β is necessary for the induction of perforin-mediated killing (Liang 2003).

Despite the observed loss of influenza-induced NK cell cytotoxic function, the expression of activation markers CD25 and B220 was increased on pulmonary NK cells in CR mice compared to AL mice in response to infection (Figures 3.2.16 and 3.2.17). Similarly, NK cells from CR mice exhibited a dramatic increase in the percentage of NK cells producing IFN-γ, as determined by intracellular staining (Figure 3.2.19). Importantly, the overall increase in these activities occurred late in the innate immune response to influenza, after maximal NK cell cytotoxicity in the lungs of AL mice was observed. This suggests that CR mice may try to compensate following a failure in NK cell activity; however, CR mice were not able to prevent mortality.

Accessory cells, like macrophages and DCs, produce large quantities of cytokines that mediate NK cell activation, inflammation, and the induction of adaptive immunity (Walzer 2005). Given alterations in the availability of NK cell-activating cytokines and differential effects on NK cell activation and effector functions, it was important to evaluate the contribution of accessory and inflammatory cells to the innate immune response to influenza infection in AL and CR mice.

An increase in CD11b+ (monocyte/macrophage marker) cells in the lungs of CR mice in response to influenza infection (**Table 2**), along with an increase in systemic IL-6 (Table 3.2.6), suggest that young adult CR mice might mount a hyper-inflammatory response as a secondary effort to control influenza infection. Such a response could be associated with the observed incresase in lung pathology and mortality in CR mice. The cellular infiltrate in the lungs of CR mice also included an increased percentage and

number of NK cell-activating pDCs, determined as CD11c+CD11b- cells expressing B220, which corresponded with the increased intracellular production of IFN-γ by NK cells in these mice.

Table 2. Summary of flow cytometry data from the lungs of young CR mice compared to AL at baseline and 3 days p.i.: CD11b+ cells and pDCs.

	CD11b+		**B220 on CD11c+**	
	%	**#**	**%**	**#**
Baseline	↓	↓	=	=
3 days p.i.	↑*	↑	↑*	↑*

*P<0.05, **P<0.01, ***P<0.001

Taken together, these data provide preliminary evidence of a compensatory mechanism in which young adult CR mice increased NK cell activation and the production of IFN-γ, possibly in an effort to stimulate adaptive immunity, after innate immunity had failed to control the influenza virus. Despite these efforts, CR mice were unable to halt the progression of infection, as indicated by a continued decrease in body weight and increased mortality through 7 days p.i. An increased inflammatory response in young adult CR mice, although indicative of an increased immune response to influenza infection, could also contribute to increased lung pathology and mortality.

Future studies. Although the preponderance of evidence suggests that CR maintains immune function at an advanced age, CR had not been evaluated in the context of an acute infection. Therefore, these pivotal studies are the first to demonstrate that CR impairs the immune response of young and aged mice when challenged with an infection.

Clearly, the effects of CR on immunity can no longer be viewed without considering the potential consequences of CR in response to infection.

Future studies must examine young adult AL and CR mice and aged AL and CR mice in parallel to further determine the independent and combined effects of advanced age and CR on the immune response to influenza infection. Such studies should evaluate the expression or production of inflammatory and regulatory cytokines, as a hyper-inflammatory response to influenza infection may be specific to young adult CR mice. Indeed, there are no current data on whether aged CR mice might mount a compensatory or inflammatory response to influenza infection similar to the response observed in young adult CR mice, or whether such mechanisms might be involved in the increased severity of influenza infection in young adult and aged CR mice.

An important observation was also made regarding alterations in food intake and weight loss in CR compared to AL mice during infection that requires an extended discussion and provides an opportunity for future study. Calorically-restricted mice maintained normal dietary intake through the first day p.i., while AL demonstrated immediate anorexia and weight loss in response to infection. This delay in the anorectic effect of infection in CR mice may be due to starvation signals which are maintained in CR mice throughout their lifetime (Speakman 2007). Further, CR mice maintained their body weight for an additional day, even after the cessation of food intake. These observations, in view of what is already known about alterations in the metabolism of CR mice, lead us to hypothesize that young adult AL and CR mice differ in their metabolic response to influenza infection. For example, weight loss and recovery in AL mice is likely to be mediated principally through the mobilization of fat stores. In contrast, CR

mice maintain a limited amount of body fat and might lose weight through protein catabolism, a process that requires additional time, but ultimately results in increased weight loss, immune suppression, and a more devastating outcome in response to infection.

Therefore, the results presented thus far allow for the proposal of a metabolic mechanism for the decrease in the innate immune response of young adult CR mice to influenza infection that is mediated by a decrease in body fat and corresponding alterations in energy status, neuroendocrine function, and micronutrient deficiency (**Figure 5**). The detailed discussion that follows provides a rationale for this proposed mechanism and highlights multiple avenues for future study.

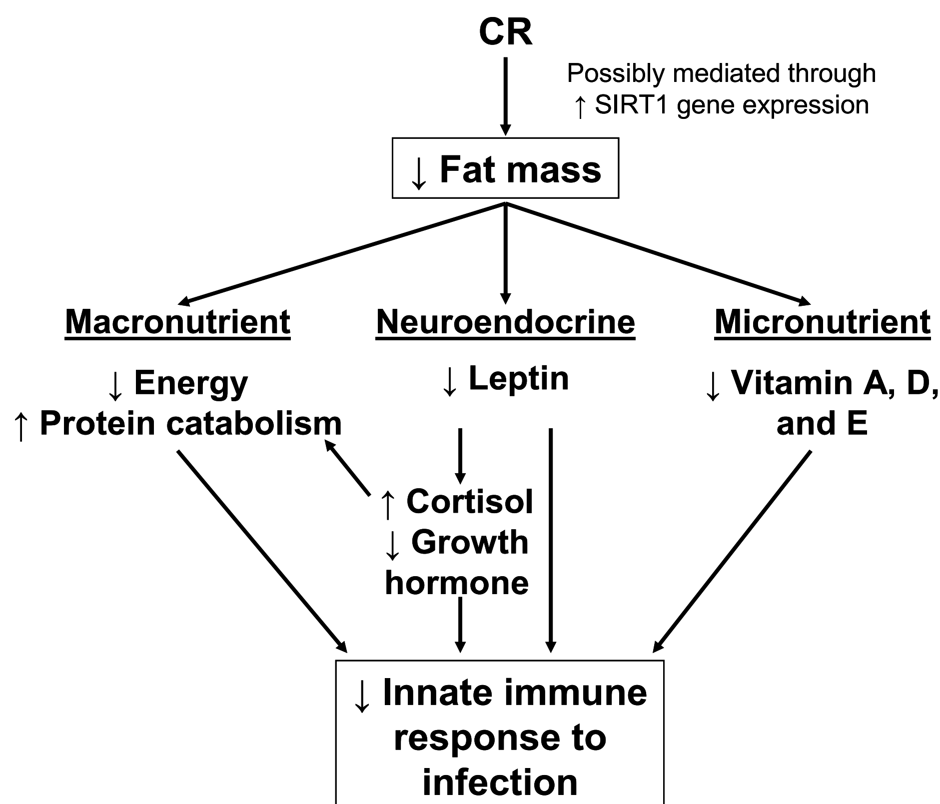


Figure 5. Proposed metabolic mechanism for the decreased innate immune response of young adult CR mice to influenza infection.

A hallmark of CR is a decrease in total body fat. Decreased fat mass may be central to the life-extending benefits of CR, by promoting decreased blood glucose, increased insulin sensitivity, decreased core body temperature, and a reduction in energy metabolism and resultant oxidative stress. However, the immune response to infection requires energy, and low body fat may also predispose CR mice to protein catabolism, which is associated with an impaired immune response to infection and an increase in inflammation mediated by the release of acute phase proteins (Biolo 1997). When caloric intake is chronically low, elevated cortisol and low circulating insulin promote the release of amino acids from muscle in response to infection, which is characteristic of clinical marasmus, or wasting associated with protein-energy malnutrition (Shetty 2006). A similar metabolic state is observed in CR mice, as CR mice demonstrate an increase in cortisol and a decrease in insulin, compared to AL-fed controls (Berner 2004). In humans, the proportion of body weight that can be lost before death varies depending on the baseline weight; however, depletion of 50% or more of lean body mass is incompatible with survival (Hoffer 1999). In our studies, young and aged AL mice can lose up to 30% of their baseline body weight in response to influenza infection and still recover (Ritz 2006b, reviewed in Ritz 2006a). In contrast, CR mice, which weigh approximately 25% less than their age-matched controls, succumbed to infection upon losing 15% of their initial body weight.

Decreased fat mass in CR mice is possibly mediated through alterations in gene expression. In yeast and drosophila, the effects of CR on longevity are controlled by the gene Sir2 (Guarente 2005). The mammalian orthologue for Sir2 is SIRT1, which is expressed throughout mammalian somatic and germline tissues. SIRT1 promotes DNA

repair and cell survival (Cohen 2004) and increases the expression of peroxisome proliferator activating receptor (PPAR)-γ in skeletal muscle (Lee 1999, Yamaza 2002). PPAR-γ is an important regulator of energy metabolism and promotes insulin sensitivity and a reduction in blood glucose, as observed in CR mice. However, PPAR-γ activation in adipose tissue increases adipogenesis and fat storage, a process that is clearly reduced in CR mice. It has recently been proposed that SIRT1 expression may result in site-specific repression of PPAR-γ in white adipose tissue and a resultant decrease in fat storage (Bordone 2005), although this concept has not been proven. Alternatively, it may be that despite increased PPAR-γ activity and survival signals expressed in CR mice, fat storage in CR mice is reduced due to a simple lack of energy substrate.

In addition to storing energy, adipose tissue is metabolically active, mediating important neuroendocrine and immune effects. A master regulator of these effects is leptin, and the inability of CR mice to mount a functional innate immune response to influenza infection may be explained, in part, by a deficiency in leptin. Leptin is a hormone produced by adipocytes that regulates appetite and energy expenditure. Leptin suppresses energy intake via a feedback mechanism, and therefore, leptin deficient *ob/ob* and leptin receptor deficient *db/db* mice exhibit an obese phenotype. In wild-type mice, plasma leptin concentrations directly correlate with adiposity. Therefore, CR mice that exhibit very little body fat are likely to be leptin deficient, further supported by the observation that both starvation and short-term fasting are associated with a decrease in circulating leptin (Berner 2004). Unlike studies involving *ob/ob* and *db/db* mice, however, CR mice have restricted access to food, which prevents obesity. As the release of leptin in response to fat mobilization during infection in AL mice may contribute to

infection-induced anorexia, the observed delay in anorexia in young adult CR mice in response to infection could be related to a leptin deficiency. The effects of lower leptin output in response to decreased body fat are designed to improve survival under hostile conditions, such as starvation, by shifting energy utilization towards vital processes. This may limit the energy available to fight infection (Biolo 2004).

Leptin also mediates direct effects on immunity (**Table 3**). Leptin is released during an infection, and the leptin receptor (Ob-Rb) is expressed by immune cells, including hematopoietic cells, T cells, B cells, NK cells, and macrophages (Bernotiene 2006, Tian 2002). Leptin is structurally related to IL-6 and functions as an acute phase reactant (Bernotiene 2006, Biolo 2004). Consistently, *ob/ob* mice demonstrate a defect in T cell and NK cell function, an increased susceptibility to infection, and a decreased susceptibility to autoimmune disease (McGillis 2005). The effects of leptin on inflammation are somewhat elusive, as leptin deficiency may be associated with a pro-inflammatory state during the innate immune response to infection, but a long-term anti-inflammatory state during the adaptive immune response or in experimental models of autoimmunity (Bernotiene 2006). In *ob/ob* mice, both obesity and immune impairment are reversed by the administration of exogenous leptin (Bernotiene 2006, Lord 1998). Similarly, leptin deficiency induced in wild-type mice by 48-hr fasting results in immunosuppression and an increase in the susceptibility to infection that can be rescued by leptin injection (Lord 1998, Mancuso 2006).

Table 3. Effects of leptin deficiency and administration in mice.

Leptin Effects	**Deficiency**	**Admin.**	**References[1]**
Energy intake	↓	↑	**Bernotiene 2006, Matarese 2004**
Energy expenditure	↑	---[2]	**Matarese 2004**
Susceptibility to infection	↑	↓	**Bernotiene 2006, Matarese 2004,** Mancuso 2006
T cell #, function	↓	↑	**Bernotiene 2006, Matarese 2004, McGillis 2005**, Lord 1998
Thymocyte apoptosis	↑	↓	**Bernotiene 2005, Matarese 2004**
NK cell #, function	↓	↑[3]	Tian 2002
B cell function	↓	---	**Bernotiene 2005**
Phagocyte function	↓	↑	**Bernotiene 2006**, Mancuso 2006
Inflammation:			
Innate	↑	↓	**Bernotiene 2006**
T cell-mediated	↓	↑	**Bernotiene 2006, Matarese 2004**, Lord 1998
Autoimmunity	↓	---	**Bernotiene 2006, Matarese 2004, McGillis 2005**
Stress (cortisol)	↑	---	**Bernotiene 2006, Berner 2004**

[1] Scientific reviews were included when available and are indicated in bold;

[2] ---, result not reported; [3] *in vitro.*

The innate immune response to acute infection may be particularly susceptible to reduced activity due to leptin deficiency. Leptin deficiency results in impaired phagocytic function, as well as impaired NK cell development and activation (Mancuso 2006, Bernotiene 2006, Tian 2002). Leptin receptor deficient *db/db* mice exhibited a decrease in the percentage and number of NK cells in both the spleen and lung, as well as a reduction in PolyI:C-induced NK cell cytotoxicity compared to controls. Further, exogenous leptin was reported to increase NK cell cytotoxcity *in vitro* (Tian 2002).

Leptin deficiency may also influence immunity indirectly through the stress response. Starvation is a stressor, and CR is consistently associated with an increase in plasma corticosterone (Berner 2004). Nearly all immune cells express glucocorticoid receptors, and glucocorticoids generally function as immunosuppressants. An increase in glucocorticoids has been observed in humans with leptin deficiency, *ob/ob* mice, and in response to leptin deficiency induced by fasting (Bernotiene 2006). Stress-induced changes in the immune response include an increase in susceptibility and severity of infection, decreased NK cell number and function, decreased production of NK cell-activating IL-12 by accessory cells, and increased plasma IL-6 (Glaser 2005, Tseng 2005). These effects are strikingly similar to our observations in young CR mice in response to influenza infection.

In addition to altering neuroendocrine functions, a decrease in fat mass may also be expected to result in infection-induced deficiencies in fat-soluble micronutrients, such as vitamins A, D, and E. The consensus in the literature is that stores of antioxidant vitamins are depleted in response to infection, including influenza (Hennet 1992, Han 2000, Mileva 2002, Meydani 2005). Although CR mice are maintained on a fortified diet

to meet normal micronutrient intake, data are not available on whether the nutritional status of CR mice is equivocal to that of AL-fed controls. Further, it is likely that a decrease in fat mass in CR mice is associated with a decrease in the storage of fat-soluble nutrients, such that their availability in response to infection is limited.

Vitamin D has received much recent attention as a modulator of immune function. The 1,25-$(OH)_2$ product of vitamin D metabolism mediates neuroendocrine functions by engaging the vitamin D receptor (VDR), which is expressed by many cell types, including cells of the immune system. Immune cells known to express the VDR include macrophages, dendritic cells, NK cells, activated T cells, and B cells (Bouillon 2006). Vitamin D appears to play a particular role in activating the innate immune response, but decreases adaptive immunity by decreasing antigen presentation (Bouillon 2006). Vitamin D deficiency is associated with increased susceptibility to infection, but has not been studied in influenza. Interestingly, the regulation of vitamin D status may be related to leptin, as leptin deficient *ob/ob* mice demonstrate an increase in circulating vitamin D that is normalized by treatment with exogenous leptin (Matsunuma 2004).

The proposed mechanism of decreased innate immunity mediated by a decrease in fat stores and alterations in energy, leptin, and micronutrients provides multiple avenues for future study. A detailed characterization of the metabolic costs of influenza infection, including energy expenditure and the mobilization of energy stores, is clearly warranted and would provide important information relevant to the general susceptibility of both AL and CR mice to infection. The effects of micronutrient and leptin deficiencies in the innate immune response to influenza infection can be readily studied through micronutrient supplementation and the administration of leptin by injection. In a recently

awarded grant, we proposed re-feeding studies in which CR mice will receive AL diets to determine whether an increase in body weight will restore immunocompentency in these mice. Recent evidence confirms that mice will resume AL feeding after long-term CR, following a short hyperphagic response (Speakman 2007). It has also been suggested that short-term re-feeding increases leptin levels in fasted mice without an increase in body weight (Kim 2003). Therefore, it is of interest to determine whether CR mice can recover the ability to mount an innate immune response through re-feeding without weight gain. If successful, short-term re-feeding in the face on an infectious challenge would avoid the detrimental effects of CR on the immune response without forfeiting the long-term benefits associated with CR and the maintenance of a low body weight, reminiscent of the old adage, "feed a cold."

If applicable to the human circumstance, these studies will have clear implications for elderly individuals who are at an increased risk for low energy intake resulting from social, physical, economic, and emotional obstacles to eating (Pirlich 2001). Low body weight in the elderly, sometimes referred to as frailty, predicts all-cause mortality (Landi 1999, Grabowski 2001), and weight loss is associated with a poor clinical prognosis, including an increased incidence of infectious disease (Sullivan 1990). Numerous reports suggest that optimal body weight may actually be increased in the elderly compared to the younger adult population (Stevens 1998). For example, according to a recent 7-yr prospective trial, disability-free life expectancy was highest among elderly individuals with a BMI of 25-30 kg/m^2, generally considered overweight (Al Snih 2007).

4.4 ***Supplementation with AHCC increases the innate immune response to influenza infection.*** Nutritional modulation can take one of two basic forms: restriction or supplementation. In the CR model, calories were restricted, resulting in a negative outcome in response to infection. Based on these observations, future studies are now planned that will elucidate the effects of re-feeding CR mice, i.e., supplementing calories, on the immune response to influenza infection. While these studies will determine whether a supplementation strategy can rescue NK cell activity in CR, they will not provide insight on the application of a nutritional strategy intended to enhance the NK cell response to influenza infection in young, immunocompetent mice. Therefore, it was important to first identify and evaluate a nutritional intervention with the potential to enhance the innate immune response to primary influenza infection.

The use of medicinal mushroom preparations for immune support has a long tradition in Asian cultures. More recently, such products have gained popularity in the U.S. and abroad, such that the estimated world market is now over $6 billion (Lindequist 2005). One such product is active hexose correlated compound (AHCC), a fermented mushroom extract, manufactured in Sapporo, Japan, and previously reported to enhance NK cell activity in response to tumors and bacterial infection. However, the effects of this agent on NK cell activity in response to a virus infection had never been evaluated.

The active compound in AHCC is an α-1,4-glucan (**Figure 6**), a polysaccharide compound similar in structure to pathogen-associated molecular patterns known to induce immunity through recognition by immune cell receptors, such as the TLRs. The activities of mushroom- and yeast cell wall-derived β-glucans are better characterized and appear to activate innate immunity by binding to C-type lectins, such as Dectin-1,

expressed on the surface of macrophages, dendritic cells, NK cells, and γ/δ-T cells (Brown 2003). It has been speculated that the effects of α-1,4-glucans on innate immunity may also be mediated by C-type lectins (personal communication, Hajime Fujii, Amino Up Chemical Company).

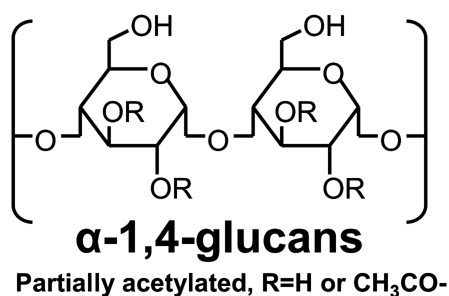


Figure 6. The chemical structure of the active compound in AHCC.

To determine if the effects of AHCC could be extended to virus infection, studies described in Chapter 3.3 of this thesis were conducted in young (6-8 wk) C57BL/6 male mice fed an AL diet supplemented with 1g of AHCC/kg body weight/ d for 1 wk prior to and during primary influenza infection. The supplementation strategy, including dose, was based on a published protocol that demonstrated enhanced NK cell cytotoxicity and bacterial clearance in young mice stressed by head-down tilt and challenged with *Klebsiella pneumoniae* (Aviles 2004). Similarly, when challenged with influenza virus, AHCC-supplemented mice demonstrated increased survival (Figure 3.3.1), NK cell cytotoxcity (Tables 3.3.2 and 3.3.3), and virus clearance (Table 3.3.1), as well as a decrease in weight loss during influenza infection (Figure 3.3.2). AHCC supplementation resulted in an increase in the percentage and number of NK cells in the lungs at 2 days p.i. (Table 3.3.4), the peak of NK cell cytotoxicity in this study. Further,

supplemented mice exhibited a decrease in CD11b+ cellular infiltration in lung (Table 3.3.6) and a reduction in lung pathology (Figure 3.3.3).

The most dramatic result from this study was the ability of AHCC supplemented mice to maintain body weight compared to control mice during influenza infection (Figure 3.3.2). Mice supplemented with AHCC lost a maximum of 1.8g, or 7%, of their initial body weight, whereas control mice lost 5.9g, or 23%. It is unlikely that AHCC supplementation maintained body weight during infection by providing additional dietary energy. On average, supplemented mice received 25 mg of AHCC, providing 4 kcal/g, or approximately 0.1 kcal/d of supplemental energy from AHCC. This accounted for less than 1% of the total daily caloric intake of these mice. It is also important to note that supplemented mice did not gain any weight during the 7 days of supplementation before infection.

Future studies. As discussed previously, the elderly are at an increased risk for morbidity and mortality associated with influenza infection, as well as at increased risk for CR. Dietary supplement use, however, is increasing among the nation's elderly and may provide an opportunity to enhance immunity in this at-risk population. In fact, the percentage of those over the age of 65 using dietary supplements is higher than for any other age group in the U.S. and has doubled in recent years (Kelly 2005).

AHCC has previously been reported to increase the number of NK cells in aged mice (Ghoneum 1992), and preliminary studies have begun to assess the effects of AHCC supplementation on NK cell cytotoxicity in aged mice in response to influenza infection (**Appendix III**). Future studies should determine whether AHCC enhances NK cell activity in aged mice and assess what effects AHCC supplementation may have on the

outcome of influenza infection in aged mice. The ultimate application of these studies would be a series of human clinical trials to determine the safe and effective uses of AHCC in promoting immunity in at-risk and healthy populations.

Future studies have also been proposed and funded to conduct a dose-response analysis to determine the lowest effective dose of AHCC and to determine the mechanism of action in improving the immune response to influenza infection.

Finally, it is of interest to consider whether AHCC might rescue the innate immune response to influenza infection in CR mice. Supplementation with AHCC improved the innate immune response to infection, increased NK cells in lung, and reduced inflammation. Supplementation also maintained body weight, independent of a direct effect on energy intake. Therefore, if excessive weight loss contributes to the increased mortality in influenza-infected CR mice due to limited energy stores, it is possible that preventing weight loss by increasing the innate immune response to influenza infection with AHCC could improve the outcome of primary influenza infection in these mice.

4.5 Conclusions and Limitations

Natural killer cell-mediated innate immunity is clearly important in the immune response to primary influenza infection and can be altered, both positively and negatively, by nutritional interventions. Specifically, the studies described in this thesis, if extended to the human circumstance, have produced several important and novel findings:

1. Natural killer cells are essential for controlling lung virus early during influenza infection.

2. There is an age-associated defect in the NK cell response to influenza infection that is marked by both an inability to expand the NK cell population a decrease in NK cell cytotoxicity.

3. The loss of NK cell-mediated innate immunity results in an increase in the severity of influenza infection, which is associated with an increase in lung virus and a decrease in body weight.

4. Caloric restriction, ***independent of advanced age***, is associated with a decrease in NK cell cytotoxicity and an increased severity of influenza infection.

5. Nutraceuticals may be a viable option for bolstering NK cell function in response to influenza infection.

6. The dietary supplement AHCC increased NK cell activity in response to influenza infection, decreased lung virus titers, and maintained body weight, independent of a direct effect on energy intake.

These studies provide ample opportunities for future research, including a detailed characterization of metabolism during influenza infection in young adult and aged AL and CR mice.

An important limitation to all of the studies presented in this thesis is that they were conducted in only one strain of mice using only one dose of a single, highly-virulent strain of influenza A virus that results in an infection in the lower respiratory tract. Therefore, these results cannot necessarily be extended to less virulent influenza strains, other viruses, or additional infectious agents. It is, however, encouraging that evidence

now suggests that AHCC supplementation enhances the NK cell response to at least one bacterial and one virus infection.

These were the first studies conducted in CR mice in response to an acute infection of *any kind*. Therefore, it remains entirely unknown whether CR exacerbates morbidity and mortality in all viruses or in response to other infectious agents. Finally, young adult CR mice exhibited signs of a hyper-inflammatory response to infection that might not be seen at a lower dose of influenza virus. Future studies should evaluate the innate immune response of CR mice to a less virulent strain or lower dose of influenza virus and consider the susceptibility of CR mice to other common infectious agents, including Norwalk virus and HSV, as well as *Staphylococcus aureus* and *Streptococcus pneumoniae*.

4.6 ***Closing statement.*** At the height of the Spanish flu pandemic of 1918, Dr. William Crawford Gorgas, Surgeon General of the United States Army, advised, “Food will win the war…help by choosing and chewing your food well…” (Barry 2004). Nearly a century later, the emergence of the H5N1 strain of influenza virus prompts us to revisit the role of nutrition in the immune response to primary influenza infection. The threat of another highly-virulent influenza pandemic intensifies the need to characterize the immune response to primary virus infection and determine interventions—nutritional, pharmaceutical, or otherwise—that may prevent or improve the outcome of infection. The series of studies presented in this thesis clearly indicate that nutrition is a potent modulator of the innate immune response to primary influenza infection, including examples of how nutrition may both optimize and interfere with host defense.

List of References

Achdout H, Arnon TK, Markel G, et al. Enhanced recognition of human NK receptors after influenza virus infection. J Immunol 2003;171:915-23.

ACIP: Prevention and control of influenza: Recommendations of the Advisory Committee on Immunization Practices 2005;54(MM713).

Adler H, Beland JL, Del-Pan NC, et al. In the absence of T cells, natural killer cells protect from mortality due to HSV-1 encephalitis. J Neuroimmunol 1999;93:208-13.

Al Snih S, Ottenbacher KJ, Markides KS, et al. The effect of obesity on disability vs mortality in older Americans. Arch Intern Med 2007;167(8):774-80.

Albright JW, Bream JH, Bere EW, Young HA, Winkler-Pickett R, Ortaldo JR. Aging of innate immunity: functional comparisons of NK/LAK cells obtained from bulk cultures of young and aged mouse spleen cells in high concentrations of interleukin-2. Exp Gerontol 2004;39:73-82.

Andoniou CE, Andrews DM, Degli-Esposti MA. Natural killer cells in viral infection: more than just killers. Immunol Rev. 2006; 214:239-50.

Andrews DM, Scalzo AA, Yokoyama WM, et al. Functional interactions between dendritic cells and NK cells during viral infection. Nature Immunol 2003;4(2):175-81.

Aviles H, Belay T, Vance M, Sun B, Sonnenfeld G. Active hexose correlated compound enhances the immune function of mice in the hindlimb-unloading model of spaceflight conditions. J Appl Physiol 2004;97:1437-44.

Avula CPR, Fernandes G. Inhibition of H2O2-induced apoptosis of lymphocytes by calorie restriction during aging. Microscopy Res Tech 2002;59:282-92.

Bajenoff M, Breart B, Huang AYC, et al. Natural killer cell behavior in lymph nodes revealed by static and real-time imaging. J Exp Med 2006;203(3):619-31.

Ballas ZK, Rasmussen W. Lymphokine-activated killer (LAK) cells. IV. Characterization of murine LAK effector subpopulations. J Immunol 1990;144:386-95.

Barger JL, Walford RL, Weindruch R. The retardation of aging by caloric restriction: its significance in the transgenic era. Exp Gerontol 2003;38:1343-51.

Barry JM. The great influenza: the epic story of the deadliest plague in history. Viking: New York, NY, 2004.

Beck MA, Handy J, Levander OA. Host nutritional status: the neglected virulence factor. Trends Microbiol 2004;12:417-23.

Beigel JH, Farrar J, Han AM, et al. Avian influenza A (H5N1) infection in humans. New Engl J Med 2005;353:1374-85.

Bender BS, Johnson MP, Small PA. Influenza in senescent mice: impaired cytotoxic T-lymphocyte activity is correlated with prolonged infection. Immunol 1991;72:514-9.

Bender BS, Small PA. Influenza: pathogenesis and host defense. Semin Respir Infect 1992;7:38-45.

Bender BS, Taylor SF, Zander DS, Cottey R. Pulmonary immune response of young and aged mice after influenza challenge. J Lab Clin Med 1995;126:169-77.

Berner YN, Stern F. Energy restriction controls aging through neuroendocrine signal transduction. Age Res Rev 2004;3:189-98.

Bernotiene E, Palmer G, Gabay C. The role of leptin in innate and adaptive immune responses. Arthritis Res Ther 2006;8(5):217-27.

Bernstein E, Kaye D, Abrutyn E, Gross P, Dorfman M, Murasko DM. Immune response to influenza vaccination in a large healthy elderly population. Vaccine 1999;17:82-94.

Biolo G, Toigo G, Ciocchi B, et al. Metabolic response to injury and sepsis: changes in protein metabolism. Nutr 1997;13(9 Suppl):52S-7S.

Biron C, Brossay L. NK cells and NKT cells in innate defense against viral infections. Curr Opinion Immunol 2001a;13:458-64.

Biron CA, Sen GC. Interferons and other cytokines. In: Fields Virology, 5th ed. Knipe DM, Howley PM, eds. Lippincott Williams & Wilkins: Philadelphia, PA, 2001b;12:321-51.

Biron CA, Turgiss LR, Welsh RM. Increase in NK cell number and turnover rate during acute viral infection. J Immunol 1983;131(3):1539-45.

Bluher M, Kahn BB, Kahn CR. Extended longevity in mice lacking the insulin receptor in adipose tissue. Science 2003;299(5606):572-4.

Bordone L, Guarente L. Calorie restriction, SIRT1 and metabolism: understanding longevity. Nature Rev Molec Cell Biol 2005;6:298-305.

Bot A, Reichlin A, Isobe H, et al. Cellular mechanisms involved in protection and recovery from influenza virus infection in immunodeficient mice. J Virol 1996;70:5668-72.

Bouillon R, Verstuyf A, Mathieu C, et al. Vitamin D resistance. Best Pract Res Clin Endocrinol Metab 2006;20(4):627-45.

Boulo S, Akarsu H, Ruigrok RWH, Baudin F. Nuclear traffic of influenza virus proteins and ribonucleoprotein complexes. Virus Res 2007;124:12-21.

Briedis DJ. Orthomyxoviruses. In: Fundamentals of molecular virology. Acheson NH, ed. John Wiley & Sons: Hoboken, NJ, 2007.

Brown GD, Herre J, Williams DL, et al. Dectin-1 mediates the biological effects of β-glucans. J Exp Med 2003;197(9):1119-24.

Brydon EWA, Morris SJ, Sweet C. Role of apoptosis and cytokines in influenza virus morbidity. FEMS Microbiol Rev 2005;29:837-50.

Bukowski JF, Woda BA, Habu S, et al. Natural killer cell depletion enhances virus synthesis and virus-induced hepatitis *in vivo*. J Immunol 1983;131(3):1531-8.

Burikhanov RB, Wakame K, Igarashi Y, Wang S, Matsuzaki S. Suppressive effect of active hexose correlated compound (AHCC) on thymic apoptosis induced by dexamethasone in rat. Endocrine Reg 2000;34:181-8.

Carroll JL, Nielen LL, Pruett SB, Mathis JM. The role of natural killer cells in adenovirus mediated p53 gene therapy. Molec Cancer Ther 2001;1:49-60.

Choi JM, Gogo S, Ikeda I, Sugaro M. Age-related changes in lipid metabolism in rats: the consequences of moderate food restriction. Biochim Biophys Acta 1988;963:237-42.

Civitarese AE, Carling S, Heilbronn LK, et al. Calorie restriction increases muscle mitochondrial biogenesis in healthy humans. PLoS Med 2007;4(3):e76.

Cohen HY, Miller C, Bitterman KJ, et al. Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. Science 2004;305:390-2.

Conn CA, McClellan JL, Maassab HF, Smitka CW, Majde JA, Kluger MJ. Cytokines and acute phase response to influenza virus in mice. Am J Physiol 1995;268:R78-84.

Conti B, Sanchez-Alvarez M, Winsky-Sommerer R, et al. Transgenic mice with a reduced core body temperature have an increased life span. Science 2006;314:825-8.

Cousens LP, Orange JS, Su HC, Biron CA. Interferon-alpha/beta inhibition of interleukin 12 and interferon-gamma production in vitro and endogenously during viral infection. Proc Nat Acad Sci 1997;94:634-9.

Dawson TC, Beck MA, Kuziel WA, Henderson F, Maeda N. Contrasting effects of CCR5 and CCR2 deficiency in the pulmonaryinflammatory response to influenza A virus. Am J Pathol 2000;156:1951-9.

de Cabo R, Furer-Galban S, Anson RM, et al. An in vitro model of caloric restriction. Exp Gerontol 2003;38:631-9.

Diebold SS, Kaisho T, Hemmi H, Arika S, Reis eSousa C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science. 2004; 303:1529-31.

Dirks AJ, Leeuwenburgh C. Caloric restriction in humans: potential pitfalls and health concerns. Mech Age Devel 2006;127:1-7.

DiSanto JP. Natural killer cell developmental pathways: a question of balance. Ann Rev Immunol 2006;24:257-86.

Dokun AO, Kim S, Smith HRC, et al. Specific and nonspecific NK cell activation during virus infection. Nature Immunol 2001;2(10):951-6.

Donnelly CA. Epidemiological determinants of spread of casual agent of severe acute respiratory syndrome in Hong Kong. Lancet 2003;361:1761-6.

Durbin JE, Fernandez-Sesma A, Lee C-K, et al. Type I IFN modulates innate and specific antivial immunity. J Immunol 2000;164:4220-8.

Effros RB, Walford RL, Weindruch R, Mitcheltree C. Influences of dietary restriction on immunity to influenza in aged mice. J Gerontol 1991;46(4):B142-7.

Effros RB, Walford RL. Diminished T-cell response to influenza virus in aged mice. Immunol 1983;49:387-92.

Ferlazzo G, Munz C. NK cell compartments and their activation by dendritic cells. J Immunol 2004;172(3):1333-9.

Fernandes G, Fleshcer E, Venkatraman J, Modulation of cellular immunity, fatty acid composition, fluidity, and Ca+ influx by food restriction in aging rats. Aging: Immunol Infect Dis 1990;3:117-26.

Fernandes G, Yunis EJ, Good RA. Influence of diet on survival of mice. Proc Nat Acad Sci 1976;73(4):1279-83.

Fontana L, Meyer TE, Klein S, Holloszy JO. Long-term calorie restriction is highly effective in reducing the risk for atherosclerosis in humans. Proc Nat Acad Sci 2004;101(17):6659-6663.

French AR, Holroyd EB, Yang Liping, et al. IL-18 acts synergistically with IL-15 in stimulating natural killer cell proliferation. Cytokine 2006;35:229-34.

Gao Y, Zhang D, Sun B, Fujii H, Kosuna K, Yin Z. Active hexose correlated compound enhances tumor surveillance through regulating both innate and adaptive immune responses. Cancer Immunol Immunother 2005;55(10):1258-66.

Garcia-Sastre A, Biron CA. Type 1 interferons and the virus-host defense: a lesson in détente. Science 2006;312:879-82.

Gardner EM, Bernstein ED, Dran S, Munk G, Abrutyn E, Murasko DM. Characterization of antibody responses to annual influenza vaccination over four years in a healthy elderly population. Vaccine 2001;19:4610-7.

Gardner EM, Bernstein ED, Popoff KA, Abrutyn E, Gross P, Murasko DM. Immune response to influenza vaccine in healthy elderly: lack of association with plasma beta-carotene, retinol, α-tocopheral, or zinc. Mech Age Devel 2000;117:29-45.

Gardner EM, Murasko DM. Age-related changes in Type 1 and Type 2 cytokine production in humans. Biogerontol 2002;3:271-289.

Gardner EM. Caloric restriction decreases survival of aged mice in response to primary influenza infection. J Gerontol Biol Sci 2005;60A(6):688-94.

Gasser S, Raulet DH. Activation and self-tolerance of natural killer cells. Immunol Rev 2006;214:130-42.

Gavazzi G, Herrmann F, Krause K. Aging and infectious diseases in the developing world. Clin Infect Dis 2004;39:83-91.

Ghoneum M, Torabi M, Gill G, Wojdani A. Active hemicellulose compound (AHCC) enhances NK cell activity of aged mice in vivo (abstract). FASEB J 1992;6:A1213.

Ghoneum M, Wimbley M, Salem F, McKlain A, Attallah N, Gill G. Immunomodulatory and anticancer effects of active hexose correlated compound (AHCC). Int J Immunother 1995;11(1):23-8.

Glaser R, Kiecolt-Glaser JK. Stress-induced immune dysfunction: implications for health. Nature Rev Immunol 2005;5:243-51.

Goldsby RA, Kindt TJ, Osborne BA, Kuby J, eds. Innate Immunity In: Immunology. 5th ed. New York, NY: WH Freeman, 2003.

Goonewardene IM, Murasko DM. Age-associated changes in mitogen-induced lymphoproliferation and lymphokine production in the long-lived brown-norway rat: effect of caloric restriction. Mech Age Devel 1995;83:103-16.

Govaert TM, Sprenger MJ, Dinant GJ, Aretz K, Masurel N, Knottnerus JA. Immune response to influenza vaccination of elderly people. A randomized double-blind placebo-controlled trial. Vaccine 1994;12:1185-9.

Grabowski DC. High body mass index does not predict mortality in older people: analysis of the Longitudinal Study of Aging. J Am Geriatr Soc 2001;49(7):968-79.

Gredilla R, Barja G. Minireview: the role of oxidative stress in relation to caloric restriction and longevity. Endocrinol 2005;146:3713-7.

Gross PA, Hermogenes AW, Sacks H, et al. The efficacy of influenza vaccine in elderly persons: a meta-analysis and review of the literature. Ann Intern Med 1995;123:518-27.

Guarente L, Picard F. Calorie restriction—the SIR2 connection. Cell 2005;129:473-82.

Han E-S, Hickey M. Microarray evaluation of dietary restriction. J Nutr 2005;135(6):1343-6.

Han LL, Alexander JP, Anderson LJ. Respiratory syncytial virus pneumonia among the elderly: an assessment of disease burden. J Infect Dis 1999;179:25-30.

Han SN, Meydani M, Wu D, et al. Effect of long-term dietary antioxidant supplementation on influenza virus infection. J Gerontol Biol Sci 2000;55A(10):B496-503.

Hara M, Tanaka K, Hirota Y. Immune response to influenza vaccine in healthy adults and the elderly: association with nutritional status. Vaccine 2005;23:1457-1463.

Hayakawa Y, Huntington ND, Nutt SL, Smyth MJ. Functional subsets of mouse natural killer cells. Immunol Rev 2006;214:47-55.

Heberman RB, Ortaldo JR. Natural killer cells: their role in defenses against disease. Science 1981;214:24-30.

Heilbronn LK, de Jonge L, Frisard MK, et al. Effect of 6-month calorie restriction on biomarkers of longevity, metabolic adaptation, and oxidative stress in overweight individuals. JAMA 2006;295(13):1539-48.

Heilbronn LK, Ravussin E. Caloric restriction and aging: review of the literature and implications for studies in humans. Am J Clin Nutr 2003;78:361-9.

Hennett T, Ziltener HJ, Frei K, Peterhans E. A kinetic study of immune mediators in the lungs of mice infected with influenza A virus. J Immunol 1992;149:932-9.

Hoffer LJ. Metabolic consequences of starvation In: Modern nutrition in health and disease, 9th ed. Shils ME, Olson JA, Shike J, Ross CA, eds. Lippincott Williams Wilkins: Philadelphia, PA; 1999:650.

Honda K, Takaoka A, Taniguchi T. Type I interferon gene induction by the interferon regulatory factor family of transcription factors. Immunity 2006;25:349-60.

Hunter CA, Gabriel KE, Radzanowski T, Nayer LE, Remington JS. Type I interferons enhance production of IFN-gamma by NK cells. Immunol Lett 1997;59:1-5.

Hunzeker J, Padgett DA, Sheridan PA, et al. Modulation of natural killer cell cytotoxicity by restraint stress during an influenza A/PR8 infection in mice. Brain Behav Immun 2004;18:526-35.

Hyodo Y, Matsui K, Hayashi N, et al. IL-18 up-regulates perforin-mediated NK activity without increasing perform messenger RNA expression by binding to constitutively expressed IL-18 receptor. J Immunol 1999;162:1662-8.

Ingram KG, Croy BA, Woodward BD. Splenic natural killer cell activity in wasted, protein-energy malnourished weanling mice. Nutr Res 1995;15:231-43.

Insel P, Turner RE, Ross D, eds. Nutrition. Jones & Bartlett: Sudbury, MA, 2002.

Jakab GJ, Warr GA, Astry CL. Alterations of pulmonary defense mechanisms by protein depletion diet. Infect Immun 1981;Nov:610-22.

James CM, Abdad MY, Mansfield JP, et al. Differential activities of alpha-beta IFN subtypes against influenza virus in vivo and enhancement of specific immune responses in DNA vaccinated mice expressing hemagglutinin and nucleoprotein. Vaccine 2007;25:1856-67.

Janeway CA, Travers P, Walport M, Schlomick MJ, eds. Immunology: the immune system in health and disease, 6th ed. Garland: New York, NY, 2005.

Jefferson T. Influenza vaccination: policy versus evidence. BMJ 2006;333:912-5.

Jiang J, Gross D, Nogusa S, et al. Depletion of T cells by Type I interferon: differences between young and aged mice. J Immunol 2005;175:1820-6.

Johnson BJ, Costelloe EO, Fitzpatrick DR, et al. Single-cell perforin and granzyme expression reveals the anatomical localization of effector T cells in influenza virus-infected mice. PNAS 2003;100(5):2657-62.

Julkunen I, Melen K, Nyqvist M, Pirhonen J, Saraneva T, Matikainen S. Inflammatory responses in influenza A virus infection. Vaccine 2001;19:S32-7.

Julkunen I, Sereneva T, Pirhonen J, Ronni T, Melen K, Matikainen S. Molecular pathogenesis of influenza A virus infection and virus-induced regulation of cytokine gene expression. Cytokine Growth Factor Rev 2001;12:171-80.

Kassim SH, Rajasagi NK, Zhao X, et al. In vivo ablation of CD11c-positive dendritic cells increases susceptibility of herpes simplex virus type 1 infection and dimishes NK and T-cell responses. J Virol 2006;80(8):3985-93.

Keller R, Bachi T, Okumura K. Discrimination between macrophage-and NK-type tumorcidal activities via anti-asialo GM1 antibody. Exp Cell Biol 1983;51(3):158-64.

Kelly JP, Kaufman DW, Kelley K, et al. Recent trends in use of herbal and other natural products. Arch Int Med 2005;165(3):281-6.

Kim Y-W, Scarpace PJ. Repeated fasting/refeeding elevates plasma leptin without increasing fat mass in rats. Physiol Behav 2003;78:459-64.

Kloting N, Bluher M. Extended longevity and insulin signaling in adipose tissue. Exp Gerontol 2005;40:878-3.

Koka R, Burkett PR, Chien M, et al. Interleukin (IL)-15Rα-deficient natural killer cells survive in normal but not IL-Rα-deficient mice. J Exp Med 2003;197(8):977-84.

Kutza J, Gross P, Kaye D, Murasko DM. Natural killer cell cytotoxicity in elderly humans after influenza infection. Clin Diag Lab Immunol 1996;3(1):105-8.

Kutza J, Kaye D, Murasko DM. Basal natural killer cell activity of young vs. elderly humans. J Gerontol Biol Sci 1995;50A:B110-6.

Kutza J, Murasko DM. Age-associated decline in IL-2 and IL-12 induction of LAK cell activity of human PBMC samples. Mech Age Devel 1996;90:209-22.

Lamb RA, Krug RM. Orthomyxoviridae: the viruses and their replication. In: Fields Virology, 5th ed. Knipe DM, Howley PM, eds. Lippincott Williams & Wilkins: Philadelphia, PA, 2001.

Lane MA, Baer DJ, Rumpler WV, et al. Calorie restriction lowers body temperature in rhesus monkeys, consistent with a postulated anti-aging mechanism in rodents. Proc Nat Acad Sci 1996;93(9):4159-64.

Lane MA, Black A, Handy A, et al. Caloric restriction in primates. Ann NY Acad Sci 2001;928:287-95.

Lane MA, Mattison J, Ingram DK, Roth GS. Caloric restriction and aging in primates: relevance to humans and possible CR mimetics. Microscopy Res Tech 2002;59:335-8.

Lane MA, Tilmont EM, De Angelis H, et al. Short-term calorie restriction improves disease-related markers in older male rhesus monkeys (Macca mulatta). Mech Age Devel 2000;112(3):185-96.

Lee C-K, Klopp RG, Weindruch R, Prolla TA. Gene expression profile of aging and its retardation by caloric restriction. Science 1999;285:1390-3.

Lee HK, Lund JM, Ramanathan B, et al. Autophagy-dependent viral recognition by plasmacytoid dendritic cells. Science 2007;315:1398-1401.

Lendi F, Zuccala G, Gambassi G, et al. Body mass index and mortality among older people living in the community. J Am Geriatr Soc 1999;47(9):1072-6.

Lesourd B. Nutrition: a major factor influencing immunity in the elderly. J Nutr Health Aging 2004;8:28-37.

Lesourd B. Nutrition and immunity in the elderly: modification of immune responses with nutrition treatments. Am J Clin Nutr 1997;66:478S-84S.

Liang S, Wei H, Sun R, Tian Z. IFNα regulates NK cell cytotoxicity through STAT1 pathway. Cytokine 2003;23:190-9.

Lindequist U, Niedermeyer THJ, Julich W-D. The pharmacological potential of mushrooms. eCAM 2005;2(3):285-99.

Liu B, Mori I, Hossain MJ, et al. Interleukin-18 improves the early defence system against influenza virus infection by augmenting natural killer cell-mediated cytotoxicity. J General Virol 2004;85:423-8.

Liu B, Mori I, Hossain MJ, et al. Local immune responses to influenza virus infection in mice with a targeted disruption of perforin gene. Microbial Pathogenesis 2003;34:161-7.

Lord GM, Matarese G, Howard JK, et al. Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression. Nature 1998;394:897-901.

Lutz CT, Moore MB, Bradley S, et al. Reciprocal age related change in natural killer cell receptors for MHC class I. Mech Age Devel 2005;126:722-31.

Mancuso P, Huffnagle GB, Olszewski MA, et al. Leptin corrects host defense defects after acute starvation in murine pneumococcal pneumonia. Am J Respir Crit Care Med 2006;173:212-8.

Massaro D, Massaro GD, Baras A, et al. Calorie-related rapid onset of alveolar loss, regeneration, and changes in mouse lung gene expression. Am J Physiol Lung Cell Mol Physiol 2004;286:896-906.

Masoro EJ, Compton C, Yu BP, Bertrand H. Temporal and compositional dietary restrictions modulate age-related changes in serum lipids. J Nutr 1983;113:880-92.

Masoro EJ. Hormesis and the anti-aging action of dietary restriction. Exp Gerontol 1998;33:61-6.

Masoro EJ. Overview of caloric restriction and ageing. Mech Age Devel 2005;126:913-22.

Matarese G, La Cava A. The intricate interface between immune system and metabolism. Trends Immunol 2004;25(4):193-200.

Matsui Y, Uhara J, Satoi S, Kaibori M, Yamada H, Kitade H, Imamura A, Takai S, Kawaguchi Y, Kwon AH, Kamiyama Y. Improved prognosis of postoperative hepatocellular carcinoma patients when treated with functional foods: a prospective cohort study. J Hepatol 2002;37:78-86.

Matsunuma A, Kawane T, Maeda T, et al. Leptin corrects increased gene expression of renal 25-hydroxyvitamin D_3-1α-hydroxylase and -24-hydroxylase in leptin-deficient, ob/ob mice. Endocrinol 2004;145(3):1367-75.

Matsushita K, Kuramitsu Y, Ohiro Y, Obara M, Kobayashi M, Li YQ, Hosokawa M. Combination therapy of active hexose correlated compound plus UFT significantly reduces the metastasis of rat mammary adenocarcinoma. Anti-Cancer Drugs 1998;9:343-50.

Mazari L, Lesourd BM. Nutritional influences on immune response in healthy aged persons. Mech Age Devel 1998;104:25-40.

McCay CM, Cromwell MF, Maynard LA. The effect of retarded growth upon the length of the lifespan and ultimate body size. J Nutr 1935;10:63-79.

McGillis JP. White adipose tissue, inert no more! Endocrinol 2005;146(5):2154-6.

McKenna K, Beignon AS, Bhardwaj N. Plasmacytoid dendritic cells: linking innate and adaptive immunity. J Virol 2005;79(1):17-27.

Mendoza-Nunez VM, Sanchez-Rodriquez MA, Retana-Uglade R, et al. Undernutrition without malnutrition as a protective factor to prevent DNA damage in the elderly. Nutr Res 2005;25:271-80.

Merry BJ. Oxidative stress and mitochondrial function with aging: the effects of calorie restriction. Aging Cell 2004;3:7-12.

Messaoudi I, Warner J, Fischer M, et al. Delay of T cell senescence by caloric restriction in aged long-lived nonhuman primates. Proc Nat Acad Sci 2006;103(51):19448-53.

Mestas J, Hughes CCW. Of mice and not men: differences between mouse and human immunology. J Immunol 2004;172:2731-8.

Meydani SN, Han SN, Wu D. Vitamin E and immune response in the aged: molecular mechanisms and clinical implications. Immunol Rev 2005;205:269-84.

Meyer KC. The role of immunity in susceptibility to respiratory infection in the aging lung. Respir Physiol 2001;128:23-31.

Mileva M, Bakalova R, Tancheva L, et al. Effect of vitamin E supplementation on lipid peroxidation in blood and lung of influenza virus infected mice. Comp Immunol Microbiol Infect Dis 2002;25:1-11.

Miller R. Age-related changes in T cell surface markers: a longitudinal analysis in genetically heterogeneous mice. Mech Age Devel 1997;96:181-96.

MMWR, Update: influenza activity - United States, 2004-05 season. 2004;54:328.

Moretta L, Ferlazzo G, Bottino C, et al. Effector and regulatory events during natural killer cell-dendritic cell interactions. Immunol Rev 2006;214:219-28.

Moskophidis D, Kioussus D. Contribution of virus-specific CD8+ cytotoxic T cells to virus clearance or pathologic manifestations of influenza virus infection in a T cell receptor transgenic mouse model. J Exp Med 1998;188:223-32.

Murasko DM, Bernstein ED, Gardner EM, Gross P, Munk GD, S., Abutyn E. Role of humoral and cell-mediated immunity in protection from influenza disease after immunization of healthy elderly. Exp Gerontol 2002;37:427-439.

Murasko DM, Gardner EM. Immunology of aging. In: Hazzard WR, Blass JP, Halter JB, Ouslander JG, Tinetti ME, eds. Principles of Geriatric Medicine & Gerontology, 5th ed. McGraw-Hill: New York, NY, 2003;35-52.

Murasko DM, Goonerwardene IM. T-cell function in aging: mechanisms of decline. Annu Rev Gerontol Geriatr 1990;10:71-96.

Murasko DM, Jiang J. Resposne of aged mice to primary virus infections. Immunol Rev 2005;205:285-96.

Murasko DM, Weiner P, Kaye D. Decline in mitogen induced proliferation of lymphocytes with increasing age. Clin Exp Immunol 1987;70:440-448.

Neff-La Ford HD, Vorderstrasse BA, Lawrence BP. Fewer CTL, not enhanced NK cells, are sufficient for viral clearance from the lungs of immunocompromised mice. Cell Immunol 2003;226:54-64.

Nelson HK, Shi Q, Van Dael P, et al. Host nutritional selenium status as a driving force for influenza virus mutations. FASEB J 2001;15:1846-8.

Neuzil KM, Graham BS. Cytokine release and innate immunity in respiratory viral infection. Virology 1996;7:255-64.

Newman KC, Riley EM. Whatever turns you on: accessory-cell-dependent activation of NK cells by pathogens. Nature Rev Immunol 2007;7:279-91.

Nguyen KB, Salazar-Mather TP, Dalod MY, et al. Coordinated and distinct roles for IFN-alpha/beta, IL-12, and IL-15 regulation of NK cell response to viral infection. J Immunol 2002;169:4279-87.

Niiya T, Akbar F, Yoshida O, et al. Impaired dendritic cell function resulting from chronic undernutrition disrupts the antigen-specific immune response in mice. J Nutr 2007;137:671-5.

Nikolich-Zugich J, Messaoudi I. Mice and flies and monkeys too: caloric restriction rejuvenates the aging immune system of non-human primates. Exp Gerontol 2005;40:884-93.

O'Connor GM, Hart OM, Gardiner CM. Putting the natural killer cell in its place. Immunol 2005;117:1-10.

Orange JS, Biron CA. Characterization of early IL-12, IFN-alpha/beta, and TNF effects on antiviral state and NK cell responses during murine cytomegalovirus infection. J Immunol 1996;156:4749-56.

Pahlavani MA. Caloric restriction and immunosenescence: a current perspective. Front Biosci 2000;5:D580-7.

Patel NV, Finch CE. The glucocorticoid paradox of caloric restriction in slowing brain aging. Neurobiol Aging 2002;23:707-17.

Phelan JP, Rose MR. Why dietary restriction substantially increases longevity in animal models but won't in humans. Age Res Rev 2005;4:339-50.

Pien GC, Satoskar AR, Takeda K, et al. Cutting edge: selective IL-18 requirements for induction of compartmental IFN-γ responses during viral infection. J Immunol 2000;165:4787-91.

Pirhonen J, Sareneva T, Kurimoto M, et al. Virus infection activates IL-1β and IL-18 production in human macrophages by a caspase-1-dependent pathway. J Immunol 1999;162:7322-9.

Pirlich M, Lochs H. Nutrition in the elderly. Best Pract Res Clin Gastroenterol 2001;15:869-84.

Plett PA, Gardner EM, Murasko DM. Age-related changes in interferon-α/β receptor expression, binding, and induction of apoptosis in natural killer cells from C57BL/6 mice. Mech Age Devel 2000;118:129-44.

Plett PA, Murasko DM. Genetic differences in the age-associated decrease in inducibility of natural killer cells by interferon-alpha.beta. Mech Age Devel 1999;112:197-215.

Po JL, Gardner EM, Anaraki F, Katsikis PD, Murasko DM. Age-associated decrease in virus-specific CD8+ T lymphocytes during primary influenza infection. Mech Age Devel 2002;123:1167-81.

Pozzetto B, Odelin MF, Bienvenu J, Defayolle M, Aymard M. Is there a relationship between malnoutrition, inflamation, and post-vaccinal antibody response to influenza viruses in the elderly? J Med Virol 1993;41:39-43.

Price GE, Gsszewska-Mastarlarz A, Moskophidis D. The role of alpha/beta and gamma interferons in development of immunity to influenza A virus in mice. J Virol 2000:3996-4003.

Prlic M, Blazar BR, Farrar MA, Jameson SC. In vivo survival and homeostatic proliferation of natural killer cells. J Exp Med 2003;197(8):967-76.

Provinciali M, Muzzioli M, Fabris N. Timing of appearance and disappearance of IFN and IL-2 induced natural immunity during ontogenic development and aging. Exp Gerontol 1989;24:227-36.

Rebrin I, Kamzalov S, Sohal RS. Effects of age and caloric restriction on glutathione redox state in mice. Free Rad Biol Med 2003;35(6):626-35.

Redmond HP, Gallagher HJ, Shou J, Daly JM. Antigen presentation in protein-energy malnutrition. Cell Immunol 1995;163:80-7.

Renshaw M, Rockwell J, Engleman C, et al. Cutting edge: impaired toll-like receptor expression and function in aging. J Immunol 2002;169:4697-701.

Ritz BW, Gardner EM. Malnutrition and energy restriction differentially affect viral immunity. J Nutr 2006a;136:1141-4.

Ritz BW, Nogusa S, Ackerman EA, Gardner EM. Supplementation with active hexose correlated compound increases the innate immune response of young mice to primary influenza infection. J Nutr 2006b;136:2868-73.

Roeker EB, Kemnitz JW, Ershler WB, Weindruch R. Reduced immune responses in rhesus monkeys subjected to dietary restriction. J Gerontol Biol Sci Med Sci 1996;51:B276-9.

Roth GS, Ingram DK, Lane MA. Caloric restriction in primates and relevance to humans. Ann NY Acad Sci 2001;928:305-15.

Sabatino F, Masoro EJ, McMahan Z, Kuhn RW. Assessment of the role of the glucocorticoid system in aging processes and in the action of food restriction. J Gerontol Biol Sci 1991;46(5):B171-9.

Samuel CE. Antiviral actions of interferon. Interferon-regulated cellular proteins and their surprisingly selective antiviral activities. Virology 1991;183:1-11.

Sareneva T, Matikainen S, Kurimoto M, Julkunen I. Influenza A virus-induced IFN-α/β and IL-18 synergistically enhance IFN-γ gene expression in human T cells. J Immunol 1998;160:6032-8.

Saxena RK, Saxena QB, Adler WH. Interleukin-2 induced activation of natural killer activity in spleen cells from old and young mice. Immunol 1984;51:719-26.

Schneider EL, Reed GD. Life extension. New Engl J Med 1985;312:1159-68.

Scrimshaw NS, San Giovanni JP. Synergism of nutrition, infection, and immunity: an overview. Am J Clin Nutr 1997;66:464S-77S.

Shetty P. Malnutrition and undernutrition. Medicine 2006:34(12):524-9.

Shils ME, Olsen JA, Shike M, Ross AC, eds. Modern nutrition in health and disease, 10th ed. Lippincott Williams & Wilkins: Philadelphia, PA, 2005.

Simonsen L, Reichert TA, Viboud C, et al. Impact of influenza vaccination on seasonal mortality in the US elderly population. Arch Int Med 2005;165:265-72.

Simonsen L, Reichert TA, Viboud C, et al. Impact of influenza vaccination on seasonal mortality in the US elderly population. Arch Int Med 2005;165:265-72.

Smith AG, Sheridan PA, Harp JB, Beck MA. Diet-induced obese mice have increased mortality and altered immune responses when infected with influenza virus. J Nutr 2007;137:1236-43.

Sohal RS, Mockett RJ, Orr WC. Mechanisms of aging: an appraisal of the oxidative stress hypothesis. Free Rad Biol Med 2002;33(5):575-86.

Solana R, Alonso MC, Pena J. Natural killer cells in healthy aging. Exp Gerontol 1999;34:435-443.

Solana R, Mariani E. NK and NK/T cells in human senescence. Vaccine 2000;18:1613-20.

Son Y-I, Dallal RM, Mailliard RB, et al. Interleukin-18 (IL-18) synergizes with IL-2 to enhance cytotoxicity, interferon-γ production, and expansion of natural killer cells. Cancer Res 2001;61:884-8.

Spaulding CC, Walford RL, Effros RB. The accumulation of non-replicative non-functional, senescent T cells with age is avoided in calorically restricted mice by an enhancement of T cell apoptosis. Mech Age Devel 1997;93:25-33.

Speakman JR, Hambly C. Starving for life: what animal studies can and cannot tell us about the use of caloric restriction to prolong human lifespan. J Nutr 2007;137:1078-86.

Sprunt DH, Flanigan CC. The effect of malnutrition on the susceptibility of the host to viral infection. J Exp Med 1956;104:687-706.

Stein-Streilein J, Guffee J, Fan W. Locally and systemically derived natural killer cells participate in defense against intranasally inoculated influenza virus. Reg Immunol 1988;1(2):100-5.

Stevens J. Studies of the impact of age on optimal body weight. J Nutr Biochem 1998;9:501-10.

Sullivan DH, Patch GA, Walls RC, Lipschitz DA. Impact of nutrition status on morbidity and mortality in a select population of geriatric rehabilitation patients. Am J Clin Nutr 1990;51:749-58.

Suttles J, Schwarting GA, Stout RD. Flow cytometric analysis reveals the presence of asialo GM1 on the surface membrane of alloimmune cytotoxic T lymphocytes. J Immunol 1986;136(5):1586-91.

Swiergiel AH, Smagin GN, Dunn AJ. Influenza virus infection of mice induces anorexia: comparison with endotoxin and interleukin-1 and the effects of indomethacin. Pharmacol Biochem Behav 1997;57(1/2):389-96.

Tamura S, Kurata T. Defense mechanisms against influenza virus infection in the respiratory tract mucosa. Jap J Infect Dis 2004;57:236-47.

Thompson WW, Shay DK, Weintraub E, et al. Influenza-associated hospitalizations in the United States. JAMA 2003;292(11):1333-40.

Thompson WW, Shay DK, Weintraub E, et al. Mortality associated with influenza and respiratory syncytial virus in the United States. JAMA 2003;289(2):179-86.

Tian L, Cai Q, Bowen R, Wei H. Effects of caloric restriction on age-related oxidative modifications of macromolecules and lymphocyte proliferation in rats. Free Rad Biol Med 1995;19(6):859-65.

Tian Z, Sun R, Wei H, Gao B. Impaired natural killer (NK) cell activity in leptin receptor deficient mice: leptin as a critical regulator in NK cell development and activation. Biochem Biophys Res Comm 2002;298:297-302.

Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat Rev Immunol 2003;3:133-46.

Tseng RJ, Padgett DA, Dhabhar FS, et al. Stress-induced modulation of NK activity during influenza viral infection: role of glucocorticoids and opioids. Brain Behav Immun 2005;19:153-64.

Turturro A, Witt WW, Lewis S, et al. Growth curves and survival characteristics of the animals used in the biomarkers of aging program. J Gerontol Biol Sci 1999;54A(11):B492-501.

Umezawa M, Hanada K, Naiki H, et al. Effects of dietary restriction on age-related immune dysfunction in the senescence accelerated mouse. J Nutr 1990;120:1393-1400.

Van der Sluijs KF, Van Elden LJR, Arens R, et al. Enhanced viral clearance in interleukin-18 gene-deficient mice after pulmonary infection with influenza A virus. Immunol 2005;114:112-20.

Van Dommelen SLH, Sumaria N, Schreiber RD, et al. Perforin and granzyme have distinct roles in defensive immunity and immunopathology. Immunity 2006;j25:835-48.

van Duin D, Mohanty S, Thomas V, et al. Age-associated defect in human TLR-1/2 function. J Immunol 2007;178:970-5.

Van Reeth K. Cytokines in the pathogenesis of influenza. Vet Microbiol 2000;74:109-16.

Verdery RB, Ingram DK, Roth GS, Lane MA. Caloric restriction increases HDL_2 levels in rhesus monkeys (*Macca mulatta*). Am J Physiol 1997;273:E714-9.

Verdijk RM, Mutis T, Esendam B, et al. Polyriboinosinic polyribocytidulic acid (PolyI:C) induces stable maturation of functionally active human dendritic cells. J Immunol 1999;163:57-61.

Walford RL, Mock D, Verdery R, MacCallum T. Calorie restriction in Biosphere 2: alterations in physiologic, hematologic, hormonal, and biochemical parameters in humans restricted for a 2-year period. J Gerontol 2002;57A(6):B211-24.

Walzer T, Dalod M, Robbins SH, Zitvogel L, Vivier E. Natural-killer cells and dendritic cells: "I'union fait la force". Blood 2005; 106:2252-8.

Wanagat J, Allison DB, Weindruch R. Caloric intake and aging: mechanisms in rodents and a study in nonhuman primates. Toxicol Sci 2000;52S:35-40.

Wang S, Ichimura K, Wakame K. Preventive effects of active hexose correlated compound (AHCC) on oxidative stress induced by ferric nitrilotriacetate in the rat. Dokkyo J Med Sci 2001;28(2-3):745-52.

Wang S, Wakame K, Igarashi Y, Kosuna K, Matsuzaki S. Beneficial effects of active hexose correlated compound (AHCC) on immobilization stress in rat. Dokkyo J Med Sci 2001;28(1):559-65.

Ward CL, Dempsey MH, Ring CJA, et al. Design and performance testing of quantitative real time PCR assays for influenza A and B viral load measurement. J Clin Virol 2004;29:179-88.

Warner HR. Longevity genes: from primitive organisms to humans. Mech Age Devel 2005;126:235-42.

Webster RG. Immunity to influenza in the elderly. Vaccine 2000;18:1686-1689.

Weindruch R, Devens BH, Raff HV, Walford RL. Influence of dietary restriction and aging on natural killer cell activity in mice. J Immunol 1983;130:99306.

Weindruch R, Gottesman SRS, Walford RL. Modification of age-related immune decline in mice dietarily restricted from or after midadulthood. Proc Nat Acad Sci 1982;79:898-902.

Weindruch R, Walford RL, Fligiel S, Guthrie D. The retardation of aging in mice by dietary restriction: longevity, cancer, immunity and lifetime energy intake. J Nutr 1986;16:641-54.

Weindruch R. Dietary restriction, tumors, and aging in rodents. J Gerontol Bio Sci 1989;44(6):67-71.

Weindruch R. Immunogerontologic outcomes of dietary restriction started in adulthood. Nutr Rev 1995;53(4):S66-71.

Woodward B, Hillyer L, Hunt K. T cells with a quiescent phenotype (CD45RA+) are overabundant in the blood and involuted lymphoid tissues in wasting protein and energy deficiencies. Immunol 1999;96:246-53.

Wright PF, Webster RG. Orthomyxoviruses. In: Fields Virology, 5th ed. Knipe DM, Howley PM, eds. Lippincott Williams & Wilkins: Philadelphia, PA, 2001.

Wright S, Bonavida B. Studies on the mechanisms of natural killer cytotoxity. III. Activation of NK cells by interferon augments the lytic activity of released natural killer cytotoxic factor (NKCF). J Immunol 1983;130:2960-4.

www.cdc.gov. Avian influenza in humans, April 23, 2007.

www.who.int. Situation updates: avian influenza, April 23, 2007.

Yagita A, Matsuzaki S, Wakasugi S, Sukegawa Y. H-2 haplotype-dependent serum IL-12 production in tumor-bearing mice treated with various mycelial extracts. In Vivo 2002;16:49-54.

Yamaza H, Chiba T, Higami Y, Shimokawa I. Lifespan extension by caloric restriction: an aspect of energy metabolism. Microscopy Res Tech 2002;59:325-30.

Ye SF, Ichimura K, Wakame K, Ohe M. Suppressive effects of active hexose correlated compound on the increased activity of hepatic and renal ornithine decarboxylase induced by oxidative stress. Life Sci 2003;74:593-602.

Yu BP, Chung HY. Stress resistance by caloric restriction for longevity. Ann NY Acad Sci 2001;928:39-47.

Yu BP. Aging and oxidative stress: modulation by dietary restriction. Free Rad Biol Med 1996;21(5):651-68.

Zhang X, Hillyer LM, Woodward BD. The capacity of non-inflammatory (steady-state) dendritic cells to present antigen in the primary response is preserved in acutely protein- or energy-deficient weanling mice. J Nutr 2002;132:2748-56.

Appendix I: Animal diets

NIH-31/NIA Fortified Diet
&
NIH31 Average Nutrient Composition

Ingredients:

Ground wheat, ground corn, ground oats, wheat middlings, fish meal, soybean meal, corn gluten meal, dehydrated alfalfa meal, soybean oil, dicalcium phosphate, brewers dried yeast, salt, calcium carbonate, choline chloride, menadione sodium bisulfite complex (source of vitamin K activity), thiamine mononitrate, calcium pantothenate, vitamin E supplement, vitamin A acetate, riboflavin, vitamin B12 supplement, niacin, vitamin D3 supplement, pyridoxine HCL, folic acid, biotin, magnesium oxide, ferrous sulfate, manganous oxide, copper sulfate, zinc oxide, calcium iodate, cobalt carbonate.

Average Nutrient Composition

		NIH-31/NIA Fortified	NIH-31
Protein	%	18.74	18.42
Fat	%	4.41	4.47
Fiber	%	4.58	4.05
Ash	%	6.51	6.64
Nitrogen-Free Extract	%	55.04	55.91
Gross Energy	kcal/g	3.95	4.02
Digestible Energy	kcal/g	3.36	---
Metabolizable Energy	kcal/g	3.07	---
Linoleic Acid	%	1.79	---
Moisture	%	---	10.51

Amino Acids

		NIH-31/NIA Fortified	NIH-31
Arginine	%	1.10	1.06
Methionine	%	0.36	0.39
Histidine	%	0.42	0.41
Leucine	%	1.50	1.61
Lysine	%	0.96	0.95
Tryptophan	%	0.22	0.24
Valine	%	0.88	0.96
Cystine	%	0.26	0.28

Isoleucine	%	0.76		0.90
Threonine	%	0.71		0.71
Pherylalanine	%	1.53	Pherylalanine	0.92
+Tyrosine			Tyrosine	0.70

Minerals

		NIH-31/NIA Fortified	NIH-31
Calcium	%	1.03	1.06
Phosphorus	%	0.93	0.92
Sodium	%	0.30	0.26
Chlorine	%	0.48	0.42
Potassium	%	0.59	0.59
Magnesium	%	0.20	0.20
Iron	mg/Kg	336.41	300.20
Maganese	mg/Kg	156.01	152.80
Zinc	mg/Kg	48.41	50.40
Copper	mg/Kg	13.28	13.20
Iodine	mg/Kg	2.01	1.94
Cobalt	mg/Kg	0.53	0.53
Selenium	mg/Kg	0.30	---

Vitamins

		NIH-31/NIA Fortified	NIH-31
Vitamin A	IU/g	40.49	---
Vitamin A3	IU/g	---	30.73
Vitamin D3	IU/g	7.00	4.19
Vitamin E	mg/Kg	52.15	38.30
Choline	mg/g	2.60	1.96
Niacin	mg/Kg	116.16	92.20
Pantothenic Acid	mg/Kg	55.07	39.50
Pyridoxine	mg/Kg	13.16	10.20
Riboflavin	mg/Kg	11.04	7.80
Thiamine	mg/Kg	123.44	77.30
Menadione	mg/Kg	111.01	22.00
Folic Acid	mg/Kg	2.13	1.70
Biotin	mg/Kg	0.38	0.13
Vitamin B12	mcg/Kg	93.80	53.00
Vitamin C	mg/g	---	---
Carotene	mg/Kg	---	---

Appendix II: PCR sequences

Sequences were provided by the lab of Melinda A. Beck, University of North Carolina at Chapel Hill:

Cytokine/Surface marker	Amplicon Size
IL-1α	---
IL-1β	90
IL-2	131
IL-4	---
IL-5	83
IL-6	80
IL-10	73
IL-12 (p40)	93
IL-15	86
IFN-γ	72
TNF-α	88
TGF-β	106
MCP	78
MIP-1α	67
RANTES	85
IFN-α	122
IFN-β	76
Influenza M1	187
IL-18	---
CD3	---
CD4	---
CD8	---
CD25	---
MIP-1β	65
GAPDH	---

Cytokine/Surface marker	**Forward Primer**
IL-1α	GTCGGCAAAGAAATCAAGATGG
IL-1β	GGCCTCAAAGGAAAGAATCTATACC
IL-2	CTCCTGAGCAGGATGGAGAATT
IL-4	GGCATTTTGAACGAGGTCACA
IL-5	GAGCACAGTGGTGAAAGAGACCTT
IL-6	TATGAAGTTCCTCTCTGCAAGAGA
IL-10	TTTGAATTCCCTGGGTGAGAA
IL-12 (p40)	AGCTAACCATCTCCTGGTTTGC
IL-15	TCATATTGACACCACTTTATACACTGACA
IFN-γ	AGCAACAGCAAGGCGAAAA
TNF-α	CTGTCTACTGAACTTCGGGGTGAT
TGF-β	GCAACATGTGGAACTCTACCAGAA
MCP	TTGGCTCAGCCAGATGCA
MIP-1α	CAAGTCTTCTCAGCGCCATATG
RANTES	TCCAATCTTGCAGTCGTGTTTG
IFN-α	TGCAACCCTCCTAGACTCATTCT
IFN-β	TGAATGGAAAGATCAACCTCACCTA
Influenza M1	GGACTGCAGCGTAGACGCTT
IL-18	TCTGCAACCTCCAGCATCAG
CD3	GACAAGATGGCAGAAGCCTACA
CD4	TTGTGCATGTCACACATGAAGC
CD8	AAGCAATGCCCGTTCCC
CD25	CTTATATTGCAAATGTGGCACAATC
MIP-1β	CCG AGC AAC ACC ATG AAG C
GAPDH	GCAGTGGCAAAAGTGGAGATTG

Cytokine/Surface marker	**Reverse Primer**
IL-1α	TCAATGGCAGAACTGTAGTCTTCG
IL-1β	GTATTGCTTGGGATCCACACTCT
IL-2	CGCAGAGGTCCAAGTTCATCT
IL-4	AGGACGTTTGGCACATCCAT
IL-5	CAGGAAGCCTCATCGTCTCATT
IL-6	TAGGGAAGGCCGTGGTT
IL-10	ACAGGGGAGAAATCGATGACA
IL-12 (p40)	CCACCTCTACAACATAAACGTCTTTC
IL-15	GCAATTCCAGGAGAAAGCAGTT
IFN-γ	CTGGACCTGTGGGTTGTTGA
TNF-α	GGTCTGGGCCATAGAACTGATG
TGF-β	GACGTCAAAAGACAGCCACTCA
MCP	CCTACTCATTGGGATCATCTTGC

MIP-1α	TCTTCCGGCTGTAGGAGAAGC
RANTES	TGATGTATTCTTGAACCCACTTCTTCT
IFN-α	CCAGCAGGGCGTCTTCCT
IFN-β	CTCTTCTGCATCTTCTCCGTCA
Influenza M1	CATCCTGTTGTATATGAGGCCCAT
IL-18	CCTGGGCCAAGAGGAAGTG
CD3	CGTGCCCCTTGCCTCTC
CD4	AGGCATCATGGGAAGCTGAG
CD8	TGAGGGTGGTAAGGCTGCA
CD25	GGGACAATCTGATCAAGGAGAATC
MIP-1β	CAG AAG GCA GCC ACG AGC
GAPDH	CCATTCTCGGCCTTGCTGT

Cytokine/Surface marker	**Probe**
IL-1α	CCTGACTTGTTTGAAGACCTAAAGAACTGTTA CAGTGA
IL-1β	ATGAAAGACGGCACACCCACCCTG
IL-2	CTGAAACTCCCCAGGATGCTCACCTTC
IL-4	CTCCGTGCATGGCGTCCCTTCT
IL-5	TGTCCGCTCACCGAGCTCTGTTGA
IL-6	CCAGCATCAGTCCCAAGAAGGCAACT
IL-10	TGAAGACCCTCAGGATGCGGCTG
IL-12 (p40)	TGCTGGTGTCTCCACTCATGGCCA
IL-15	CTTTCATCCCAGTTGCAAAGTTACTGCAATG
IFN-γ	CCTCAAACTTGGCAATACTCATGAATGCATCC
TNF-α	ATGAGAAGTTCCCAAATGGCCTCCCTC
TGF-β	ACCTTGGTAACCGGCTGCTGACCC
MCP	AACGCCCCACTCACCTGCTGCTACT
MIP-1α	AGCTGACACCCCGACTGCCTGC
RANTES	TTGGCACACACTTGGCGGTTCCTT
IFN-α	CTGCATCAGACAGCCTTGCAGGTCATT
IFN-β	AGGGCGGACTTCAAGATCCCTATGGA
Influenza M1	CTCAGTTATTCTGCTGGTGCACTTGCCA
IL-18	CAAAGAAAGCCGCCTCAAACCTTCCA
CD3	TGAGATCGGCACAAAAGGCGAGAGG
CD4	AATGAAGACTGAGAGGCTGCGGGAGTC
CD8	ACCCAGAGACCCAGAAGGGCCTGAC
CD25	AGAGAAATCTGGGATGAGGGAACCTCAGTG
MIP-1β	CTG CGT GTC TGC CCT CTC TCT CCT CT
GAPDH	TGACTCCACTCACGGCAAATTCAACG

Appendix III: Supplementation of aged mice with AHCC

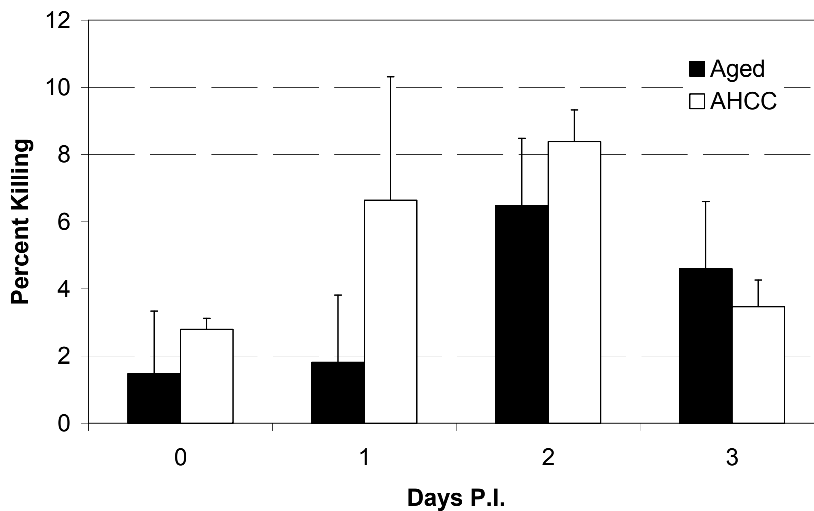


Figure 1. Influenza-induced NK cell cytotoxicity in the lungs of aged mice and aged mice supplemented with AHCC.

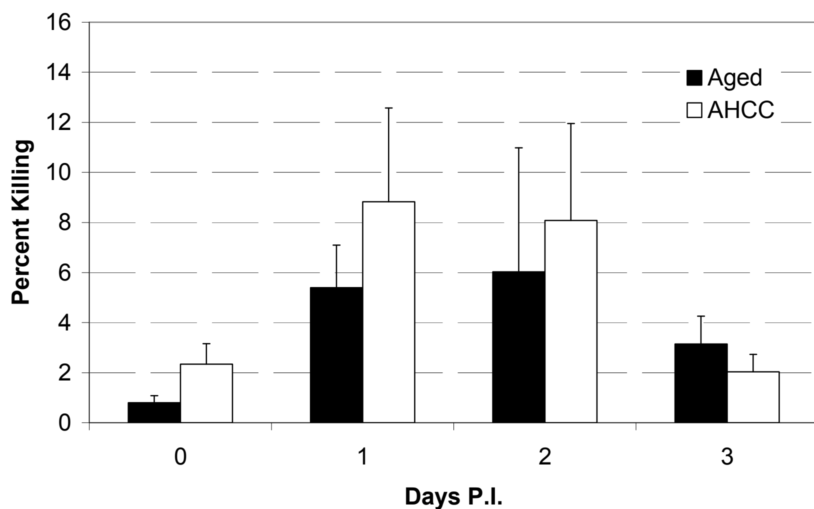


Figure 2. Influenza-induced NK cell cytotoxicity in the lungs of aged mice and aged mice supplemented with AHCC. n=3 mice per group per day. Not repeated.

Vita

Barry William Ritz was born on March 21, 1975, the son of Barre Lee and Carol Blessing Ritz. In 1993, Barry graduated as the Valedictorian of Eastern High School, Wrightsville, PA, and entered The Pennsylvania State University. He served as President of Lion Ambassadors and as a member of Alumni Council. He was a member of *Omicron Delta Kappa* National Honor Society and *Parmi Nous*. Barry graduated with a B.S. in Life Science and Nutrition Science in 1997. In 2001, after several years spent in the nutrition industry, he began his graduate degree at Drexel University. Barry married Kendall Sinclaire Guyer of Bethlehem, PA, on May 25, 2002, and at the time of this writing was expecting his first child. He earned his M.S. in 2004, and presented his thesis entitled *Functional recovery of peripheral blood mononuclear cells in modeled microgravity: no effect of vitamins C and E*. Barry was the founding president of the Bioscience & Biotechnology Graduate Student Association. As a graduate student, Barry published an invited review,[1] two original manuscripts,[2,3] and made multiple presentations at national and international meetings, including the *NASA Cell Science Conference* (Galveston, TX, 2005); *Experimental Biology* (San Diego, CA, 2005, San Francisco, CA, 2006, and Washington, DC, 2007); and the *14th International Symposium on AHCC Research* (Sapporo, Japan, 2006). On June 8, 2007, he orally defended his PhD dissertation entitled *Nutritional modulation of the innate immune response to influenza infection*. Barry will be remaining at Drexel as a Research Associate.

[1]Ritz BW,Gardner EM..Malnutrition and energy restriction differentially affect viral immunity.J Nutr 2006;136:1141-4.
[2]Ritz BW, Lelkes PI, Gardner EM. Functional recovery of peripheral blood mononuclear cells in modeled microgravity. FASEB J 2006;20:305-7.
[3]Ritz BW, Nogusa S, Ackerman EA, Gardner EM. Supplementation with active hexose correlated compound increases the innate immune response of young mice to primary influenza infection. J Nutr 2006;136:2868-73.