Impacts of Aging and Inflammation on *Mycobacterium tuberculosis* Control

DISSERTATION

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By

Cynthia Hsin-Tzu Canan

Biomedical Sciences Graduate Program

The Ohio State University

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Dissertation Committee:

Joanne Turner PhD Advisor

Prosper Boyaka PhD

Larry Schlesinger MD

Susheela Tridandapani PhD
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Abstract

The global demographic is shifting at a dramatic rate. Between the years 2000 and 2050, the number of individuals 60 years and older is expected to double, accounting for more than 20% of the total population worldwide. This shift in the distribution of the population is primarily a consequence of decreased fertility rates in women and increased life expectancy of elderly individuals. Although people are getting older, there is little evidence to suggest that they are also getting healthier. The World Health Organization recently reported that there has been no significant change in the rates of mild to moderate disability, including susceptibility to infectious disease, of elderly individuals in high-income countries. Increased susceptibility to infection in old age is made even more evident by the disproportionate rate of influenza, pneumonia and *Mycobacterium tuberculosis* infections with advanced age. Disease progression in the elderly is often prolonged and exacerbated leading to increased morbidity and mortality. In this work, we sought to gain further understanding of immune changes that can lead to disease susceptibility with advanced age.

Aging is often accompanied by a low-grade, systemic inflammation term inflammaging, which has been implicated in several age-associated diseases including coronary heart
disease and Alzheimer’s disease. Recently, inflammaging has been proposed to alter immune function in elderly individuals, although direct support for this concept is lacking. In this work, we demonstrate that old mice can also experience inflammaging, suggesting an evolutionarily conserved phenotype. We also demonstrate that inflammation in old age is associated with altered immune function. By modulating inflammation in both young and old mice, we were able to further define the impact of inflammation during *Mycobacterium tuberculosis* infection. Overall, this collective work expands upon our understanding of inflammation induced immune changes and how susceptibility to infectious disease in old age may be altered to enhance the lifespan and health span of elderly individuals.
Dedication

To my husband, Ben.

Thank you.
Acknowledgments

I would like to take this opportunity to thank my advisor, Dr. Joanne Turner. Thank you for taking the time and energy (A LOT of time and energy) into helping me become a better thinker and a better scientist. Thank you for fostering a great scientific environment and always being available when I was in need of assistance. You have such patience and such an amazing ability to mentor. Most of all, I cannot express how grateful I am that you allowed me to become a part of your research team.

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Vita

June 2005..................................................Diploma, Dublin Scioto High School

June 2009..................................................B.S. Biology, Ohio State University

2009-2011.................................Research Technician, Ohio State University

2011-2016.........................Graduate Research Associate, Dept of Microbial Infection and Immunity, The Ohio State University

Publications


Fields of Study

Major Field: Biomedical Sciences

Emphasis: Immunology
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<th>Full Form</th>
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<tr>
<td>7-AAD</td>
<td>7-aminoactinomycin</td>
</tr>
<tr>
<td>ALF</td>
<td>alveolar lining fluid</td>
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<td>APC</td>
<td>antigen presenting cell</td>
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<tr>
<td>BAL</td>
<td>bronchoalveolar lavage</td>
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<tr>
<td>BM</td>
<td>bone marrow-derived</td>
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<tr>
<td>CIITA</td>
<td>class II major histocompatibility complex transactivator</td>
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<tr>
<td>CFU</td>
<td>colony forming units</td>
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<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
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<tr>
<td>COX</td>
<td>cyclooxygenase</td>
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<tr>
<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>CSF</td>
<td>colony stimulating factor</td>
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<tr>
<td>DAMP</td>
<td>damage-associated molecular pattern</td>
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<tr>
<td>DC</td>
<td>dendritic cell</td>
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<tr>
<td>ETP</td>
<td>early thymocyte progenitors</td>
</tr>
<tr>
<td>G-CSF</td>
<td>granulocyte-colony-stimulating factor</td>
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<tr>
<td>GFP</td>
<td>green fluorescence protein</td>
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<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony-stimulating factor</td>
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<tr>
<td>HSC</td>
<td>hematopoietic stem cell</td>
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<tr>
<td>HSV</td>
<td>herpes simplex virus</td>
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<tr>
<td>IFNγ</td>
<td>interferon-gamma</td>
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<tr>
<td>IDO</td>
<td>indoleamin-2,3-dioxygenase</td>
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<tr>
<td>Abbreviation</td>
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<tr>
<td>RT-qPCR</td>
<td>real-time quantitative polymerase chain reaction</td>
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<td>RU</td>
<td>relative unit</td>
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<tr>
<td>TB</td>
<td>tuberculosis</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>TEC</td>
<td>thymic epithelial cells</td>
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<tr>
<td>TFR</td>
<td>total fertility rate</td>
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<tr>
<td>TLR</td>
<td>toll-like receptor</td>
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<tr>
<td>TNFα</td>
<td>tumor necrosis factor-alpha</td>
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<tr>
<td>Treg</td>
<td>regulatory T cells</td>
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<tr>
<td>WNV</td>
<td>West Nile virus</td>
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Chapter 1: An introduction to an aging world

**The global aging population**

The elderly population is increasing at a remarkable rate. Currently, among the 7 billion people worldwide, it is estimated that roughly 9% of the total population are among the elderly (aged 65 and older). It is projected that this number will almost double by the year 2050 accounting for 1.6 billion people worldwide. In contrast to the growth of the aging population over the next 35 years, the total youth population (<20) is projected to drop by 438 million. This has resulted in a dramatic shift in global population growth trends where for the first time in documented history, elderly individuals 65 years and older will outnumber children under the age of 5.

**Declining fertility rates: the cause of current population shifts**

The major driving factor behind population aging is declining fertility rates. The total fertility rate (TFR) refers to the average number of children that a woman will have in her lifetime. The global fertility rate is currently 2.5 children per woman compared to the
1970s where the global average TFR was 4.5 children per woman. This global average however, masks wide regional differences with more developed regions (Europe, northern America, Australia/New Zealand and Japan) having a current TFR of 1.7 and less developed regions (Africa, Asia (except Japan), Latin America, and the Caribbean including Melanesia, Micronesia and Polynesia) at a higher TFR of 2.6. This suggests that several developed, and some developing countries are currently below the replacement rate (2.1) which is the necessary fertility rate for a community to sustain its population. This suggests that countries which have been below the replacement rate are expected to experience a total population decline. For example, China, with a TFR of 1.6 in 2015 is expected to experience a 60 million population decline by 2050. In the United States, the TFR peaked after World War II at around 3.5 but has continued to decline, reaching a TFR of 2.5 in the 1970s and is expected to drop to an all-time low of 1.9 by the year 2050. This suggests that the United States will begin to experience population decline within the next century.

The economic impact of an aging nation

As a result of decreasing fertility rates, and increasing life expectancy, the percentage of the population that is in the labor force has seen a drastic decline and is projected to further decrease over the next 35 years. If this projection holds true, the aging of America would have created a situation in which relatively few workers will be supporting a growing population of elderly individuals. Consequently, governmental programs such as
Social Security will experience a decline in the total number of workers who are paying taxes to support the services that older people need\(^6\). To contend with the decrease in taxable workers, policy makers are now suggesting solutions including reduction in retirement benefits and/or raising the normal retirement ages in order to combat the exhaustion of these programs\(^6\).

The tremendous shift in aging demographics will also put new pressure on the healthcare system. Due to projected changes to government programs including Social Security, and Medicaid, the annual out-of-pocket costs for elderly individuals is estimated to more than double by 2040, putting the elderly under significant financial strain\(^7\). Furthermore, older Americans are disproportionally affected by chronic conditions as well as infectious diseases resulting in a per person health care spending that is 3-5 times more than those younger than 65 years old\(^8\). Additionally, it is projected that insufficient healthcare providers will be available for elderly individuals. By the year 2030, the total number of geriatricians is projected to total 7,750 or roughly one geriatrician for every 4,300 older Americans\(^9\), suggesting inadequate opportunities for health maintenance and health improvements. Health related consequences of aging will have significant financial and social impacts, therefore it is imperative that we try to understand the immune changes that occur with old age in order to find and discover opportunities for effective interventions and to promote healthy aging.
Immune changes in old age

Aging is accompanied by immunosenescence or alterations in the immune system. The immune system is a combination of biological structures, processes and specific functions within an organism that protects itself against disease. To recognize foreign objects, the innate immune system, an evolutionarily conserved host defense mechanism \(^1\), distinguishes between self and nonself using a limited number of receptors or pattern recognition receptors (PRR) and secreted proteins that are encoded in the germline which can recognize common features of many pathogens, termed pathogen-associated molecular patterns (PAMP) \(^2\). The innate immune system also contributes to the induction of the adaptive immune response against specific pathogen control \(^3\). The adaptive immune system can modify and improve its recognition of its target antigen, allowing for adapted responses specific for the control of a particular foreign entity. However, with increasing age, altered cellular function is thought to contribute to increased age-associated susceptibility to infection.

Elderly individuals are more susceptible to infection as increased rate of urinary tract \(^4\), influenza \(^5\), pneumonia \(^6\) and *Mycobacterium tuberculosis* infections \(^7\) are seen with advanced age. In addition, morbidity and mortality rates increase in old age due to the inability to recover from infection \(^8-10\). A recent study suggests that 87.9% of all annual influenza-associated death was among individuals 65 years and older \(^11\). Furthermore, when West Nile virus (WNV) first appeared in the United States, a significant number of individuals with greater severity of disease occurred in either the elderly or those with...
immunocompromised immune systems\textsuperscript{22}. Initial studies examining WNV infection in mice demonstrated that aged mice were 4-6 times more likely to succumb to infection\textsuperscript{23}. Protecting the elderly from infectious disease is made harder by the diminished response they have to vaccination\textsuperscript{24}. Alterations in immune function with increasing age were recognized decades ago and although research has henceforth provided us with numerous studies and observations, the literature is unfortunately littered with contrary results making it hard to truly understand age-associated immune changes. Here, we try to summarize the current concepts and understandings of the fundamental changes in immune function with old age.

\textit{Adaptive immune function in old age}

The adaptive immune system has received the greatest attention in the aging field due to early observations showing impaired T and B cell function. In the 1960s, B cells from old mice were shown to have decreased responsiveness to sheep red cells demonstrated by a decrease in the number of antibody forming cells after vaccination\textsuperscript{25}. Later, cytotoxic T cell function was examined in influenza infected old mice. Effros, R.B. \textit{et al.} showed that \textit{in vitro}, cytotoxic T cells, which peaked at day 5 in young infected mice, were delayed in old mice and appeared at days 7-9 in old mice, suggesting a shift in the kinetics of the response\textsuperscript{26}. Furthermore, the magnitude of the peak response at day 5 in young mice was significantly higher compared to the peak response in the elderly at days 7-9. Early studies of impaired adaptive immune function were further supported in human studies
examining mitogen-induced proliferative responses in different age groups. T cell mitogens, phytohaemagglutinin and concanavalin A as well as the B cell pokeweed mitogen were used to stimulate peripheral blood mononuclear cells (PBMC). T cell mitogen-induced proliferation started to decline at age 40 in response to at least one of the mitogens, with both becoming significantly decreased starting at age 60 and older. B cell mitogen-induced proliferation started to decline, although not significantly, at age 60 and finally became significantly decreased in the 80 and 90 year old age groups. This study concluded that mitogen-induced proliferative responses decreased with increasing age, with the largest differences becoming apparent between the ages of 60 and 90 years old. Current studies examining T and B cell function in old age have continued with more sophisticated techniques delving into the complexities of the adaptive immune system in the elderly.

*T cell alterations with advanced age*

T cell progenitors arise from hematopoietic stem cells (HSC) within the bone marrow and travel to the thymus for further differentiation and maturation. Of all the different lymphatic organs, the thymus is the most structurally consistent across species. It is divided into distinct sections with an inner cortex and a peripheral medulla separated by a vascular corticomedullary junction. Depending on their developmental stage, T cell precursors may be found in different parts of the thymus where they differentiate from CD4-CD8- (double negatives) through CD4+CD8+ (double positives) to either CD4+ or
CD8+ single positive T cells through the process of positive and negative selection. After development, naïve T cells exit the thymus where they circulate through secondary lymphoid organs in order to protect the body from non-self molecules. Upon encountering their specific cognate antigens, activation of distinct T cells will lead to specific effector function such as cytokine production or the release of cytotoxins. Activated T cells may then develop into memory T cells which can persist long-term within the body after the resolution of an infection to ensure a faster and stronger immune response during a second encounter.

**Structural changes of the thymus in old age**

One of the most prominent changes of the immune system with increased age is the regression, or involution of the thymus. Thymic involution is conserved amongst almost all vertebrates suggesting an evolutionary trait. Thymic involution in old age is characterized by a loss of tissue mass and organized architecture and an increase in deposition of adipocytes. Thymic involution is often thought to occur during puberty based on evidence of chemical or surgical castration of male mice leading to restoration of thymic size and function. In support of this notion, the addition of androgens and estrogens in uncastrated mice results in decreased T cell generation and maturation. However, recent accounts suggest that thymic involution occurs earlier in life, with changes being evident even before puberty or soon after birth. This finding is consistent with observations in other species including mice and horses. These new
lines of evidence suggest that levels of sex hormones might contribute to the diminishing state of the thymus with age but they are not the only factors responsible. Interestingly, after the initial loss of thymic structure and function, involution proceeds at a lower rate suggesting that thymic involution is not uniform throughout life.

**Age associated alterations in thymus microenvironment**

Thymopoiesis, or the process by which a thymocyte develops and differentiates into a mature T lymphocyte, involves a series of sequential developmental steps which have exhibited age-related alterations in almost every single step. T cell development begins with the generation of T cell precursors from HSC within the bone marrow. In old age, several studies have demonstrated that HSCs exhibit a bias towards increased myeloid progenitors with a reduced capacity towards lymphoid progenitors resulting in a reduced number of T cell precursors, or early thymocyte progenitors (ETP), seeding the thymus as well as reduced proliferation and differentiation potential. Single positive T cells from elderly individuals also exhibit decreased CD3 expression which may contribute to impaired TCR dependent activation upon thymus emigration. However several studies also suggest that altered T cell function in old age is not an intrinsic property but driven from changes in the microenvironment of the thymus.

The microenvironment of the thymus is profoundly important in T cell development because the thymus provides necessary signals to drive T cell differentiation and survival
including Notch signaling\textsuperscript{50}, IL-7 production \textsuperscript{51,52} and positive and negative selection \textsuperscript{53}. However, the thymic integrity is disrupted with age leading to alterations including increased adipose tissue and increased senescent thymic epithelial cells (TEC) \textsuperscript{54-57}. TECs in old age express decreased MHC class II and changes in cortical and medullary markers \textsuperscript{58,59} as well as decreased IL-7 production \textsuperscript{60}. IL-7 supplementation of older mice \textsuperscript{61,62} and rhesus macaques \textsuperscript{63,64} has been shown to increase naïve T cell output. Furthermore, addition of exogenous keratin growth factor to stimulate TECs has shown enhanced thymic cellularity and improved immune function in old mice \textsuperscript{65}. This suggests that T cell impairment can be rescued by the restoration of the thymus structure and function. In addition, fetal thymus transplant under the kidney capsule of young and old mice displayed similar numbers and proportions of developing T cells \textsuperscript{66,67}. Furthermore, intrathymic injections of young ETP failed to develop in older animals but fully developed in the thymus of young recipients \textsuperscript{68}. When peripheral T cells were depleted in naïve aged C57BL/6 mice by irradiation and reconstituted with young bone marrow cells, the phenotype and cytokine profile of the newly generated CD4\textsuperscript{+} T cells in aged animals were indistinguishable from that found in age-matched control \textsuperscript{69}. This suggests that the acquired T cell defect in old age may be due to the inability of the aged thymic microenvironment to support and maintain proper thymopoiesis.
Changes in peripheral T cell homeostasis

Naïve T cells circulate through secondary lymphoid organs in search of their cognate antigen. Some cells will find their cognate antigen, undergo T cell activation and move into the periphery \(^{70}\), and in some cases, differentiates into long-lived memory T cells. Other naïve T cells never encounter their cognate antigen and so they continue to circulate in the periphery or die to make space for newly emerging cells from the thymus \(^{71}\). In order to maintain this dynamic homeostatic environment, T cells are tightly regulated to preserve a constant pool circulating in the periphery. Even in old age, the absolute number of circulating T cells remains similar to young individuals \(^{72}\). However, age-associated thymic involution is thought to lead to a 10-fold reduction in T cell output \(^{31,73,74}\). This drastic decline in new T cells leads to the loss of at least half of naïve CD8+ T cells \(^{72,75}\) but only results in a slow reduction of total circulating naïve CD4+ T cells in old mice \(^{76,77}\). The ability of the aging environment to maintain a constant level of circulating T cells is thought to be due to the ability of naïve CD4+ T cells in the periphery to increase their lifespan, \(^{78}\) as well as increased total number of CD8+ memory cells \(^{79}\) (both from persistent infections as well as virtual memory cells). Experiments examining the survival time of CD4+ T cells from non-transgenic mice by the transfer of CD4+ T cells from aged mice to young hosts concluded that aged naïve cells had a longer survival time leading to a larger accumulation in the peripheral secondary lymphoid organs within the young mice \(^{78}\). In support of this notion, other studies have demonstrated that the pro-apoptotic protein Bim, a known T cell regulator, is reduced in old age and contributes to increased CD4+ T cell survival in the periphery \(^{80}\). Although
the absolute number of T cells is maintained in the periphery in old age by longer survival of naïve T cells and increased CD8+ memory cells, this consequently leads to decreased T cell receptor (TCR) diversity which suggests decreased ability to respond to a wider variety of antigens\textsuperscript{81,82}.

**Changes in CD4+ T cell function with increasing age**

In order to generate a proper immune response, CD4+ T cells must bind to their cognate antigen as displayed by an antigen presenting cell. In old age, the absolute number of naïve CD4+ T cells capable of forming a synapse with antigen presenting cells (APC) falls approximately to half compared to cells from a young mouse\textsuperscript{83}. In addition, when cells do form a proper synapse, signaling of the TCR is compromised with altered cytoskeletal rearrangement and delayed recruitment of downstream signaling molecules to the lipid rafts\textsuperscript{84}. Alterations continue downstream of signaling as demonstrated in both *in vitro* and *in vivo* conditions. A well-documented TCR engagement dysfunction downstream of CD4+ T cells in old age is decreased IL-2 production, a key cytokine required for survival and proliferation of T cells\textsuperscript{85}. Transgenic mouse models have shown that CD4+ T cells from old mice produce less IL-2 and have decreased proliferation and effector function, including a poor activation phenotype and decreased cytokine production\textsuperscript{86}. When exogenous IL-2 was added to cultures *in vitro*, expansion and effector function of CD4+ T cells from old mice were restored\textsuperscript{86}, but impairment in the generation of functional memory could not be overcome by exogenous IL-2 treatment.
This suggests that IL-2 may contribute to some but not all T cell dysfunction in old age. Interestingly, memory CD4+ T cells respond well to antigen in old age when those memory cells are generated from CD4+ T cells at a younger age. This suggests that the age of the mouse and/or naïve T cell is important during the first encounter with its cognate antigen. Since CD4+ T cells from aged hosts have increased survival time and decreased turnover to maintain T cell number in the periphery, circulating T cells may be “older” which could contribute to the impairment of CD4+ T cells effector and memory function in old age. In support of this idea, when peripheral CD4+ T cells were depleted in a transgenic mouse model, newly generated naïve CD4+ T cells displayed robust responses to antigen ex vivo and in vitro including increased cytokine production and robust generation of antigen-specific B cell expansion.

**Changes in CD8+ T cell function in old age**

Similar with CD4+ T cells, impairments have been observed and documented in CD8+ T cell function with old age. On an organism level, CD8+ T cell impairment has been documented in several infection models. CD8+ T cells show impaired cytotoxic killing compared to young mice after influenza virus infection, which is correlated with delayed viral clearance and prolonged viral infections. Similarly, after WNV infection, old mice are more likely to succumb to mortality which is thought to be the result of impaired CD8+ T cell proliferation, differentiation and function. In studies of *Listeria monocytogenes* and respiratory syncytial virus (RSV) infections, where CD8 T cells play
a critical role in mediating clearance, greater mortality was observed in old mice when compared to young mice \(^\text{89,90}\). On a cellular level, CD8\(^+\) T cells exhibit decreased proliferative capacity \(^\text{91-94}\), decreased upregulation of activation markers \(^\text{89,95}\), decreased production of cytokines and lytic proteins \(^\text{89}\) as well as decreased lytic abilities \(^\text{26}\).

Extensive studies have delved into the impact of persistent viral infections on CD8\(^+\) T cells in old age. These viruses, including herpes simplex virus (HSV) and cytomegalovirus (CMV) are ubiquitous, where calculations extrapolated from antiviral antibodies in serum predict that almost 6.75 billion individuals harbor multiple (upwards of 8-12) latent viral infections \(^\text{96}\). Persistent viral infections provide a large amount of epitopes for the immune system to detect, and continual interaction between reactivating latent viruses and naïve T cells can lead to “memory inflation” where a significant proportion of the T cell pool is specific to persisting viruses in both humans and mice \(^\text{97,98}\). CMV infection of young mice has shown that long term viral persistence (but not shorter than 12 months) can lead to impairment in CD8\(^+\) T cell responses to new pathogen challenge \(^\text{99,100}\). However, these studies demonstrate that the decrease in naïve CD8\(^+\) T cell numbers, commonly observed in old age, was not seen in CMV long term infected young mice. Further support has been shown in human studies where loss of naïve CD8\(^+\) T cells was correlated strictly with aging and no difference was seen in CMV (-) or CMV (+) individuals \(^\text{101,102}\). These studies suggest that persistent viral infections play a role in modulating CD8\(^+\) T cell response to new pathogens but do not affect total CD8\(^+\) T cell numbers in old age.
**B cell alterations with advanced age**

Cells within the hematopoietic compartment of the bone marrow, such as stromal cells, are lost with advanced age and replaced with adipocytes\textsuperscript{103,104}. Loss of stromal cells, which is important in promoting the survival of HSCs, greatly reduces the frequencies of HSCs capable of generating B cells \textit{in vitro}\textsuperscript{105}. Subsequently, the pool of available pre-B cells becomes smaller\textsuperscript{106,107}. In addition, the expression of crucial genes and transcriptional regulators that are important in B cell development within the bone marrow is decreased in old age\textsuperscript{107-109}. Consequently, this results in a decrease in B cell output from the bone marrow as demonstrated by \textit{in vitro} labeling in old mice\textsuperscript{110-112}. B cell output from the bone marrow in old mice is significantly reduced compared to the output observed in young mice\textsuperscript{112,113}, however, similar to the peripheral total T cell numbers, follicular B cells are thought to remain relatively constant with old age. This has been shown to be the result of decreased B cell turnover\textsuperscript{112,114}, increased expansion of memory B cells\textsuperscript{115-117} and decreased repertoire diversity\textsuperscript{118,119}.

The most extensive research regarding B cell function in old age has been in the study of vaccine efficacy. In general, vaccination experiments in the elderly have shown reduced antibody responses as well as cell-mediated immunity to viral infections. Besides defects in cell maturation and peripheral B cell pool distribution which could affect B cell function, the generation of new plasma cells in old age is thought to be hampered due to decreased formation of germinal centers leading to decreased somatic hypermutation, and antibody affinity maturation\textsuperscript{120,121}. A study examining antibody responses to influenza
vaccine determined that roughly 90% of adults were able to produce viral hemagglutinin neutralizing antibodies while only 57% of elderly subjects could make hemagglutinin neutralizing antibodies. The report concluded that not only did B cells from aged subjects produce fewer antibodies, the antibodies that were produced had decreased neutralization abilities. Another study examining immune responses to live attenuated yellow fever vaccine showed that elderly individuals had significantly elevated viremia associated with delayed early antibody titers following vaccination. However, roughly 30 days post-vaccination, the antibody titers were similar between young and elderly individuals. Taken together, these studies suggest that antibody responses to new antigen develop more slowly and are less protective while memory B cells responding to previously encountered antigens produce fewer antibodies and have decreased function.

Innate immune function in old age

Changes within the innate immune system in old age have not been well characterized. This is in part due to the lack of available data, since major research focused on age-associated dysfunction within innate immune cells did not occur until the late 1980s. Initial studies were focused on natural killer cell dysfunction including their reduced cell lysis activity in old age. Following discoveries of natural killer cell dysfunction in old age, widespread aging research was initiated in other cell types within the innate immune system. Although the general consensus is that cellular function is reduced in old age, contradictions within specific cell types have been observed.
Neutrophils are the most abundant type of leukocytes found in the human body and are one of the first inflammatory cell types to migrate towards the site of inflammation or pathogen invasion, through a process called chemotaxis. Neutrophils are known to have three main mechanisms to combat invading pathogens including ingestion of pathogen through phagocytosis and the generation of reactive oxygen species (ROS), release of soluble antimicrobial substances known as degranulation and the generation of neutrophil extracellular traps (NET). Microorganisms which are phagocytosed cause the formation of phagosomes, a pathogen containing vacuole within the cell, and undergo phagosome maturation where fusion with antimicrobial mediators occurs. Degranulation occurs when stored proteinases and antimicrobial peptides fuse with the plasma membrane causing extracellular release of contents during neutrophil activation. Lastly, neutrophils can control invading pathogens by a form of cell death known as NETosis where decondensed chromatin fibers decorated with granules and enzymes are released into the extracellular space. However, neutrophil function is significantly altered in old age.

Interestingly, alterations of neutrophils in old age are not a consequence of myelopoiesis or the regulated formation of myeloid cells, including neutrophils, in the bone marrow. Although it has been noted that the proliferation of neutrophil precursors was reduced in response to one lineage-specific cytokine, granulocyte-colony-stimulating factor (G-CSF), newly generated neutrophil numbers remain similar compared to circulating
neutrophils in young hosts. Generation of neutrophils in old age is thought to be compensated by other growth factors including granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-13, where proliferative responses are not altered with advanced age\(^\text{132}\).

Furthermore, the number of circulating neutrophils during acute inflammation is not significantly different in elderly hosts\(^\text{133}\). Instead, defects of neutrophil function are thought to start with chemotaxis and adhesion.

**Chemotaxis and adhesion**

Neutrophils move to a site of infection by following a gradient of chemotactic molecules or chemokines. To enter the site of infection, neutrophils roll along and attach to the endothelial cells through CD15 and integrins, including CD11a and CD11b, enabling sustained adhesion and eventual transmigration towards the site of injury\(^\text{125,126}\). Historically, *in vitro* reports of chemotaxis and adhesion in neutrophils from old mice did not show significant differences compared to young mice\(^\text{134,135}\). Adhesion studies of neutrophils from elderly humans have revealed increased adhesion to nylon fibers\(^\text{136}\) but no differences to endothelium\(^\text{137,138}\). In addition, the level of neutrophil receptors including CD15, CD11a and CD11b are reported to be unaltered\(^\text{138}\) or slightly increased with old age\(^\text{137,138}\), contributing to the notion of unaffected adhesion abilities in neutrophils from elderly donors. Increased levels of neutrophil receptors at basal level are
thought to compensate for in vitro defects in receptor mediated responses\textsuperscript{137} resulting in a net gain/loss in function via multiple mechanisms. Recent studies however, have suggested that neutrophil defects in old age are due to a decrease in accuracy of neutrophil migration or a decrease in chemotaxis but preserved chemokinesis (induced motility) towards a variety of inflammatory stimuli\textsuperscript{139}. In addition, old mice intratracheally infected with Pseudomonas aeruginosa demonstrated inaccurate neutrophil migration where high numbers of neutrophils were stranded in the parenchyma compared to the neutrophils of young mice which were recruited to the alveolar space for better control of infection\textsuperscript{140}. Taken together, new lines of evidence suggest that poor neutrophil migration in old age might be due to inaccurate neutrophil migration and not chemokinesis.

**Antimicrobial responses**

Unlike reports for chemotaxis, phagocytosis by neutrophils is consistently thought to be impaired in old age. Studies measuring the phagocytic abilities of neutrophil for opsonized bacteria, yeast and zymosan have all shown a significant reduction in phagocytic ability by neutrophils from the elderly\textsuperscript{141,142}. Although the mechanism of reduced phagocytosis in neutrophils from the elderly is unknown, several reports have eluded to contributing factors for decreased function. Emmanuelli et al. showed that phagocytosis of unopsonized bacterial targets by neutrophils occurred at the same rate between young and old donors,\textsuperscript{142} suggesting that neutrophil receptors necessary for
innate recognition of bacterial components is not altered with age. Since serum immunoglobulin are within normal ranges and complement levels were shown to be elevated in the elderly\textsuperscript{137}, it has been suggested that receptors necessary for opsonization may have compromised signaling function. In addition, Roa \textit{et al.} demonstrated that actin polymerization, essential for phagocytosis, was impaired in neutrophils from elderly donors\textsuperscript{143} which may contribute to defective neutrophil phagocytosis in old age. Recently Qian \textit{et al.} demonstrated that neutrophils from elderly humans exhibited impaired responses to Toll-like receptor (TLR) 1 stimulation which correlated with a decrease in intracellular glucose accumulation\textsuperscript{144}. Defective bioenergetics or cellular transformation of energy may impact several neutrophil functions including phagocytosis during old age. Interestingly, despite reports of reduced ability for phagocytosis in neutrophils from aged hosts, ROS production has been reported to be unaltered in response to stimulation by zymosan or lipopolysaccharide (LPS) with advanced age\textsuperscript{145,146}.

Unlike the consistent reports for phagocytosis, oxidative burst and degranulation from neutrophils in old age have generated much conflicting data\textsuperscript{138,139,147-149}. Interestingly, a recent paper by Wenisch \textit{et al.} demonstrated that superoxide generation by neutrophils was decreased in response to \textit{Staphylococcus aureus} but not \textit{Escherichia coli}\textsuperscript{150}. This suggests that conflicting data in the field of neutrophil function may be a result of differences in the ability of neutrophils from the aged hosts to distinguish between gram-negative and gram-positive bacteria.
NETs, are chromatin mesh containing antimicrobial proteins such as neutrophil elastase released out of the cell in order to trap and eliminate pathogens. *In vitro*, neutrophils from aged mice produce fewer NETs in response to *Staphylococcus aureus*, LPS and IL-8 stimulation\textsuperscript{151,152}. *In vivo*, fewer NETs were present at the site of *Staphylococcus aureus* skin infection which contributed to the increased dissemination of *Staphylococcus aureus* to other organs\textsuperscript{153}. Although the mechanisms for decreased NET formation in aged hosts are unknown, decreased NADPH in neutrophils associated with increased age\textsuperscript{151} is thought to play a role since NADPH is known to be important in NET formation\textsuperscript{154}. Taken together, neutrophil function in old age is thought to be impaired with decreased phagocytosis, altered degranulation (depending on pathogen) and decreased net formation.

**Monocyte alterations with advanced age**

Monocytes constitute a highly motile component of the innate immune system which can differentiate into professional APCs including macrophages or dendritic cells. There are at least three types of monocyte subpopulations: classical (CD14\textsuperscript{+}/CD16\textsuperscript{-}), non-classical (CD14\textsuperscript{low}/CD16\textsuperscript{high}) and intermediate monocytes (CD14\textsuperscript{high}/CD16\textsuperscript{low})\textsuperscript{155}. When stimulated using different TLR ligands, different monocyte populations exhibit different cytokine profiles reinforcing their distinct role and function\textsuperscript{156}. 
In old age, alterations in phenotype distribution occur within the monocyte population. An expansion of CD16+ monocytes is associated with increased age\textsuperscript{157,158}, as well as decreases in cell surface receptor expressions including CD62L\textsuperscript{159,160} which may impact monocyte adherence and migration to sites of inflammation.

**Cytokine production in response to toll-like receptor signaling**

TLRs can recognize conserved molecules associated with different groups of pathogens leading to activation of monocyte function. Cytokines are important molecules in cell signaling pathways which are released to affect their own function as well as those of other cells\textsuperscript{161}. Unfortunately, TLR stimulated cytokine responses in monocytes isolated from elderly host are not well defined due to conflicting data in the field. Stimulation of peripheral blood monocytes with different TLRs exhibit decreased,\textsuperscript{162,163} increased\textsuperscript{159}, or no changes\textsuperscript{157} in proinflammatory cytokine production.

**Macrophage alterations in old age**

Macrophages are professional phagocytes found throughout the body with various names and tissue specific function driven by the local microenvironment\textsuperscript{164}. Macrophages have many diverse functions within the immune system including a role in eliminating invading pathogens through phagocytosis, releasing mediators including cytokines and chemokines, as well as a role in activating the adaptive immune system through antigen
Macrophages also play an important role in tissue homeostasis by clearing apoptotic cells, tissue repair and resolution of inflammation \(^{166,167}\).

**Phagocytosis**

The phagocytic process starts when a phagocytic receptor on the macrophage cell surface binds to its respective ligand initiating intracellular signaling pathways. Activation of phagocytic receptors causes the rearrangement of the actin cytoskeleton \(^{168,169}\) which allows for the fusion of the cell membrane around the target pathogen into a vacuole known as a phagosome. As phagosomes mature, they fuse with lysosomes in a process known as phagolysosome fusion \(^{170}\) in order to help breakdown various particles engulfed by the cell. Lysosomes are vesicles found within cells that contain hydrolytic enzymes including cathepsins, lysosomal alpha-glucosidase and lysosomal acid phosphatase \(^{171}\). Resulting peptide fragments from the pathogen can then be loaded into major histocompatibility complex (MHC) class II molecules and migrate to the cell surface for antigen presentation.

Conflicting results have been reported regarding the phagocytic function between young and elderly hosts. An early study by De la Fuente in 1985 concluded that in elderly mice, peritoneal macrophages have decreased ability to phagocytose latex beads compared to macrophages from young mice \(^{172}\). This study was further supported using peritoneal macrophages from a different mouse strain \(^{173}\) as well as a macrophages isolated by the
subcutaneous sponge model\textsuperscript{134}, both of which showed decreased phagocytosis in macrophages from old mice. Microglia phagocytic function has also been shown to decrease with increasing age as less amyloid beta is internalized from old mice compared to young mice\textsuperscript{174}. However, other studies in old rats have shown that alveolar macrophages have an increased ability to phagocytose \textit{Klebsiella pneumoniae}\textsuperscript{175} and microglia from old rats have increased phagocytic activity based on increased uptake of quantum dots\textsuperscript{176}. Taken together, these conflicting results suggest that alterations in macrophage phagocytic function in old age may be tissue and pathogen specific.

\textbf{Cytokine production in response to toll-like receptor signaling}

It has been reported that splenic and thiglycollate stimulated peritoneal macrophages secreted lower amounts of proinflammatory cytokines including tumor necrosis factor-alpha (TNF\textalpha), IL-6 and IL-12 in response to several TLRs in aging mouse models\textsuperscript{177-179}. Decreased cytokine production was correlated with decreasing expression of TLR2, TLR4 and TLR9 in old age\textsuperscript{178}. However, a study examining alveolar macrophage cytokine production in response to influenza infection demonstrated delayed but enhanced TNF\textalpha and IL-1\textalpha message expression in macrophages isolated from old mice\textsuperscript{180}. Furthermore, a human study using monocyte-derived macrophages (MDM) has also demonstrated increased cytokine production in response to infection with WNV\textsuperscript{181}. Taken together, this suggests that macrophage cytokine production in old age is generally
thought to be decreased in response to TLR stimulation, however a few studies in both mice \(^{180}\) and humans \(^{181}\) have observed the opposite effects.

**Antigen presentation**

Consistent with other aspects of macrophage function in old age, conflicting data is also reported in antigen presentation. Discrepancies in antigen presentation responses were first documented in the 1980s when examining the ability of peritoneal macrophages from old mice to induce proliferation of lymphocytes. While one study found no differences in lymphocyte proliferation \(^{182}\) another report saw a decreased ability to induce proliferation \(^{183}\). Although conflicting results are reported in macrophage antigen presentation in old age, several recent studies support decreased function with aging. One particular study examining the ability of macrophages from old mice to stimulate lymphocytes observed diminished lymphocyte cytotoxicity and CD8\(^+\) T cell interferon-gamma (IFN\(\gamma\)) production in comparison with macrophages from young mice \(^{184}\). Further support for reduced antigen presentation was reported in CD8\(^+\) T cells from young mice which exhibited reduced clonal expansion and decreased IFN\(\gamma\) production when stimulated with peritoneal macrophages from old mice \(^{185}\). Decreased antigen presentation from peritoneal macrophages from old mice was correlated with a decrease in costimulatory molecule (CD80/CD86) expression as well as decreased cytokine and chemokine secretion \(^{185}\).
Dendritic cell alterations with advanced age

Dendritic cells (DC) are known as the messengers between the innate and the adaptive immune system because their primary function is to process, migrate to the draining lymph nodes and present antigens to adaptive immune cells. Besides antigen presentation, dendritic cells also have the ability to induce cytokine production \textsuperscript{186}. Information about alterations in dendritic cell function in old age is conflicting with some studies citing impaired responses while others show preserved or enhanced responses.

Activation, migration and priming

One early paper looking at DC function in old age utilized mice of various ages including 6, 46 and 95 week old mice and concluded that DC priming was not defective in old age based on comparable T cell proliferation magnitude and kinetics \textsuperscript{187}. More recently, several reports supported this observation of preserved function. In one study, activation of splenic and bone-marrow derived DCs in response to different TLR agonists including TLR2, TLR3, TLR4, TLR5 and TLR9 resulted in similar mean fluorescence intensity (MFI) of CD40 and CD86 co-stimulatory molecules from young and aged mice \textsuperscript{188}. The same study also showed that in an infection model with lymphocytic choriomeningitis virus (LCMV), where virus-specific young CD8+ T cells were adoptively transferred into young and old mice, young CD8+ T cells were equally well primed with similar IFN\(\gamma\) production, expansion and acquisition of activation markers including CD62L and CD27...
regardless of whether the host DC was from young or aged mice. In contrast, when T cells from young or aged mice were adoptively transferred into young T cell deficient animals, and infected with LCMV, only the aged T cells demonstrated impaired anti-viral responses suggesting that the defect in old age is T cell dependent. In another recent study, the absolute number of DCs from the spleen and mediastinal lymph node was reported to remain consistent in young and old mice. Basal expression levels of co-stimulatory molecules including CD80 and CD40 were not significantly different on DCs from multiple organs including the spleen, mediastinal lymph node and the lung. In vitro, DCs from young and old mice were both equally capable of inducing T cell proliferation when using antigen specific and cross-presentation experiments. Furthermore, in an in vivo influenza infection model, migration of DCs into the lungs was comparable between young and old mice and upregulation of CD40 and CD80 on DCs were also similar following influenza infection of young and old mice.

However, several new studies also challenge the notion that DCs are unaffected in old age. When examining DC induced CD4+ T cell function in an adoptive transfer model, old mice had decreased CD44 expression as well as decreased IFNγ production as indicated by an intracellular staining assay. This decrease in CD4+ T cell function was correlated with decreased MHC-peptide complexes in DCs from old mice as well as decreased levels of CD40, suggesting decreased antigen presentation and decreased activation. A defect in DCs in old age was further supported by a study using Listeria monocytogenes. Prior to infection, basal expression levels of MHC II and CD40 were decreased in CD8+ splenic DCs. After infection, DCs from elderly mice had decreased
migratory abilities towards the spleen and splenic DCs continued to express decreased levels of MHC II, CD40 and CD86 compared to cells from adult mice. In an *Encephalitozoon cuniculi* oral infection model, mucosal CD11c+ DCs were less able to prime splenic T cells as seen by decreased proliferation of Ag-specific T cells and decreased cytolytic activities against parasite-infected macrophages. In addition, while IFNγ transcript levels increased in young mice after *E. cuniculi* infection in both the spleen and the lymph node (LN), IFNγ did not rise above background levels in old mice in either organ. Furthermore, monocyte-derived myeloid DCs from elderly humans cultured *in vitro* have shown reduced migration towards a chemokine gradient as well as reduced phagocytosis of apoptotic cells. Therefore, with numerous studies supporting both diminished and unaltered dendritic cell activation and priming in old age, more research characterizing cell phenotype and responses to different pathogens will be necessary in order to truly understand the age-associated effects.

**Cytokine production**

In mice, one report suggested that in response to several TLR agonists, splenic and bone marrow-derived (BM) DCs showed preserved IL-6, TNFα and IL-12p40 production. Using only a TLR4 agonist, LPS, another reported similar findings where no differences were observed between TNFα and IL-12p70 production in young and old stimulated BMDCs. While another report agreed with the preserved function of IL-12p70 production after LPS stimulation, IL-6 and TNFα production was significantly decreased
in DCs from draining LNs of old mice\textsuperscript{190}. Similar findings were observed in LPS stimulated bone marrow-derived CD11c+ cells, where no differences were observed in IL-12p70 but TNF\textgreek{a} and IL-6 production was decreased\textsuperscript{194}. One study in rhesus macaques also observed a defect in DCs with age as stimulation with TLR2/6 or TLR4 showed decreased frequency of TNF\textgreek{a} and IL-6 positive cells. In humans, it has been reported that monocyte-derived DCs cultured with GM-CSF and stimulated with LPS or a TLR7 agonist have shown increased IL-6 and IL-12p40/70 production\textsuperscript{192} while myeloid DCs from PBMCs have shown decreased IL-6 and TNF\textgreek{a} production in response to a broad range of TLR activators including TLR2, TLR3, TLR5 and TLR7\textsuperscript{195}. Taken together, these data suggests that IL-12 production by DCs seems to be preserved in old age however, other cytokines, including TNF\textgreek{a} and IL-6 production may be decreased or unaltered depending on the dendritic cell source.

**Natural killer cell alterations with advanced age**

Natural killer (NK) cells are known for their ability to recognize and eliminate viral-infected and malignant cells. NK cells mediate immune control mainly through two mechanisms including (1) cell cytotoxicity and (2) the secretion of cytokines and chemokines. Recently, new emerging data suggest that NK cells may be involved in other roles including anti-microbial defense\textsuperscript{196}, resolution of inflammation\textsuperscript{197,198} and modulation of the adaptive immune system\textsuperscript{199,200}. Thus, NK cell dysfunction in old age may have a more significant impact on elderly health.
**Natural killer cell cytotoxicity**

One of the most well studied mechanisms for NK induced cell death is through granule release \(^{201}\). NK cells confer host protection through the release of cytotoxic proteins including perforin and granzymes into the immunological synapse between an NK cell and the target cells \(^{201}\). Perforin is thought to trigger a membrane-repair response in the target cell membrane resulting in endocytosis of perforin and granzymes \(^{202}\). Endocytosed granzymes can then induce cell apoptosis by direct or indirect caspase activation \(^{203}\) as well as caspase-independent killing mechanisms such as cleavage of proteins necessary for DNA repair \(^{204}\). Research regarding NK cell cytotoxicity in old age has yielded conflicting findings including decreased \(^{205,206}\), unchanged \(^{207,208}\) or increased \(^{209,210}\) lytic function. These conflicting data are thought to stem from different subject inclusion criteria, the NK cell source (PBMCs vs purified NK cells) and assay protocols. Despite the contradictory results, the general consensus is that when looking within a purified NK cell population, single cell cytotoxicity is decreased with increasing age \(^{205,211}\).

**Cytokine production**

NK cells are also known to mediate their host defense function by secreting a multitude of immunoregulatory proteins including TNFα, IFNγ and IL-8 \(^{212}\). These cytokines help to amplify innate immune responses by enhancing cytotoxic activities of macrophages as well as drive the maturation of immature DCs for efficient antigen presentation \(^{213,214}\).
Furthermore, NK cells have recently been shown to be involved in T cell priming. In a study by Martin-Fontecha et al., NK cells were shown to migrate to the LNs in a CXCR3 dependent manner. Recruitment of NK cells provided an early source of IFNγ necessary for Th1 polarization. When NK cells were depleted, T cells capable of producing IFNγ were reduced by 90% compared to control mice. NK cells isolated from elderly subjects were still able to produce cytokines in response to different stimulus, however, the levels of cytokine generated were significantly reduced compared to those produced by NK cells from younger individuals.

Inflammaging or inflammation in aging

Interestingly, changes in the adaptive and innate immune system with increasing age are thought to be the result of an age-associated phenomenon, termed inflammaging. Inflammaging, characterized by a low-grade, chronic increase in circulating proinflammatory mediators such as cytokines and acute phase proteins, has been implicated in several age related diseases including type-2 diabetes mellitus, osteoporosis, rheumatoid arthritis and Alzheimer’s disease. Although a correlation has been observed between inflammaging and altered immune function in old age research examining the direct effects of inflammation on immune function in old age is lacking. Interestingly, although characteristics of inflammaging are easy to detect, the mechanism for the generation of inflammation in old age is still unknown, though several theories have been proposed.
Molecular mechanisms of inflammaging

The mitochondria, which is the primary site for respiration and energy production, is necessary for regulation of normal cellular function including cellular metabolism, apoptosis-programmed cell death, as well as signaling through mitochondrial ROS. In normal physiological conditions, ROS are generated as a byproduct of the metabolism of oxygen and have important roles in cellular signaling including the induction of host defense genes and mobilization of ion transport. While ROS are byproducts of normal cellular function, excessive amounts can cause deleterious effects. Enzymes like catalase and superoxide dismutase can ameliorate the damaging effects of certain ROS, including hydrogen peroxide and superoxide, by converting these species to water. However, this conversion is not completely efficient, and residual peroxide can persist within the cell increasing in concentration with advanced age. Highly sustained levels of ROS can lead to oxidation and irreversible changes in DNA, amino acids in protein, lipids and deactivation of specific enzymes which can cause dysregulation of cell and organ function and to overall system decline. ROS and the damage that it causes are known to promote inflammatory responses through TLR and inflammasome activation. TLRs are now known to be able to respond to damage-associated molecular patterns (DAMPs) including ROS modified molecules, leading to activation and production of pro-inflammatory mediators like IL-1, IL-6 and TNFα. Inflammasomes, which are multiprotein oligomers, function to promote innate immune responses through the maturation of the inflammatory cytokines IL-1β and IL-18. Recently, Nalp3, a specific inflammasome, has been shown to be directly activated by the presence of...
sustained amounts of ROS \textsuperscript{234-236}. This suggests that increasing ROS with aging may lead to the chronic inflammation observed in old age.

Additional mechanisms for the generation of inflammation in old age are telomere shortening and cellular senescence. Telomeres are the repetitive nucleotide sequences found at the ends of a chromosome, protecting it from degradation \textsuperscript{237}. When DNA is duplicated during chromosome replication and cellular division, the enzyme necessary for duplication cannot process the ends of a chromosome, so in each duplication the end of the chromosome is truncated. Telomeres, in short, act as a buffer at the ends of each chromosome to protect the genes from truncation. Current evidence suggests that telomere attrition, known to be increased in old age, can trigger cellular senescence \textsuperscript{238,239} which is associated with the secretion of proinflammatory cytokines, chemokines and proteases termed the senescence-associated secretory phenotype (SASP), contributing to the chronic inflammation associated with aging \textsuperscript{240,241}.

Interestingly, the relationship between the cause and effect of inflammaing is thought to be bidirectional. This suggests that inflammation can drive mitochondrial damage which can induce inflammation dependent telomere dysfunction \textsuperscript{242} leading to further inflammation generated mitochondrial damage. This “chicken or the egg” dilemma suggests that, once initiated, inflammaing becomes a vicious cycle where increased inflammation can induce the generation of more inflammation through dysfunction and damage. Similarly, it has been proposed that inflammaing is a result of altered immune function in old age but at the same time altered immune function continues to perpetuate
inflammaging. Unfortunately, the effects of age-associated inflammation and their ability to alter immune function are not well characterized. To truly understand if inflammaging can alter immune function in old age, we propose to modulate the inflammatory environment in a naïve old mouse. Furthermore, in order to recognize differences in immune control, we used *Mycobacterium tuberculosis*, an infectious disease that is relevant in old age, to challenge and study a specific immune response.

*Tuberculosis in old age*

*Mycobacterium tuberculosis* (*M.tb*), the causative agent of the disease tuberculosis (TB), is estimated to have infected approximately 2 billion people worldwide, resulting in a total mortality of 1.5 million individuals in 2014. Despite the fact that TB mortality has fallen 47% since 1990, and nearly all TB cases can be managed, WHO reported that in 2015, TB has surpassed HIV as the leading cause of global mortality, making it the deadliest infectious disease worldwide. Within the globally infected population, elderly individuals 50 years and older have the highest TB notification rates, demonstrating the susceptibility of elderly individuals to *M.tb* infection on a global scale. However, distributions of TB rates are quite different depending on the regions of the world. Within developed countries, new TB notifications occur predominately in the elderly, while in developing countries, TB notifications occur primarily in individuals 25-44 years of age. High TB incidence rates in young adults are thought to be due to compounding factors, including comorbidity such as HIV. In support of this, the Global Burden of
Disease Report recently estimated that the highest rate of mortality occurs in elderly populations when examining data from HIV-negative individuals. These observations suggest that elderly individuals have the highest risk for TB incidence and mortality in a healthy population. Further support has been provided by TB reviews in Asia and in America, documenting increased TB rates in the elderly. Based on WHO’s Global Tuberculosis report in 2014, 12 out of the 22 highest TB burden countries have life expectancies over 65 years of age making TB a very relevant disease to study in the elderly. Since the global population is aging at a dramatic rate, TB is expected to become a major public health concern.

Impact of age on clinical diagnosis and presentation of M.tb infection

Unfortunately, TB can be difficult to diagnose in the elderly because of differences in clinical presentations and inconclusive results of laboratory tests. Symptoms that are often used to identify TB such as fever, hemoptysis (coughing of blood from the respiratory tract), and night sweats are less common in the elderly compared to young adults. On the other hand, nonspecific symptoms of TB become more prevalent with old age including chronic fatigue, cognitive impairment, and weight loss which are often confused with normal aging or age-related illnesses such as diabetes mellitus and malnutrition. Furthermore, laboratory diagnoses including sputum smear and Mendel-Mantoux skin test (purified protein derivative (PPD)) are less accurate with increasing age. Additionally, because radiologic analyses demonstrate that
elderly individuals display higher frequencies of lower lung involvement of *M.tb* lesions, TB is frequently misdiagnosed as pneumonia or lung cancer in old age \(^{252,257}\).

Complications with diagnosis have led to concerns for under reported/undetected cases among older individuals. Indeed, retrospective analysis conducted by Lee *et al*, showed that during their study period, initial diagnosis of TB was made correctly in over 94% of younger patients but only in 66% of elderly patients \(^{252}\). This trend was echoed in a study in Cambodia, where active case finding, by door to door TB survey and screening yielded double the cases found in individuals 65 years and older compared to passive diagnosis \(^{258}\). Furthermore, complications with misdiagnosis leading to delayed initiation of treatment as well as increased adverse drug effects \(^{252,259}\) lead to higher frequencies of morbidity and mortality in *M.tb* infected elderly individuals \(^{256}\).

**Immune responses during *M.tb* infection**

To understand how the immune response is changed in old age, first we have to examine the current understanding of *M.tb* infection in young adults and young mice. *M.tb* is predominately a pulmonary pathogen, although *M.tb* can be found in various locations within the body. *M.tb* is transmitted by aerosol droplets that are inhaled into the distal airspace, including the terminal bronchioles and alveoli of the lung. The lung environment at the time of infection with *M.tb* is thought to be an important factor in determining the course of infection. Components of the pulmonary environment,
including surfactant proteins and hydrolases can directly affect the initial contact of *M.tb* with its primary host, alveolar macrophages (AM) \(^{260-262}\). Once engulfed by AMs, *M.tb* survive by subverting cellular killing mechanisms, including phagolysosome fusion, or the maturation and acidification of the *M.tb* containing phagosomes \(^{263-265}\).

*M.tb* growth appears to remain unchecked until the onset of adaptive immunity, where antigen specific IFNγ secreting T cells can activate infected macrophages \(^{266-269}\). Activated AMs will generate reactive oxygen and nitrogen intermediates to limit bacterial replication \(^{266,267}\). Published literature has consistently shown the importance of Th1 T cell immune responses including IFNγ and IL-12 \(^{270,271}\). Additionally, CD4+ T cells are known to be essential mediators in controlling bacterial growth, while CD8+ T cells play a less important role but are still critical for optimum host defense following infection \(^{272-275}\). Interestingly, compared to other pathogens such as influenza \(^{276}\) or inanimate antigens such as the live attenuated yellow fever virus \(^{277}\), the onset of adaptive immunity is delayed after initial infection with *M.tb* \(^{278}\). In mice, antigen specific CD4+ and CD8+ T cells are not detected in the lymph node until 9-11 days post-infection \(^{279}\). In humans, it can take approximately 8 weeks for antigen specific responses to be detected by a PPD skin test or in peripheral blood \(^{280-283}\).

As *M.tb* infection continues, cellular signals, including TNFα \(^{284}\), will eventually cue healthy host innate and adaptive immune cells to form a granuloma around the infected macrophages, separating them from surrounding tissue to try and limit the dissemination of infection \(^{285}\). Cellular organization is an important factor in determining the
effectiveness of a granuloma at containing the infection. Small and concise foci are considered a protective phenotype, while a large and more diffused lesion may lead to poor control and dissemination\textsuperscript{286}. \textit{M.tb} can remain in a latent or non-replicating state within the granuloma for the duration of the host’s lifespan or the bacteria may somehow overcome the granuloma’s protective barrier, replicate and disseminate leading to active disease\textsuperscript{287,288}.

**Immune responses to \textit{M.tb} in old age**

Because so much of our knowledge in understanding \textit{M.tb} immune control is elucidated in mouse models, the influence of age on susceptibility to \textit{M.tb} infection has generally been studied in old mice. Early studies of susceptibility of old mice to \textit{M.tb} infection were performed by Orme and colleagues\textsuperscript{289,290}. Intravenous infection of mice with \textit{M.tb} demonstrated increased susceptibility with increasing age, shown as significant bacterial growth in the lung, liver and spleen of old mice\textsuperscript{289}. However, intravenous infection models of \textit{M.tb} do not truly replicate the natural infection which normally occurs via the pulmonary route\textsuperscript{291}. While extrapulmonary TB is more common with advanced age including TB meningitis and joint infection\textsuperscript{256,259,292}, pulmonary TB still remains the most common site of infection in elderly individuals, occurring in approximately 75\% of cases\textsuperscript{251}. Further research by our lab (unpublished observations) and others using low-dose aerosol infection models have confirmed that old mice are more susceptible to \textit{M.tb}
infection. In these studies, in vivo infection of old mice led to early mortality compared to uninfected old mice and infected young mice.

**Lung environment in old age**

As previously discussed, the microenvironment of the lung at the initial time of bacterial contact may dictate infection outcome and bacterial control. Recently, a study demonstrated that the alveolar lining fluid (ALF) components are significantly altered in both young and old mice and humans. Specifically, ALF from old mice exhibited increased levels of pro-inflammatory cytokines including TNFα and IL-1β. Additionally, components known to have immunomodulatory roles against M.tb infection including surfactant proteins A and D, ALF hydrolases and complement proteins were significantly altered in old age. Furthermore, proteins from the lungs of old mice demonstrated increased oxidative damage compared to proteins isolated from the lungs of young mice. Although more studies are needed in order to confirm the modulatory roles of ALF components in M.tb infection with old age, this study suggests that M.tb may already be altered in the microenvironment prior to contact with any immune cells.

**Transient resistance to M.tb infection in old age**

In vivo M.tb infection in old mice leads to eventual increased mortality compared to young mice, however, we have previously shown that old mice demonstrate an early
resistance phenotype where the *M. tb* bacterial load within the lung is reduced for the first 2-3 weeks of infection suggesting enhanced innate responses in old mice after *M. tb* infection\(^{290,295}\). This early resistance phenotype is usually lost by D35 post-infection\(^{295}\) where bacterial burden increases compared to young infected mice. Our laboratory has been able to identify that early resistance in old age is mediated by CD8+ T cells and enhanced IFNγ within the lungs of old mice.

Intrinsic properties of CD8+ T cells isolated from the lungs of naïve old mice were shown to be significantly altered compared to cells from young mice. Phenotypically, CD8+ T cells isolated from the lungs of naïve old mice exhibited increased memory markers (CD4\(^{4hi}\)CD45RB\(^{lo}\))\(^{296}\), increased receptors for Th1 cytokines\(^{296}\) as well as increased levels of IL-15 receptor (CD122)\(^{295}\). Functionally, CD8+ T cells isolated from naïve old mice nonspecifically produce IFNγ in response to Th1 cytokines including IL-2, IL-12 and IL-18, in the absence of APCs\(^{296}\). Production of IFNγ from CD8+ T cells isolated from naïve old mice was also increased compared to cells from young mice when co-cultured in transwells with *M. tb* infected APC. This increased IFNγ production was an intrinsic property of cells from old mice since CD8+ T cells could produce IFNγ regardless of the source of infected APCs (isolated from young or old mice).

Interestingly, IL-12 was shown to be essential for IFNγ production by CD8+ T cells isolated from naïve old mice since co-culture with αIL-12 abrogated IFNγ production\(^{297}\). Furthermore, when CD8+ T cells were cultured with infected APCs lacking β2m (MHC class I), IFNγ production was not diminished compared to CD8+ T cell cultures with
wildtype APCs. These data demonstrate that IFNγ production by CD8+ T cells was dependent on IL-12 but independent of antigen simulation.

Further confirmation of alterations in CD8+ T cell function was evident in *M. tb* aerosol infected old mice. While no differences were observed in total CD8+ T cell infiltration into the lungs of *M. tb* infected young and old mice, a greater proportion of CD8+ T cells from infected old mice were capable of producing IFNγ post-infection. IFNγ message expression from total lung cells was also observed to be increased as early as D5 post-*M. tb* infection in old mice. Finally, when either CD8+ gene disrupted (KO) or IFNγ KO old mice were infected with *M. tb*, the early resistance phenotype was lost during infection. These data support the idea that increased IFNγ production by CD8+ T cells from old mice generates early resistance against *M. tb* infection via an innate immune mechanism occurring prior to lung infiltration of antigen-dependent adaptive immune cells.

**T cell immune responses to *M. tb* infection in old mice**

Previous sections within this chapter have summarized recent published assessments of T cell dysfunctions within naïve old mice. Similar T cell alterations were thought to be evident in *M. tb* infected old mice leading to increased mortality. However, recent analysis using a more physiologically relevant *M. tb* infection model challenges historical views regarding T cell function during *M. tb* infection in old age.
As previously noted, CD4+ T cells are known to be essential in the control and maintenance of *M. tb* infection in young mice. Some of the first studies examining T cell function in *M. tb* infected old mice were performed by Orme and colleagues using the intravenous infection method. While old mice exhibited delayed accumulation of both CD4+ and CD8+ T cells in the spleen following intravenous infection, by day 30 post-infection, old mice were fully capable of generating antigen specific CD4+ T cells. CD4+ T cells from naïve old mice reportedly had an increased basal expression of adhesion molecules that failed to further enhance receptor density upon *M. tb* infection, suggesting that delayed accumulation of T cells post-infection in the spleen may be due to defects in cell surface expression. However, T cells from old mice exhibited functional defects since *M. tb* specific T cells from old mice failed to generate protection against *M. tb* challenge when adoptively transferred to young mice. In contrast, transfer of *M. tb* specific T cells from young mice into old mice generated protection, suggesting that the environment of old mice is fully capable of supporting T-cell mediated immunity. However, since T cells were isolated from *M. tb* infected old mice at 15 days post-infection, and it has been established that T cell accumulation is delayed in intravenous infected old mice, it is possible that only a minimal number of antigen-specific T cells were transferred into young mice resulting in failed protection. Therefore, this model may reflect fewer *M. tb* specific T cells and not less responsive T cells. Together, these studies suggest that increased *M. tb* burden and mortality in old age may be the result of defective T cell function.
While the intravenous infection model has provided us with valuable information on *M.tb* disease progression in old age, intravenous delivery systems poorly mimic the dominant and natural route of infection through the pulmonary tract in humans\(^{251}\). Intravenous injection delivers *M.tb* directly into the bloodstream leading to large numbers of bacteria detected in organs such as the spleen and liver before reaching the lung\(^{291}\). Accumulation of antigen specific CD4+ T cells and generation of IFN\(\gamma\) message expression within infected lungs are significantly altered depending on the type of infection model\(^{291}\). Studies utilizing an aerosolized *M.tb* delivery system through the pulmonary tract are therefore thought to be a better and more realistic infection model.

Indeed, studies using the aerosol model of infection display alternate T cell responsiveness compared to intravenous infection in old mice. Specifically, old mice exhibit competent immune cell accumulation in the lungs after aerosol infection with *M.tb*. Our laboratory has also observed that the number of CD8+ T cells that label positive for a *M.tb* peptide tetramer is unaltered in old mice (unpublished observations), which suggests similar generation of *M.tb*-specific CD8+ T cells entering or proliferating in the lung. CD4+ and CD8+ T cells isolated from the lungs of *M.tb* infected old mice were also fully capable of, and sometimes even better at, producing IFN\(\gamma\) in response to T cell receptor cross-linking\(^{295}\). Furthermore, the frequency of antigen specific IFN\(\gamma\) secreting cells was not significantly different when CD4+ T cells were isolated from the draining LNs or the lungs of old mice aerosol infected with *M.tb*, compared to cells from young mice\(^{303}\).
Macrophage mediated responses to M.tb in old mice

An early study examining macrophage responsiveness to M.tb infection between young and old mice was performed using bone marrow derived macrophages. BMDMs isolated from young and old mice were infected with 4 separate isolates of M.tb, and no significant difference in the ability to control M.tb in vitro was observed\(^{304}\), suggesting that macrophages isolated from old mice are as competent as macrophages isolated from young mice in their ability to control bacterial growth when infected with M.tb. BMDMs from old mice were also able to generate a full range of cytokines and chemokine mRNA upon M.tb infection\(^{304}\). These responses included several genes known to be important for M.tb control including IL-12p40, TNFα and iNOS. However, BMDMs are not considered to be representative of resident tissue macrophages including AM, as they are artificially generated from precursor cells but some support has been generated by a study using pulmonary macrophages from rhesus macaques\(^{305}\). In this study, AMs from aged macaques were fully capable of controlling bacterial replication and could kill M.tb upon the induction of autophagy, similar to that observed in young macaques. Furthermore, no significant differences were observed between M.tb uptake in infected AMs from young and old macaques. However, differences in growth trends were evident between AMs from young and old macaques over a 7 day infection period. Nevertheless, this report concluded that AMs from old macaques did not demonstrate significant defects in bacterial control when compared to AMs from young macaques.

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Combined with the knowledge that infected BMDMs from old mice show similar bacterial control, this suggests that macrophage responses to *M. tb* infection are intact and functional in old age. Studies from our laboratory using pulmonary mouse macrophages have confirmed some of the initial findings observed in BMDMs from old mice. Specifically, *M. tb* infected pulmonary macrophages from old mice are capable of producing IL-12p40 and TNFα protein compared to pulmonary macrophages from young mice.

Although no differences have been observed in cytokine production between AMs from young or old mice infected with *M. tb*, published studies have demonstrated altered AM surface receptor expression between young and old mice. As described in the previous section, several receptors including TLR2, TLR4 and TLR9 are thought to be decreased with increasing age. Because *M. tb* is known to be recognized by TLRs, predominately through TLR2, TLR4 and TLR9 alterations in expression could significantly alter bacterial control. However, our laboratory has demonstrated that the lungs of old mice contained similar numbers of AMs that could express TLR2 and TLR9. Furthermore, the relative expression, or density, of TLR2 on the cell surface of macrophages from old mice was significantly increased. This suggests that TLR2 expression on macrophages isolated from the lungs of old mice is not impaired. Previous published studies using KO mice have shown that in young mice, TLR2 is necessary for IL-12p40 and TNFα production by macrophages in response to *M. tb*. Interestingly, when old TLR2 KO mice were infected with *M. tb*, no significant difference in cytokine production was observed. Although at this time it is unclear which receptor(s) is
compensating for TLR2-independent *M.tb* recognition in old age, we have elucidated that TLR4 and TLR9 could only partially compensate for IL-12p40 and TNFα production in the absence of TLR2\textsuperscript{306}.

Pulmonary macrophages are also known to be important in activating the adaptive immune response by presentation of *M.tb* antigens to CD4+ and CD8+ T cells. Alterations in antigen presentation with increasing age may lead to decreased control of *M.tb* infection. However, studies in our laboratory have revealed that MHCII expression on APCs from the lungs of old mice was comparable and sometimes even increased compared to APCs from young mice\textsuperscript{303}. We have also shown that when pulmonary macrophages isolated from young and old mice are incubated with *M.tb* antigen Ag85 and co-cultured with the T cell clone BB7\textsuperscript{311}, specific for Ag85 peptide, IL-2 production was similar between both groups. This suggests that macrophages from old mice are as competent as macrophages from young mice at presenting *M.tb* antigen to CD4+ T cells\textsuperscript{303}.

These studies suggest that pulmonary macrophages from old mice are fully capable of responding to *M.tb* infection in terms of cytokine production and antigen presentation. Although macrophages from old mice utilize a TLR2-independent mechanism for cytokine production in response to *M.tb*, at this time it is unclear whether altered receptor recognition affects infection outcome. Results from chapter 2 of my dissertation further extend our knowledge of macrophage phenotype and function in old age, and may even challenge our understanding of old mouse macrophage responses to *M.tb*. 

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Chapter 2: Lung inflammation in old age contributes to altered macrophage phenotype and function

Abstract:

Aging is associated with increased susceptibility to pulmonary disorders and infectious diseases. Systemic basal inflammation that occurs with increasing age is thought to contribute to this susceptibility. However, little is known about the consequences of inflammation on immune function. In this study, we demonstrate that the lungs of naïve old mice have elevated levels of pro-inflammatory cytokines (IL-1β, IL-6 and TNFα), as well as Th1 related cytokines (IL-12 and IFNγ). Pulmonary macrophages isolated from this environment had molecular markers of activation prior to stimulation. Using *Mycobacterium tuberculosis* (*M.tb*) infection of pulmonary macrophages from old mice, we were able to determine how inflammation affects their function. Macrophages isolated from the lungs of old mice had increased *M.tb* binding and phagolysosome fusion in response to *M.tb* infection compared to macrophages isolated from young mice. Altered responses in macrophages isolated from old mice were restored when old mice were given the non-steroidal anti-inflammatory drug, ibuprofen, prior to any manipulations. These data indicate that the pulmonary environment has increased inflammation with
advanced age leading to altered macrophage function but that this environment can be restored with the use of ibuprofen.

**Introduction:**

Aging is characterized by an increase in low-grade chronic inflammation, termed inflammaging, measured by circulating levels of proinflammatory cytokines including interleukin (IL)-1, IL-6 and tumor necrosis factor-alpha (TNFα). Inflammaging is now thought to contribute to several age-associated diseases including type-2 diabetes mellitus, osteoporosis, rheumatoid arthritis and Alzheimer’s disease. Inflammaging is also thought to contribute to altered immune function in old age leading to increased susceptibility to infection. However, how a cell is altered in old age is a topic of controversy. For instance, within the myeloid lineage, some studies suggest that in vitro macrophage function is enhanced as measured by increased cytokine production to stimuli, while other studies using similar stimuli have shown decreased cytokine production. These seemingly conflicting data have been attributed to differences in environmental exposures, including diet, housing conditions, and enrichment as well as the source of monocytes/macrophages and the specific stimulus used in the experiment.

In this report, we demonstrate that lungs of naïve old mice are in an inflammatory state as determined by increased mRNA message levels of several inflammatory cytokines. To understand how inflammation can impact macrophage function, we focused on the response of macrophages from old mice to stimulus with the virulent pulmonary
pathogen *Mycobacterium tuberculosis* (*M.tb*). Because resident tissue-derived pulmonary macrophages are the primary host cells for harboring *M.tb*, we sought to understand the impact of an inflammatory environment on their phenotype and function.

One known mechanism for *M.tb* control by macrophages is through the process of phagocytosis\textsuperscript{263-265}. Following phagocytosis by a professional phagocyte, ingested particles, including *M.tb*, will fuse with a lysosome, a process known as phagolysosome (PL) fusion\textsuperscript{312}, resulting in the digestion of the particle by hydrolytic enzymes contained within the lysosome. However, *M.tb* is known to escape this host defense mechanism by inhibiting phagosome maturation\textsuperscript{287,313}. Interestingly, several proinflammatory cytokines including IL-1β\textsuperscript{314} and TNFα\textsuperscript{315} are known to regulate phagosome maturation.

Furthermore pro-inflammatory cytokines including IL-1β can generate interferon-gamma (IFNγ) through the production of IL-12\textsuperscript{316}. Activation of macrophages with IFNγ can stimulate the maturation and acidification of *M.tb* containing phagosomes resulting in increased intracellular killing by infected macrophages\textsuperscript{317,318}.

In this study, we demonstrate that genes associated with phagosome maturation are upregulated in macrophages isolated from old mice. Furthermore, macrophages from old mice had an increased level of *M.tb* uptake during *in vitro* infection and a greater percentage of phagocytosed *M.tb* located within late stage phagolysosome compartments. However, increased PL fusion did not lead to enhanced bacterial control since *in vitro* *M.tb* infection of macrophages isolated from old mice failed to sustain control over time compared to macrophages isolated from young mice.
Because our data suggest that altered phenotype and function of pulmonary macrophages from old mice may be a result of "inflammaging" \(^{314-318}\), we next sought to modulate this environment by decreasing inflammation using ibuprofen, a nonsteroidal anti-inflammatory drug (NSAID). Ibuprofen works by inhibiting cyclooxygenase (COX) enzymes, which are known to convert arachidonic acid to prostaglandin and thromboxane, the mediators of pain, inflammation, fever and platelet aggregation. Decreasing inflammation in old mice using an ibuprofen-enriched diet restored the pulmonary macrophage phenotype and function to levels similarly seen in young mice. Our work suggests that inflammation induced enhanced macrophage function in old mice may not be beneficial during the very first encounter of \(M.\text{tb}\) with its host niche.

**Materials and Methods:**

**Mice**

Specific pathogen-free, female C57BL/6 mice were purchased from Charles River Laboratories or through rodent resources with the National Institute on Aging (supplied by Charles River Laboratories) at an age of 3 months or 18 months. Specifically, young mice were defined as 3 months of age as this is an age where sexual maturity has been reached \(^{319}\). Old mice were defined as 18 months which is equivalent to old age in humans (65 years or older) \(^{320}\). Furthermore, because the median lifespan of female C57BL/6 mice is 24.9 months, our studies using 18 months old mice avoided
confounding factors which may appear late in life such as frailty and spontaneous tumor generation. In addition, *M. tb* infection phenotypes have been previously characterized in 18 month old mice \(^{296,297,321}\), thus providing useful information which could be beneficial in our studies to understand changes in old mice. Mice were maintained in ventilated cages and provided with sterile food and water *ad libitum*. For diet modification studies, mice were fed either the Teklad Global 18% protein rodent control diet or the Teklad Global 18% protein rodent control diet supplemented with ibuprofen (375ppm; Harlan Laboratories) for 2 weeks before experimental manipulation. Ibuprofen dosage was chosen based on efficacy and safety shown in long-term use in old mice \(^{322}\). Mice were killed for experiments by CO\(_2\) asphyxiation. All protocols were approved by The Ohio State University’s Institutional Laboratory Animal Care and Use Committee.

**Isolation of lung cells**

Lungs of young and old mice were perfused by PBS containing 50U/ml heparin (Sigma, St. Louis, MO, USA) through the right ventricle of the heart. Lung lobes were extracted and placed in 2ml DMEM containing L-glutamine (Mediatech), supplemented with sterile-filtered 5ml HEPES buffer (1M; Sigma), 10ml MEM nonessential amino acid solution (100x; sigma), 660ul 2-ME (50mM; Sigma), and 45ml heat-inactivated fetal bovine serum (FBS; Atlas Biologicals) (complete DMEM). Lungs from individual mice were mechanically disrupted using a Gentle MACS dissociator (Miltenyi Biotec) followed by digestion for 30 minutes at 37°C, 5% CO\(_2\), in a total of 4ml complete
DMEM containing collagenase Xi from *Clostridium histolyticum* (0.7mg/ml; Sigma) and type IV bovine pancreatic DNase (30μg/ml; Sigma). Complete DMEM (5ml) was added to stop the enzymatic activity, and lung pieces were passed through a 70μm strainer to achieve a single cell suspension. Residual erythrocytes were lysed using the Gey’s lysis buffer (8mM NH₄CL, 5mM KHCO₃ in water), and suspended in complete DMEM.

**Purification of pulmonary macrophages**

Lung cells were adhered to tissue culture grade Petri dishes for 1hr at 37°C, 5% CO₂. Petri dishes were washed three times using warm DPBS (Invitrogen) to remove nonadherent cells. Adherent cells were incubated with 5ml trypsin-EDTA (Sigma) for 10 minutes at 37°C, 5% CO₂, after which, 10ml complete DMEM was added to stop the reaction. Adherent cells were harvested by vigorous pipetting and pooled and suspended in 1ml complete DMEM. Cells were counted using trypan blue (Sigma) and adjusted to working concentrations in complete DMEM. This protocol has the potential to isolate alveolar macrophages as well as interstitial macrophages but because the impact of inflammation with aging is unknown in the lung, and responses to *M.tb* infection are not well defined in pulmonary macrophages from old mice, we chose to first characterize macrophage changes in the whole lung environment. This approach allowed for a broad characterization of immune cell function without limiting data to one cell source. However, it will be important to define function in specific cell types in the future since...
based on previously published studies from our group and additional analyses here (Table 2.1), cellular populations within the pulmonary environment in old age are significantly altered. For example, the pulmonary adherent cell population in old mice has increased proportions of CD11b+ cells as well as increased receptor expression on a per cell basis, indicating that in old age, the lungs have more cells that resemble infiltrating monocytes. Furthermore, although CD11c+ cells have been previously shown to be reduced in the lungs of old mice, they are still the dominant cell subset that is responsive to \textit{M. tb} infection. Pulmonary macrophages and dendritic cells both express CD11c+ and therefore, were not analyzed independently in our studies. For simplicity, adherent cells are subsequently referred to as pulmonary macrophages.

**Real-time quantitative polymerase chain reaction (RT-qPCR)**

The lungs of naïve, young and old mice were removed and homogenized in 1ml TRIzol reagent (Invitrogen). RNA was extracted with chloroform, precipitated using isopropanol and 75% ethanol, reconstituted in DNase/RNase-free water and quantified on a NanoDrop spectrophotometer. cDNA was synthesized with random primers using an Omniscript RT Kit (Qiagen). RT-qPCR was performed on cDNA from whole lung using TaqMan gene expression assays (Applied Biosystems) for IFN\(\gamma\), TNF\(\alpha\), IL-12p40, IL-10, IL-6 and IL-1\(\beta\). TaqMan gene expression assays for class II major histocompatibility complex transactivator (CIITA), immunity-related GTPase family M protein 1 (IRGM-1) and interferon regulatory factor 1 (IRF-1) were performed on cDNA from pulmonary
macrophages that were purified as described above following RNA-extraction with TRIZol reagent. A Bio-Rad CFX96 Real-Time PCR thermal cycler was used for qPCR. The ΔΔCT method was used for quantification of the relative units (RU) of mRNA, using 18s as an endogenous normalizer and young mice as the calibrator \[RU=2^{-\Delta\Delta CT}; \Delta CT=CT \text{ gene (IFNγ, TNFα, etc.)} - CT 18S; \Delta\Delta CT= \Delta CT \text{ sample} – \Delta CT \text{ calibrator (young mouse)}\].

**In vitro bacterial infections**

Isolated pulmonary macrophages were adjusted to 2x10^5 cells/well and placed in a 24-well plate. Confocal studies used acid-treated, flame-sterilized Gold-Seal coverslips (Electron Microscopy Sciences). Cells were allowed to adhere to coverslips or tissue-culture wells overnight at 37°C, 5% CO₂. For some experiments, 2x10^5 cells/well were incubated with purified murine recombinant-IFNγ (100U/ml; Roche) for 16hr under standard conditions before a 24hr infection. All work involving live *M.tb* was conducted in the BSL3. All infections were completed at a multiplicity of infection (MOI) of five, with green fluorescent protein (GFP)-*M.tb*. This strain expresses GFP constitutively through a *Mycobacterium bovis* bacillus Calmette-Guerin heat shock protein 60 promoter and was generated by Horwitz and colleagues. GFP-*M.tb* was grown in Proskauer-Beck liquid medium containing 0.05% Tween 80 to the mid-log phase. Bacterial suspensions were frozen in aliquots at -80°C. Stock GFP-*M.tb* was thawed, and clumps were disrupted by four cycles of drawing and ejection through a 26-G needle. The
inoculum was subsequently diluted to a working concentration and added to cells in a fixed volume. Cells/M.tb were incubated at 4°C for 10 minutes and then centrifuged at 200g, 4°C for 10 minutes. The infection was allowed to proceed for 2hr at 37°C, 5% CO₂. For confocal studies, cells were washed twice with warm DPBS and then fixed for 10 minutes with 2% paraformaldehyde. After fixation, coverslips were removed from the BSL3 facility for further processing.

**Immunocytochemistry and confocal microscopy**

Infected, fixed cells on coverslips were permeabilized with methanol for 3 minutes at room temperature, washed with DPBS, and blocked overnight at 4°C with blocking buffer PBS [10% heat-inactivated FBS (Atlas Biologicals) and 5mg/ml bovine serum albumin (BSA; Sigma) in DPBS]. Primary rat anti-mouse IgG2 antibodies against lysosomal associated membrane protein 1 (LAMP-1) (Santa Cruz Biotechnology) and cathepsin D (R&D Systems) were used as lysosomal markers. Goat anti-rat IgG antibody conjugated to AlexaFluor 594 (Invitrogen) was used as the secondary antibody. Normal rat IgG2 antibody (R&D Systems) was the isotype control. Coverslips were mounted on slides using ProLong Gold Antifade Reagent containing the nuclear stain 4’6’-diamidino-2-phenylindole (Invitrogen). Coverslips were analyzed by laser-scanning confocal microscopy using an Olympus FV1000 Filter Confocal Microscope. To measure the percentage of cells containing GFP-M.tb (attached and intracellular), Z-stacks of at least six fields were taken over a depth of 10 microns at a magnification of 400x for all
experimental groups so that at least 300 cells were counted per coverslip. Similar methods were used to measure PL fusion over the same depth and magnification, with at least 100 internalized bacteria counted per coverslip and analyzed for colocalization of phagosomes containing GFP-M.tb (GFP, 488nm) with LAMP-1 or cathepsin D (lysosomal markers, AlexaFluor 594, 594nm). Microscopy data was analyzed using the Olympus FluoView Viewer software.

**Analysis of bacterial growth in macrophages**

Pulmonary macrophages isolated from the lungs were incubated with 5:1 GFP-M.tb for 2 hr at 37°C and 5% CO₂ before washing with warm complete DMEM to remove nonadherent M.tb. Infected macrophages were then cultured in complete DMEM. Bacterial growth, or colony forming units (CFU) at defined time-points postinfection was assessed using the following protocol. Supernatant from each well was removed and centrifuged down to obtain the pellet of unattached infected cells. Sterile, cold water (300μl) containing DNase I (500μm/ml; Sigma) was added to the monolayer. After 10 minutes with periodic agitation, 560μl Middlebrook 7H9 culture broth (BD Biosciences) containing the pellet of unattached-infected cells was added to the monolayer, followed by lysis with 240μl 0.25% sodium dodecyl sulfate (SDS; Fisher Scientific International) in DPBS. After 10 minutes with periodic agitation, 300μl 20% BSA (Sigma) was added to all wells to stop lysis and mixed by pipetting. The contents of each well were transferred to a 1.5ml tube with a tether cap (Fisher Scientific International) containing
two flame-sterilized glass beads. Tubes were pulsed (5x) on a vortex, and serial dilutions were plated on 7H11 agar plates, supplemented with OADC (Remel). Colonies were enumerated after 21 days incubation at 37°C.

**Flow cytometry**

All antibodies were obtained from BD Biosciences, unless stated otherwise. Isolated lungs cells were suspended in deficient RPMI (Mediatech), supplemented with 0.1% sodium azide (Sigma). Surface targets were stained with specific antibodies for 20 minutes at 4°C and subsequently washed with supplemented deficient RPMI. Specific antibodies included rat IgG2a FITC (553929), anti-CD86 FITC (553691), rat IgG2b PE (556925), and anti-major histocompatibility complex (MHC) class II PE (ab24842; Abcam, Cambridge, UK) and hamster IgG1 APC-Cy7 (561206), anti-CD11c APC-Cy7 (561241), rat IgG2a APC (553932), and anti-CD206 APC (141708; BioLegend, San Diego, CA, USA). Samples were read using the FACSCanto II flow cytometer (BD Biosciences) and analyzed with FACSDiva software (BD Biosciences).

**Statistical analysis**

Data were plotted and analyzed using the GraphPad Prism 5.0 software (GraphPad). The unpaired two-tailed Student’s *t*-test was used to determine statistical significance. The Grubbs’ test was used to identify outlying data points.
Results

The pulmonary lung environment of old mice is in an inflammatory state and macrophages isolated from this environment have increased activation markers.

Elderly individuals account for the highest TB incidence rate among all age groups in the United States\(^1^7\). This increase in susceptibility is thought to be due to inflammaging, the low-grade, chronic, systemic proinflammatory state observed in old age. However, the effects of inflammaging are not well characterized in mice, specifically in localized environments. Because the lungs are the primary site for \textit{M. tb} infection, we looked at the pulmonary environment of young and old mice by examining messenger RNA (mRNA) levels of several proinflammatory markers as well as the anti-inflammatory marker IL-10 in whole lung homogenates by RT-qPCR. Cytokines known to be upregulated in the serum of elderly humans including IL-1\(\beta\) (Fig. 2.1A), IL-6 (Fig. 2.1B) and TNF\(\alpha\) (Fig. 2.1C) were elevated in the lungs of old mice compared with young mice, although IL-1\(\beta\) and IL-6 did not reach significance because of the small group sizes and highly variable data points within old mice. Furthermore, the expression of IL-12 (Fig. 2.1D) and IFN\(\gamma\) (Fig. 2.1E) was also significantly upregulated in the lungs of old mice. Although it is unclear which mediators were involved in the induction of IL-12 and IFN\(\gamma\), both IL-1\(\beta\)\(^{316}\) and TNF\(\alpha\)\(^{325}\) are capable of modulating Th1 cytokine production suggesting a feedforward mechanism that perpetuates inflammaging. These data demonstrate that the lungs of old mice have a basal inflammatory environment. However, expression of mRNA for IL-10 (Fig. 2.1F) also had a trend for increased expression although this
observation was not statistically significant. This suggests that cell function within the lungs of old mice may be dysregulated.

Because the pulmonary environment of old mice is in a constant inflammatory state including elevated IL-1β, TNFα and IFNγ, all known activators of macrophage function, we anticipated that resident macrophages isolated directly from the lungs of old mice would express markers associated with downstream inflammatory activation that are important in immune control. These markers include IRGM-1, IRF-1 and CIITA. Pulmonary macrophages were isolated from the lungs of young and old mice, and mRNA expression was determined for genes associated with macrophage activation by RT-qPCR. Macrophages isolated from the lungs of old mice expressed significantly more mRNA for IRGM-1 (Fig. 2.2A), and IRF-1 (Fig. 2.2B). A similar trend was observed for the expression of CIITA (Fig. 2.2C), although expression was not significantly different compared with macrophages from young mice. Together, these data demonstrate that mRNA expression for known inflammatory inducible products was increased in resident macrophages isolated from the lungs of old mice.

Highly activated pulmonary macrophages isolated from the lungs of old mice have increased cytokine production, M.tb uptake and PL fusion which are refractory to further activation by IFNγ

Because IFNγ, a potent activator of macrophage function, was found in abundance in the lungs of old mice, we hypothesized that the resident pulmonary macrophages from the lungs of old mice may be dysregulated.
old mice were endogenously exposed to IFNγ and would be in a preactivated state. Macrophages from the lungs of young and old mice were stimulated with murine recombinant IFNγ for 16hr prior to *M. tb* infection. IL-12p40 was determined in culture supernatants 24hr post-infection. Although not statistically significant, the basal levels of IL-12p40 production from untreated cells were slightly increased in macrophages from old mice (Fig. 2.3). When infected with *M. tb*, macrophages isolated from old mice produced significantly more IL-12 compared to macrophages isolated from young mice. This is thought to be the result of a feedforward mechanism to induce IL-12 production by IFNγ stimulation. In line with our hypothesis, the difference in IL-12 production between young and old mice was mitigated when cells were incubated with IFNγ prior to *M. tb* infection. This difference was mainly a result of an increase in IL-12 production by macrophages from young mice. These data suggest that macrophages isolated from the lungs of old mice, which contain high concentrations of IFNγ, may be preactivated and primed to function.

To understand how the preactivated state of pulmonary macrophages from old mice might lead to altered function, we looked at immune related responses of known altered activation markers. Because macrophages isolated from the lungs of old mice have high levels of IRGM-1 and IRF-1, genes known to regulate PL fusion and mycobacterial control 329,330, we sought to determine whether the preactivated macrophages from old mice led to increased phagocytosis and *M. tb* killing in vitro. Macrophages isolated from the lungs of young and old mice were incubated with GFP-expressing *M. tb* and examined by confocal microscopy. An infected cell was defined as one that had attached or
internalized at least one GFP-\textit{M.tb} bacillus (Fig. 2.4A). The percentage of infected macrophages from the lungs of old mice was significantly increased compared to the percentage of infected macrophages from young mice (Fig. 2.4B). This suggests that macrophages from old mice phagocytosed \textit{M.tb} more readily than macrophages from young mice.

To determine whether the preactivated macrophages from old mice could overcome \textit{M.tb} PL fusion inhibition, we assayed for the colocalization of \textit{M.tb} with LAMP-1 and cathepsin D, both of which are used to identify late endosome and lysosome markers.\textsuperscript{331,332} GFP-\textit{M.tb} and LAMP-1 colocalization (PL fusion events) seen in macrophages isolated from old mice was significantly higher compared with macrophages from young mice (Fig. 2.4C-D). Similarly, when looking at cathepsin D, a lysosomal protease that is activated at low pH, significantly higher colocalization events were observed in pulmonary macrophages from old mice compared with macrophages from young mice (Fig. 2.4E).

To further extend on these findings, we determined whether IFN\textgamma activation of macrophages from old mice could modify intracellular trafficking resulting in increased PL fusion. Pulmonary macrophages from young and old mice were incubated with murine recombinant IFN\textgamma for 16hr and infected with \textit{M.tb}. PL fusion was assessed 2h post \textit{M.tb} infection. Colocalization events, signifying PL fusion, were significantly higher in untreated macrophages isolated from old mice compared to untreated (media) macrophages isolated from young mice (Fig. 2.5), as we demonstrated previously (Fig.
2.4C-E). In the presence of IFNγ, PL fusion events were significantly increased in cells isolated from young mice although no significant difference was observed in cells isolated from old mice. Taken together, these data suggest that the function of pulmonary macrophages from old mice cannot be further enhanced by IFNγ, potentially as a consequence of the increased basal levels of IFNγ in the lungs of old mice.

**Preactivated macrophages from old mice fail to control intracellular bacterial growth**

In studies of human monocyte-derived macrophages, or macrophages from young mice, increased PL fusion correlates with enhanced *M. tb* killing, which can be detected by CFU determination \(^{287}\). Because macrophages isolated from the lungs of old mice have increased PL fusion and increased *M. tb* uptake, we hypothesized that the preactivated macrophages from old mice would have enhanced bacterial control. Due to differences in initial *M. tb* uptake, the CFU data was adjusted to reflect similar binding 2 hours post-infection. Both macrophages from young and old mice were equally capable of reducing the CFU tenfold within the first 12 hours post-infection (Fig. 2.6). As the culture period extended to greater than 24 hours, however, macrophages from old mice became more permissive to *M. tb* growth than macrophages from young mice.
Ibuprofen can decrease the pulmonary inflammatory environment of old mice and restore macrophage function

Since the pulmonary environment of old mice was inflammatory in nature, we hypothesized that the inflammatory state was directly responsible for the altered phenotype and function seen in pulmonary macrophages from old mice. To test this hypothesis, we used ibuprofen, a NSAID, to modify the inflammatory environment of old mice in vivo. We used ibuprofen because of its effects as a broad acting agent with the ability to decrease IL-1β, IL-6, and TNFα. Old mice were placed on a diet supplemented with ibuprofen (or control chow) for 2 weeks prior to pulmonary macrophage isolation.

A 2 week diet modification in old mice was sufficient to decrease the basal inflammatory levels of the lungs of old mice as seen by decreased message expression of IL-1β (Fig. 2.7A), IL-6 (Fig. 2.7B), TNFα (Fig. 2.7C), IL-12 (Fig. 2.7D) and IFNγ (Fig. 2.7E). Again, decreasing expression of IL-1β and IL-6 failed to reach significance due to a small sample size and the highly variable expression within old mice. Macrophages from old mice on ibuprofen also had reduced IL-12 p40 production after M.tb infection compared to macrophages isolated from old mice on control diet (Fig. 2.8A). Interestingly, no differences in macrophage cell surface marker expression for CD86, mannose receptor (MR) or MHCII were observed between control and ibuprofen fed mice in either age group (Table 2.1). Furthermore, the ibuprofen diet modification significantly decreased the PL fusion events detected in macrophages from old mice infected with M.tb (Fig.
However, contrary to our hypothesis, macrophages isolated from old mice on ibuprofen supplemented diet did not have changes in CFU control in vitro, with a similar CFU reduction at 12 hours and subsequent CFU increase by 36hr (Fig. 2.8D). Taken together, these data suggest that an ibuprofen supplemented diet can reduce the inflammatory lung environment of old mice and reverse phenotype and function but remains inefficient at boosting control of in vitro M.tb growth in culture.

**Discussion**

Inflammaging, or the low-grade increase in inflammatory mediators during physiological aging is thought to contribute to several age related disease states, including infectious diseases. In this study, we examined the pulmonary environment of naïve, old mice and the impact of inflammaging on macrophages isolated from this environment. We determined that the lungs of old mice had elevated basal message levels for several proinflammatory cytokines similar to those observed in elderly individuals. Furthermore, we demonstrated that macrophages isolated from the lungs of old mice were in a preactivated state, with increased frequency of intracellular bacteria found within phagolysosome compartments after M.tb infection. Despite increased PL fusion, macrophages isolated from old mice infected with M.tb were unable to sustain control in vitro relative to macrophages from young mice.

Although we observed increased proinflammatory message expression in the lungs of old mice and increased activation markers from macrophages isolated from old mice, we also...
observed increased basal anti-inflammatory IL-10 message expression within the lungs. IL-10 is known to modulate macrophages by downregulating Th1 cytokine production, MHC class II and co-stimulatory molecule expression. Interestingly, IL-10 has been shown to be both upregulated and downregulated in mouse and human studies of old age. Data analyses examining genotypes associated with high IL-10 production in elderly humans is correlated with longevity, decreased frequency of tumor generation and risk of mortality from cardiovascular events. Furthermore, polymorphisms associated with low IL-10 production were significantly associated with increased cardiovascular mortality risk and frequency of myocardial infarction in old age. This suggests that IL-10 production and successful aging has a strong genetic component.

Although basal circulating IL-10 levels have not been well characterized in old age among mouse strains, we hypothesize that different mouse strains will display different IL-10 cytokine profiles at their prospective old ages. Furthermore, regulation of basal IL-10 throughout the lifespan of an organism, but most importantly when proinflammatory mediators increase with aging, might alter age-associated disease development and progression. For example, high circulating basal IL-10 in C57BL/6 mice (used in our models) might contribute to their long lived phenotype (average medium lifespan of 26 months for females) with decreased susceptibility to spontaneous tumor growth. On the other hand, short living strains with high tumor incidence such as AKR/J (average median lifespan is 10 months in females) might be associated with low IL-10 producing genotypes. Thus, we realize that our experimental results are limited by our single mouse
strain and studies using different mouse strains will be necessary to truly understand IL-10 associations with old age and aging related phenotypes.

We next showed that macrophages from old mice exhibited increased mRNA expression of proinflammatory inducible genes \(^{345-349}\) that regulate autophagy (IRGM-1), apoptosis (IRF-1) and MHC II expression (CIITA). Most importantly, these genes have been shown to modulate mycobacterial control in \textit{in vivo} infection models. IRF-1 knockout (KO) mice infected with \textit{Mycobacterium bovis} succumb to overwhelming infection \(^{329}\) while IRGM-1 KO and CIITA KO mice fail to control \textit{M.tb} infection \(^{330,350}\). Thus, increased expression of IRGM-1 and IRF-1 message in macrophages from old mice suggested an increased ability to control \textit{M.tb}, which is dependent on phagolysosome maturation \(^{330}\). We therefore hypothesized and observed that intracellular bacteria were found more frequently in phagolysosomal compartments in macrophages from old mice compared to young mice. Although we did not see a significant increase in CIITA expression, we did observe a significant increase in MHCII expression in macrophages isolated from old mice compared to young mice. This suggests that functionally, macrophages from old mice should decrease bacterial burden by increasing phagosome maturation as well as facilitate adaptive immune function through enhanced MHCII presentation.

We confirmed the activation state of macrophages isolated from old mice by the addition of endogenous IFN\(\gamma\) to cultures containing either macrophages from young or old mice. Notably, macrophages isolated from old mice failed to further induce IL-12 production or increase bactericidal activity when endogenous IFN\(\gamma\) was added to culture, which was in
direct contrast to that observed with macrophages from young mice. IFNγ was chosen because of its potent ability to activate macrophages and because of its abundance in the naïve lungs of old mice. Previously, our laboratory has demonstrated that old mice possess a unique subset of CD44$^{hi}$ CD8$^+$ T cells which can produce IFNγ in the absence of T-cell receptor engagement. IFNγ production by CD8$^+$ T cells was stimulated by IL-12 alone and facilitated by increased IL-12Rβ2 message and enhanced STAT-4 signaling. Although these CD8$^+$ T cells expressed a marker associated with activation/memory (CD44), the ability to produce IFNγ without T-cell receptor engagement suggests that they possess functional characteristics similar to innate immune cells. Since old age is associated with increased proinflammatory cytokines, such as IL-1β and IL-6, and these cytokines in turn can stimulate the production of IL-12 from dendritic cells and macrophages, this indicates that upregulation of IFNγ may be an indirect consequence of inflammaging. This could potentially lead to a feedforward mechanism of perpetual induction of proinflammatory cytokines with IL-1β and IL-12 driving IFNγ production in innate CD8$^+$ T cells and IFNγ driving IL-1β and IL-12 production in dendritic cells and macrophages in old age. Since recent human studies have also observed increased IL-12 and IFNγ in the serum of elderly volunteers, this suggests that increased Th1 cytokine expression in old age is not an artifact of our mouse model but is conserved between species. Taken together, this suggests that inflammaging may lead to mechanisms which could exacerbate inflammatory responses and contribute to further alterations in immune function.
Our observation of increased macrophage function is in contrast to some (but not all) research studies showing decreased macrophage function in old age\textsuperscript{175,176}. These functions include decreased phagocytosis, decreased cytokine production and decreased antigen presentation in macrophages from old mice (described in chapter 1). While we agree that these conflicting results stem from differences in mouse strains, macrophage source, and stimulus\textsuperscript{351} we further propose that researchers need to take into consideration basal environmental changes in old age, which is necessary to fully understand age-associated macrophage function. For instance, when macrophages from old mice are stimulated with lipopolysaccharide and decreased cytokine production is observed, is it because cytokine production was normalized to basal production? If inflammaging causes macrophages to produce increased basal levels of inflammatory cytokines and these cytokines are then measured as a response to stimuli, normalized response to basal levels in old age would appear decreased and therefore “dysfunctional”, while in fact they are functional but refractory to further stimulation. Furthermore, if IL-10 production is upregulated in macrophages from old mice in order to control a proinflammatory environment, \textit{in vitro} cultures may exhibit decreased cytokine production because of the suppression triggered by increased IL-10. Therefore, we believe it is important when studying immune function in old age, especially now knowing that age is associated with increased inflammation, to take into consideration the basal cellular and environmental activities while characterizing functional aspects of any cells from an aged host.
Despite observing increased function in pulmonary macrophages from old mice, when macrophages were infected with *M.tb* they were unable to sustain control *in vitro* relative to macrophages from young mice. This suggests that enhanced basal activation in pulmonary macrophages from old mice may not be beneficial to pathogen control. Although, it is important to note that at early timepoints (≤12hr) macrophages from old mice could control bacterial growth at similar levels, compared to macrophages from young mice (Fig 2.6), and only succumbed to higher bacterial burden at later timepoints (≥24hrs). This observation may give insight into the mechanism of decreased bacterial control in macrophages from old mice over time.

Although PL fusion is often associated with bacterial killing and decreased bacterial burden, a recent paper published by Levitte *et al.* demonstrated that *Mycobacterium marinum* (*Mm*) within PL compartments of zebrafish could survive and replicate albeit at a slower rate compared to a non-acidified phagosomes. This suggests that at early timepoints, despite increased bacterial-PL fusion events, survival of *M.tb* may not be impeded in macrophages from old mice. Furthermore, Levitte *et al.* demonstrated that increased bacterial growth was better associated with survival of *Mm* within an acidified PL compared to the avoidance of PL fusion, which suggests that increased PL fusion in macrophages from old mice may in fact be detrimental to bacterial control. In addition, Lee *et al.*, have documented a dynamic role of IFNγ on macrophage cell death during *M.tb* infection which was dependent on bacterial load. When bone marrow derived macrophages (BMDM) were infected with *M.tb* at a MOI ≥ 25, but not at MOI < 10, pretreatment of IFNγ promoted increased caspase-independent apoptosis of BMDMs.
which rapidly progressed to necrosis. Furthermore, both untreated macrophages and macrophages primed with IFNγ exhibited similar CFU post-\textit{M.tb} infection. Lack of an enhanced bacterial control in IFNγ primed macrophages was associated with increased cell death which was hypothesized to disrupt cellular components necessary for antimicrobial activities. Since macrophages isolated from old mice exhibited increased \textit{M.tb} uptake post-2 hour infection, the lack of enhanced control may be due to IFNγ-dependent increased cell death. Therefore, initial bacterial control induced by the preactivate state of macrophages from old mice may be counteracted by IFNγ induced apoptosis and necrosis leading increased bacterial replication.

Although macrophages isolated from old mice failed to control bacterial burden over time \textit{in vitro}, our previously published report show that old mice infected with \textit{M.tb in vivo} demonstrate an early resistance phenotype that is dependent on innate production of IFNγ\textsuperscript{297,355}. This suggests that the \textit{in vitro} and \textit{in vivo} environment may exhibit different abilities to control \textit{M.tb} infection. For example, increased numbers of myeloid precursors have been described within the bone marrow in old age\textsuperscript{76,356}, which is associated with increased pulmonary macrophages consistently observed in old mice from both whole lung cell isolation and bronchoalveolar lavage (unpublished observation)\textsuperscript{357}. This suggests that although macrophages from old mice fail to control bacterial burden \textit{in vitro} at a single cell level where the cells are adherent on tissue culture wells and where number of cells/well is diluted to similar concentrations compared to cells from young mice, increased pulmonary macrophage numbers \textit{in vivo} may enhance bacterial control. For instance, efferocytosis, a process by which dying cells are removed by phagocytic
cells, has been shown to be an efficient innate immune mechanism against *M.tb* infection \(^{358}\). With increased total macrophage numbers within the lungs of old mice, it is possible that uninfected macrophages can more readily detect and efferocytose infected macrophages, thereby controlling initial infection. Furthermore, no additional IFNγ was given to macrophages infected with *M.tb* *in vitro*, and since IFNγ has been shown to kill intracellular *M.tb* by PL fusion independent pathways including the generation of iNOS and autophagy \(^{359}\), missing components over time within *in vitro* experiments may significantly alter bacterial control.

Although macrophages from old mice exhibited increased function and old mice infected with *M.tb* *in vivo* exhibit an early resistance phenotype, this early resistance is lost over time as old mice succumb to increased bacterial burden \(^{295}\). This is also evident in elderly individuals where increasing age is associated with increased susceptibility to succumb to infectious diseases, including TB \(^{17}\). One potential explanation for this conflicting data may stem from the damage caused by chronic inflammation \(^{360}\). For example, excessive TNFα, IL-1β and IFNγ detected within the lungs of *M.tb* infected young mice can lead to increased mortality \(^{361-363}\) as a result of extensive inflammation and tissue destruction \(^{361,362}\). Since the pulmonary environment of old mice have increased levels of TNFα and IFNγ, and a trend for increasing IL-1β, long term infection models in old hosts may demonstrate increased tissue damage and mortality due to organ failure.

Alternatively, generation of a robust, inflammatory response may lead to a subsequent increase in anti-inflammatory responses \(^{364}\) which can interfere with activation of
adaptive immunity. In support of this notion, a human study examining the effects of IL-10 post-influenza infection demonstrated that the IFNγ:IL-10 ratio was reduced in old age which was significantly associated with patients suffering from laboratory diagnosed influenza. These studies demonstrate that paradoxically, although genotypes associated with high IL-10 are correlated with longevity, high IL-10 is also associated with susceptibility to influenza infection in old age. This suggests that old mice infected with M.tb may display increased levels of IL-10 post-infection due in part to increased basal concentrations of IL-10, which could have decreased bacterial control. This is supported by previously published data within our laboratory demonstrating that the presence of IL-10 during early infection can negatively regulate the generation of long term immunity. Together these data suggest that increased IL-10, which may be important in longevity for the context of inflammaging, could increase susceptibility of elderly individuals to infectious diseases.

Furthermore, functional aspects of the other immune cells during M.tb infection in vivo still need to be characterized in old mice in order to understand why bacterial control is lost over time. Although preliminary findings from our laboratory (described in chapter 1) demonstrated that M.tb specific-IFNγ responses by T cells from infected old mice were not significantly different compared to cells from young mice, recent findings have established IFNγ-independent M.tb bacterial control pathways which may be altered in old age. For instance, a report by Cowley et al. demonstrated that M.tb infection of IFNγ receptor-deficient BMDMs co-cultured with M.tb primed CD4 T cells exhibited increased bacterial control, that was dependent on nitric oxide (NO) production,
compared to cultures with BMDMs in the absence of CD4 T cells. Since alveolar macrophages from old rats have been shown to induce less NO in response to stimulation, increased bacterial burden over time may be a consequence of reduced CD4-dependent, IFNγ-independent NO generation. M.tb-specific CD8 T cells have also been shown to enhance protection through cytolytic activities utilizing perforin to kill target cells in vivo. Although the effects of cytotoxic T cells during M.tb infection with advanced age are unclear, the proportion of cytoplasmic perforin positive cells decline rapidly in old age suggesting a functional deficiency in cytotoxic potential. These data suggest that IFNγ-independent T cell mechanisms able to control M.tb infection may be altered in old age in vivo and further studies are necessary to understand the contribution of IFNγ-independent responses to long-term bacterial control in old age.

It is also known that neutrophils, which have been associated with an exacerbation of M.tb infection, may be altered in old age. For example, expression of IL-17, a proinflammatory cytokine which can regulate neutrophil infiltration, is increased in the lungs of old age. This suggests that increased IL-17 could recruit excessive neutrophils post-M.tb infection, leading to increased immunopathology and increased mortality in old mice. Support for excessive IL-17 and neutrophils leading to detrimental disease progression in old age have been documented in a herpes simplex virus (HSV) infection model. Stout-Delgado et al. demonstrated that systemic viral infection led to increased NKT cell IL-17 production, neutrophil accumulation and hepatocyte necrosis which were associated with mortality in old mice. When αIL-17 was given during HSV infection, old mice were rescued from increased neutrophil activity, hepatocyte necrosis
and IL-17 induced death. Taken together, these data suggest that other cell types besides macrophage and T cell functions will need to be further evaluated in the context of *M. tb* infection in old age to understand how bacterial control is lost over time.

We used ibuprofen-supplemented food diet as a mechanism to modify the inflammatory state in old mice *in vivo*. Ibuprofen acts, in part, by inhibiting the production of prostaglandins which are known to mediate pain, swelling, fever and inflammation.\(^{377}\). Furthermore, ibuprofen has been reported to be a successful drug in treating or preventing several age-associated inflammatory diseases such as Alzheimer’s\(^{322}\), sarcopenia\(^ {378}\), Parkinson’s\(^ {379}\) and cataracts\(^ {380,381}\). In this study, two weeks of ibuprofen supplemented diet was sufficient to significantly decrease the message levels of lung inflammation observed in old mice. Furthermore, PL fusion events in macrophages isolated from old mice on ibuprofen were restored to levels similarly observed in macrophages from young mice. Although we saw significant changes in the lung environment and macrophage function of ibuprofen fed old mice, we failed to see a reduction in bacterial control during *in vitro* infection.

However, it is unclear if the two week ibuprofen treatment was the ideal duration to observe alterations in inflammatory responses. Since inflammaging is thought to accumulate slowly over decades of aging with significant alterations in cellular and molecular phenotypes and function\(^ {382}\), we hypothesize that short regimens (1-2 days) of ibuprofen would not have led to profound restoration in inflammation or cellular function. This hypothesis is further supported by human studies examining short and
long-term effects of ibuprofen and association with risk of Parkinson’s disease.

Parkinson’s disease is a neurodegenerative disorder thought to be associated with increased inflammatory activities. In a recent meta-analysis study, Gagne et al. demonstrated that ibuprofen usage was associated with decreased incidence of Parkinson’s development. Furthermore, the protective effects of ibuprofen were more pronounced among regular users and long term users compared to inconsistent and short-term users of ibuprofen. This study suggests that the duration of ibuprofen may significantly alter immune phenotype and function.

In addition, although the message levels of proinflammatory cytokines were decreased in ibuprofen fed old mice compared to control old mice, our data lacked protein analysis to determine systemic and organ specific inflammatory states. This suggests that mice fed ibuprofen for two weeks may be able to reduce proinflammatory mRNA message levels but have yet translated to reduced inflammatory protein levels. Thus, although two weeks of ibuprofen treatment was sufficient to restore message levels of inflammation back to levels observed in young mice, a long-term ibuprofen regimen might have the potential to further restore cellular and molecular functions. These include functions such as autophagy, which is altered in old age and could affect M.tb bacterial control. Therefore, in order to understand the whole scope of ibuprofen induced changes in macrophages from old mice, different duration and dosage of ibuprofen treatment will be necessary.
Ibuprofen duration might also explain why we failed to see a reduction in the surface expression of MR, CD86 or MHCII (Table 2.1) on macrophages isolated from old mice on ibuprofen compared to control old mice. In addition, no differences were observed in surface expression of CD11b and CD11c between control and ibuprofen fed old mice. This suggests that the 2 week addition of ibuprofen cannot alter the cellular composition within the lungs of old mice and is unable to reduce proinflammatory induced receptors such as CD86 and MHCII\textsuperscript{385,386}. Alternatively, we hypothesize that other mediators capable of driving cellular surface expression would still occur within the lungs of old mice regardless of ibuprofen administration. These include damage-associated molecular patterns (DAMPs) that arise from cellular stress, and DNA and tissue damage\textsuperscript{232}, which are known to be increased in old age\textsuperscript{152} and are able to increase MR, CD86 and MHCII expression\textsuperscript{232,387,388}. Furthermore, ibuprofen is known to modulate pain which is known to alter immune cell function\textsuperscript{389}. This suggests more information will be necessary in order to determine if different ibuprofen regimens could affect receptor expression or if ibuprofen-independent mechanisms, such as increased DAMP in old age could modify receptor expression.

Taken together, we believe that aging is associated with increased proinflammatory mediators and age-associated inflammation which can alter immune phenotypes and \textit{M.tb} bacterial control. When old mice are given ibuprofen, immune function can be restored to levels similarly observed in macrophages from young mice by an anti-inflammatory and possibly other mechanisms, including the suppression of pain. However, contrary to our functional and previously published early infection data in old mice, macrophages from
old mice cannot control *in vitro* bacterial growth. Furthermore, although ibuprofen is able to reduce inflammation and restore function in macrophages from old mice, we again failed to observe alterations of *in vitro* *M. tb* bacterial control. Since *in vitro* cultures lack the environmental changes provided in the host, such as increased IFNγ in old mice, or ibuprofen in ibuprofen fed mice, studies *in vivo* will be necessary to determine how age-associated inflammation as well as the effects of ibuprofen could alter bacterial control in old age. In chapter 3, we further dissect and define immune changes with advanced age and how ibuprofen may alter *M. tb* bacterial control in old age using *in vivo* manipulations.
Figure 2.1: Lungs of old mice demonstrate an enhanced inflammatory state. Relative expression of IL-1β (A), IL-6 (B), TNFα (C), IL-12p40 (D), IFNγ (E) and IL-10 (F) as determined by RT-qPCR. Whole lung homogenates were collected into TRIzol® reagent and cDNA was synthesized using Omniscript RT Kit and real-time PCR was performed using TaqMan® gene expression assays. Data were combined from two independent experiments (A-B), three independent experiments (C-E) or a single experiment with pools of 5 mice (F). The Student's t test was used to determine statistical significance.
Figure 2.2: Macrophages isolated from the lungs of old mice have elevated inflammation associated activation markers. Relative expression of IRGM-1 (A), IRF-1 (B) and CIITA (C) as determined by RT-qPCR. Purified lung macrophages were collected into TRIzol® reagent and cDNA was synthesized using Omniscript RT Kit and real-time PCR was performed using TaqMan® gene expression assays. Data were combined from two independent experiments. The Student's t test was used to determine statistical significance.
Figure 2.3: IFN-γ fails to boost cytokine production in response to *M.tb* infection in pulmonary macrophages from old mice. Pulmonary macrophages from young (open bars) and old (solid bars) mice were plated onto coverslips and left untreated for 16 hours or incubated with recombinant murine IFNγ. Cells were then stimulated with media alone, or infected with 5:1 GFP-*M.tb* for 24 hours. Supernatants were collected and the concentration of IL-12p40 was analyzed by ELISA. Data were combined from three independent experiments. The Student's *t* test was used to determine statistical significance.
Figure 2.4: Pulmonary macrophages from old mice have increased *M.tb* infection and increased P-L fusion compared to macrophages from young mice. Pulmonary macrophages isolated from old (solid bars) and young (open bars) mice were added to coverslips and incubated with 5:1 GFP-*M.tb* for 2 hrs and analyzed by confocal microscopy. White arrows indicate infected pulmonary macrophages (A), quantified in (B). P-L fusion was observed in macrophages from young and old using LAMP-1 (C, D) and Cathepsin D (E). Data were combined from three independent experiments from pools of 5 mice. Statistical significance was determined by the Student's *t* test.
Figure 2.5: IFN-γ fails to enhance P-L fusion in *M.tb* infected pulmonary macrophages from old mice. Pulmonary macrophages from young (open bars) and old (solid bars) mice were plated onto coverslips and incubated with recombinant murine IFNγ or left untreated in media alone for 16 hrs. Cells were infected with 5:1 GFP-*M.tb* for 2 hrs, fixed, permeabilized, blocked and stained for Cathepsin D and analyzed for P-L fusion by confocal microscopy. Data were combined from three independent experiments. Student's *t* test was used to determine statistical significance.
Figure 2.6: Pulmonary macrophages from old mice fail to control *M.tb* infection compared to macrophages from young mice. Pulmonary macrophages isolated from old (solid bars) and young (open bars) mice were incubated with 5:1 GFP-*M.tb* for 2 hrs and plates were washed with warm DPBS. Bacterial growth at defined time points post infection was then assessed by plating lysed macrophages on 7H11 agar with OADC supplemented plates (F). Colonies were enumerated after 21 days incubation at 37°C. Data were combined from three independent experiments from pools of 5 mice. Statistical significance was determined by the Student's *t* test.
Figure 2.7: Ibuprofen decreases the inflammatory lung environment of old mice. Relative expression of IL-1β (A), IL-6 (B) TNFα (C), IL-12 (D) and IFNγ (E) as determined by RT-qPCR of whole lung homogenates from young mice on control diet (YC; open bars) and naïve old mice on either control (OC; solid bars) or ibuprofen diet (OI; grey bars). The ΔΔct method was used to analyze data, with 18S rRNA as endogenous normalizer. Data were combined from three independent experiments each using pools of 5 mice. The Student's t test was used to determine statistical significance.
Figure 2.8: Ibuprofen is able to restore macrophage function but fails to promote intracellular bacterial control. For cytokine production, cells isolated from old mice on control (OC; solid bars) or ibuprofen (OI; grey bars) diet were infected with 5:1 GFP-M.tb for 24 hrs and the concentration of IL-12 in supernatants was analyzed by ELISA (A). For P-L fusion, cells were infected with 5:1 GFP-M.tb for 2 hrs, fixed, permeabilized, blocked and stained for LAMP-1 (B) or Cathepsin D (C) and analyzed by confocal microscopy. For CFU, macrophages were incubated with 5:1 GFP-M.tb for 2 hrs then washed with warm DPBS. Bacterial growth at defined time points post infection was assessed by plating lysed macrophages on 7H11 agar with OADC supplemented plates (D). Colonies were enumerated after 21 days incubation at 37°C. Data were combined from three independent experiments (A-C) or from a single experiment (D). The Student's t test was used to determine statistical significance.
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**Table 2.1: Mean fluorescence intensity of cell-surface markers.** Pulmonary macrophages from young and old mice on control or ibuprofen diet were isolated, fixed and stained extracellularly with antibodies against CD11b, CD11c, CD86, mannose receptor (MR), or MHC II. Mean fluorescence intensity (MFI) of total CD11b and CD11c populations and MFI of CD86, MR, or MHCII on CD11c positive cells are expressed. Isotype control antibodies were used to set positive gates. MFI values were normalized by subtracting isotype control values from those obtained using specific antibodies. # denotes significant differences ($p<0.05$) detected between young control and old control groups. No significant differences were observed between control and ibuprofen treatment in either age groups.
Chapter 3: Modulating inflammation in young and old mice alters *Mycobacterium tuberculosis* bacterial control

*Abstract:*

Elderly individuals have increased susceptibility to pulmonary disorders and infectious diseases due in part to the age-associated increase in systemic basal inflammation resulting in altered cellular function. In this study, we investigated the impact of increasing inflammation in young mice and decreasing inflammation in old mice on the control of a *Mycobacterium tuberculosis* (*M.tb*) infection. LPS injected young mice showed increased trends for low-level systemic inflammation and displayed similar cellular phenotypes to those observed in naïve old mice. Upon *M.tb* infection, LPS injected young mice displayed similar early bacterial control as *M.tb* infected old mice implicating increased inflammation as a mediator of early resistance. However, *M.tb* infected old mice given ibuprofen (which decreased inflammation) had a further enhanced early control of *M.tb* infection compared to both young and old mice. This enhanced bacterial control in old mice on ibuprofen was independent of IFNγ but dependent on IL-17 and cell death.
Introduction:

The elderly population is increasing at a dramatic rate. Between the year 2000 and 2050, individuals 85 years and over are projected to increase by over 300% within the United States. This tremendous increase is a public health concern because elderly individuals are more likely to need long-term health care due to their increased susceptibility to infectious diseases and slow recovery rate from infection. Aging is accompanied by an elevated basal inflammatory state which is thought to contribute to the altered immune function in the elderly and the increased susceptibility to infectious diseases, including tuberculosis (TB). However, aging is also associated with a multitude of other changes including increased tissue lipid deposition, loss of tissue function (collagen and elastin in connective tissues), muscle atrophy and altered hormone production and responsiveness. Therefore, it is unknown if inflammation alone can induce immune changes that drive M.tb susceptibility in old age.

In chapter 2, we have shown that the lungs of old mice are in an inflammatory state compared to the lungs of young mice, consistent with the newly described phenomenon termed inflammaging. Because inflammation is increased in old mice and inflammaging is implicated in several age-associated diseases including Alzheimer’s, we hypothesized that inflammation might be responsible for the increased susceptibility of the elderly to M.tb infection. In order to understand the impact of inflammation in old age, we examined immune changes when inflammation was 1) increased in young mice and 2) decreased in old mice.
To determine if inflammation alone can mimic the phenotypes in old age, we injected low-dose lipopolysaccharide (LPS) into young mice to increase inflammation. We chose to use an agonist with broad responses as opposed to injection of specific cytokines because several pro-inflammatory cytokines including interleukin (IL)-1β, IL-6 and tumor necrosis factor-alpha (TNFα) are known to increase in old age. Furthermore, it is anticipated that inflammation in old age induces multiple uncharacterized pathways and molecules. We recognize that our inflammatory model is limited by the ability of the host to recognize LPS, since cellular activation is predominately through toll-like receptor (TLR) 4, and caspase-11 in mice, while inflammaging is thought to be due to alterations in aging mitochondria or due to telomere shorting. However, the downstream inflammatory cascades are broad and we hypothesized that they would represent some changes associated with aging. Low-dose injections of LPS in mice display mild fever and mild increases in proinflammatory cytokines such as interleukin IL-6, IL-1β and TNFα, while high dose injections can result in significant increases in proinflammatory mediators, reduced motor activity, lethargy, decreased cardiac output and lethality. Therefore, low-dose LPS injection allowed for a general increase in inflammation and inflammatory responses. Young mice injected with LPS exhibited systemic inflammation with increasing trends of several proinflammatory cytokines, as well as altered cellular function similarly observed in cells isolated from naïve old mice. Furthermore, LPS injected young mice infected with the virulent pulmonary pathogen Mycobacterium tuberculosis (M.tb) exhibited similar bacterial control compared to M.tb.
infected old mice. Our study demonstrates that inducing inflammation in young mice by LPS injection is sufficient to mimic *M.tb* bacterial control detected in old mice.

Previously, we have shown that ingestion of ibuprofen, a nonsteroidal anti-inflammatory drug (NSAID), can decrease the inflammatory lung environment observed in naïve old mice. Ibuprofen treatment also restored functional abilities of cells isolated from the lungs of old mice to cells from young mice. Therefore, we chose to use ibuprofen to modulate the inflammatory environment in order to determine how decreasing inflammation may alter bacterial control in old mice *in vivo*. Similar to our LPS injection model in young mice, we chose a broad acting anti-inflammatory agent as many inflammatory mechanisms in old age are not well characterized.

We have previously shown that old mice infected with *M.tb* displayed an early resistance phenotype compared to young mice\(^{295,296}\). Therefore, we hypothesized that *M.tb* infected old mice on ibuprofen would have increased bacterial burden compared to infected old mice on control diet. Contrary to what we hypothesized, old mice on ibuprofen infected with *M.tb* showed a further enhanced bacterial control compared to infected old mice on control diet. Enhanced *M.tb* control in old mice induced by ibuprofen was not dependent on previously characterized mechanisms of early resistance by CD8+/interferon-gamma (IFNγ)\(^{295,402}\). Instead, we found that high levels of IL-17 detected in *M.tb* infected old mice exacerbated bacterial burden compared to old mice on ibuprofen diet. Furthermore, we found that cells isolated from *M.tb* infected old mice demonstrated increased levels of
cell death. Taken together, our study further confirms that inflammatory cytokines may play an important role in the control of primary \textit{M.\textit{t}b} infection in old age.

\textit{Materials and methods:}

\textbf{Mice}

Specific pathogen-free, female and male C57BL/6, and female BALB/c mice were purchased from Charles River Laboratories or through rodent resources with the National Institute on Aging (supplied by Charles River Laboratories) at an age of 3 months or 18 months (similar to chapter 2). Mice were maintained in ventilated cages and provided with sterile food and water \textit{ad libitum}. For diet modification studies, mice were fed either the Teklad Global 18\% protein rodent control diet or the Teklad Global 18\% protein rodent control diet supplemented with ibuprofen (375ppm; Harlan Laboratories) for 2 weeks before experimental manipulation. Ibuprofen dosage was chosen based on efficacy and safety shown in studies of long-term ibuprofen use in old mice\textsuperscript{322}. Mice were killed for experiments by CO\textsubscript{2} asphyxiation. All protocols were approved by The Ohio State University’s Institutional Laboratory Animal Care and Use Committee.
Real-time quantitative polymerase chain reaction (RT-qPCR)

The lungs of young and old mice were removed and homogenized in 1ml TRIzol reagent (Invitrogen). RNA was extracted with chloroform, precipitated using isopropanol and 75% ethanol, reconstituted in DNase/RNase-free water and quantified on a NanoDrop spectrophotometer. cDNA was synthesized with random primers using an Omniscript RT Kit (Qiagen). Real-time PCR was performed on cDNA from whole lung using TaqMan gene expression assays (Applied Biosystems) for IFNγ, TNFα, and IL-12p40. A Bio-Rad CFX96 Real-Time PCR thermal cycler was used for qPCR. The ΔΔCT method was used for quantification of the relative units (RU) of mRNA, using 18s as an endogenous normalizer and young control mice as the calibrator $[ RU = 2^{-\Delta\Delta CT}; \Delta CT = CT \text{ gene (IFNγ, TNFα, etc.)} - CT \text{ 18S}; \Delta\Delta CT = \Delta CT \text{ sample} - \Delta CT \text{ calibrator (young mouse)}]$. 

M. tb infection and Colony Forming Unit (CFU) enumeration

All mice were infected with green fluorescent protein (GFP)-M. tb. This strain expresses GFP constitutively through a Mycobacterium bovis bacillus Calmette-Guerin heat shock protein 60 promoter and was generated by Horwitz and colleagues $^{324}$. M. tb stocks were grown in Proskauer-Beck liquid medium containing 0.05% Tween 80 to mid-log phase and frozen in 1 ml aliquots at –80°C. Mice were infected with GFP-M. tb Erdman using an inhalation exposure system (Glas-Col) calibrated to deliver 50–100 CFU (for low-dose aerosol) as previously described $^{296}$. At specific time points post M. tb infection mice were sacrificed and lung and spleen were aseptically removed into sterile saline. Organs were
homogenized and serial dilutions plated onto 7H11 agar supplemented with OADC as previously described. Plates were incubated at 37°C for 21 days in order to enumerate bacterial colonies and calculate the bacterial burden.

**Lung cell isolation**

For whole lung cells, mice were euthanized by CO₂ asphyxiation and lungs perfused with cold phosphate buffered saline containing 50 Units/mL of heparin through the right ventricle. Lungs from individual mice were mechanically disrupted using a GentleMACS dissociator (Miltenyi Biotec) followed by collagenase A (type XI) (0.7mg/mL, Sigma) and type IV bovine pancreatic DNase (30μg/mL, Sigma) digestion at 37°C for 30 minutes in GentleMACS C-tubes. Lung cell suspensions were passed through a 70μm nylon cell screen and residual erythrocytes were lysed with Gey’s solution. Viable cells were determined by trypan blue exclusion.

For alveolar macrophage isolation, mice were sacrifice and the trachea was exposed for bronchoalveolar lavage (BAL) isolation. A 20 gauge catheter was placed into the trachea and a syringe containing 0.5ml PBS was used for installation and aspiration of the lung. 10 cycles of instillation and aspiration was performed to collect a total of 5ml BAL fluid. BAL fluid was washed once with PBS and resuspended in 1ml PBS. Cells were counted using trypan blue and adjusted to working concentrations in PBS.
Cell purification

Single lung cell suspensions were adhered to sterile tissue culture dishes for 1 hour at 37°C. Non-adherent cells were washed and removed from the plates. CD8+ cells were obtained from the non-adherent cell fraction by magnetic cell separation (BD IMAG anti-CD8+ particles 551516) and placed directly into TRIZOL reagent (Invitrogen), homogenized, and frozen at -80°C. Purity of CD8+ cell populations was determined to be greater than 90% for all experiments by flow cytometry using an LSRII flow cytometer (BD). Adherent cells were lysed in TRIZOL, homogenized, and immediately frozen at -80°C.

Flow cytometry

Isolated lung cells were suspended in deficient RPMI supplemented with 0.1% sodium azide (Sigma). Surface targets were detected as previously described in chapter 2. For LPS studies, specific Abs and isotype controls were purchased from BD Biosciences: R-phycoerythrin (PE)-CD122 (TM-Beta 1), Allophycocyanin (APC)-CD44 (IM7), PE-Cy7-CD4 (RM4-5), PerCP-CD8+ (53-6.7). For intracellular staining studies, specific Abs and isotype controls were purchased from Biolegend: APC-Cy7-IL-17 (TC11-18H10.1), PE-IFNγ (XMG1.2), PE-Cy7-CD8+ (53-6.7), and PerCP-Cy5.5-CD4 (GK1.5). Cytokine levels were determined according to the manufacturer’s instructions for intracellular cytokine staining (Cytofix/Cytoperm fixation/permeabilization solution kit with BD GolgiStop, BD Biosciences), following a 4 hour incubation with 10μg/mL anti-CD3
(145-2C11) and 1μg/mL anti-CD28 (37.51). Samples were read using a six color BD LSRII flow cytometer and analyzed with FlowJo software (Tree Star, Inc). For apoptosis assays, BALs were washed with PBS and resuspended in binding buffer (BD Biosciences) and stained with PE annexin V and 7-aminoactinomycin D (7-AAD). Cells were further processed according to the manufacturer’s instructions and analyzed by flow cytometry.

**In vivo mouse manipulations**

For inducing inflammation, young mice were injected daily for 14 days by intraperitoneal (i.p.) injection with a dose of 20 μg/mouse LPS (Sigma) diluted in 100μl pyrogen-free 0.9% NaCl. Mice were aerosol infected with *M.tb* on D12 of the injection protocol.

For neutralizing IFNγ, mice were i.p. injected with 500μg/mouse anti-IFNγ neutralizing antibody (XMG1.2, BioXcell), or 2A3 isotype control in 100μl pyrogen-free 0.9% NaCl. For CD8+ depletion, mice were i.p. injected with 500μg/mouse anti-CD8+ (53-6.72) or HRPN isotype control in 100μl NaCl. For IL-17 neutralization, mice were i.p. injected with 500μg/mouse anti-IL-17 (17F3) or MOPC-21 isotype control in NaCl. Mice were injected every other day for 14 days, and mice were aerosol infected with *M.tb* on D12 of the injection protocol. For all neutralization and depletion experiments, NaCl vehicle control was used for the first experiment while isotype control was used for repeated experiments. No differences were observed between NaCl injection or isotype control
injection in any experimental conditions. Injections will be referred to as “vehicle control” indicating either NaCl or isotype control.

For recombinant (r)IL-17 experiments, mice were injected i.p. with 0.5μg/mouse of rIL-17 (R&D Systems) in 100μl pyrogen-free 0.9% NaCl or NaCl alone. Mice were injected every other day for 14 days and mice were aerosol infected with M.tb on D12 of the injection protocol.

Protein and eicosanoid measurements in lung homogenates

Whole-lung and liver homogenates were collected 12 days post LPS injection or 14 days post-infection with M.tb and frozen at -80°C. Samples were thawed and clarified by centrifugation to remove tissue debris. The levels of IFNγ, IL-12p40, IL-6, and TNFα in supernatants were determined using enzyme-linked immunosorbent assay (ELISA) sets according to the manufacturer’s instructions (BD Biosciences).

Prostaglandin E2 (PGE2) concentrations were quantified using an enzyme immunoassay (EIA) kit (Cayman Chemical). Levels of PGE2 were assessed in partial lung homogenates according to the manufacturer’s directions.

C-reactive protein (CRP) concentrations from lung and liver were quantified by an ELISA kit (R&D Systems), following the manufacturer’s directions.

Indoleamine-2,3-Dioxygenase (IDO) concentrations were quantified using an ELISA kit (Lifespan Biosciences), following the manufacturer’s directions.
Statistical analysis

Data were analyzed using GraphPad Prism 5.0 software (GraphPad). The unpaired two-tailed Student’s $t$-test was used to determine statistical significance. The Grubbs’ test was used to identify outlying data points.
**Results:**

Low-dose LPS injection in young mice can increase lung inflammation and recapitulate age-associated phenotypes of old mice

Young mice were intraperitoneally injected daily with 20µg of LPS for 12 days. LPS injection led to upregulation of IL-1β (Fig 3.1A), IL-6 (Fig 3.1B) but a decrease in TNFα (Fig 3.1C), within the spleen as well as upregulation of IL-1β (Fig 3.1D), IL-6 (Fig 3.1E) and TNFα (Fig 3.1F) within the lung. Furthermore, IFNγ (Fig 3.1G) and IL-12 (Fig 3.1H), cytokines known to be important for *M. tb* control and previously observed to be upregulated in old mice, were increased in the lung of LPS injected young mice, but these did not reach statistical significance. Message potential translated to protein changes as concentrations of both TNFα (Fig 3.2A) and IL-6 (Fig 3.2B) also showed a similar increasing trend compared to NaCl injected young mice. CRP, an acute-phase protein that increases in response to inflammation and has been used clinically as a diagnostic tool as a marker of inflammation, increased significantly in the liver (Fig 3.2C) compared to NaCl injected young mice. These data suggest that systemic inflammation was occurring within LPS injected young mice. An increasing trend for CRP was also observed in the lungs (Fig 3.2D) of LPS injected young mice compared to NaCl injected young mice, although the difference was not statically significant. Together these data suggest that low-dose injection of LPS into young mice can induce a mild inflammatory environment in multiple organs.
We have previously shown that old mice infected with *M.tb* displayed a transient early resistance during infection compared to young *M.tb* infected mice which is lost at later timepoints when old mice succumb to higher bacterial burden. Since we believe that inflammation is a driver of age-associated alterations, we hypothesized that inflammation in old mice led to early resistance as well as the subsequent loss of bacterial control. To test our hypothesis, we infected LPS injected young mice with *M.tb* to determine CFU burden. If age-associated alteration in bacterial control was due to inflammation, then LPS injected young mice, which displayed moderate increased inflammation (Fig 3.1-2), infected with *M.tb* would also generate the early resistance phenotype and subsequent increased bacterial burden at later timepoints. NaCl and LPS injected young mice were aerosol infected with *M.tb* and the lungs and spleen were excised on D14 and D35 post-infection for bacterial load determination. The bacterial burden in LPS injected young mice were significantly decreased in both the lung (Fig 3.3A), and the spleen (Fig 3.3B) compared to NaCl injected control on D14 post-infection, matching the old mouse early resistance phenotype. However, no differences were observed in bacterial control D35 post-infection in the lung (Fig 3.3C) or spleen (Fig 3.3D). Together these data demonstrate that inducing inflammation by LPS injection in young mice can generate changes that are responsible for the early bacterial control seen in *M.tb* infected old mice but, not the late loss of *M.tb* control we observe in old mice.
**M. tb control is further enhanced in old mice supplemented with ibuprofen diet**

Since inflammation alone in young mice can drive the early resistance phenotype we have reported in old mice, we next hypothesized that decreasing environmental inflammation in old mice would reverse age-associated phenotypes. We used ibuprofen in order to generate a broad systemic decrease in inflammation. Ibuprofen has been tested previously in old mice where it decreased lung inflammation and reverted pulmonary macrophage phenotype and function back to what was observed in young mice. 404 Young and old mice were placed on control or ibuprofen supplemented diet and aerosol infected with *M. tb*. Mice were sacrificed and the lungs excised for bacterial burden determination. No significant differences were determined at D6 (Fig 3.4A) post-infection between any experimental groups but early bacterial control between old and young mice was evident by D10 post-infection (Fig 3.4B), and most clearly by D14 post-infection (Fig 3.4C-D), similar to previously described 295. Contrary to what we hypothesized, the bacterial burden of old mice on ibuprofen diet did not revert to levels similarly seen in young mice on control diet. Instead, an additional decrease in bacterial burden was evident by D14 post-infection between old control mice and old ibuprofen mice (Fig 3.4C-E). Because the differences between old mice on control and ibuprofen diet were not evident until D14 post-infection, this suggest that ibuprofen induced bacterial control might be mediated by a mechanism that occurs later than day 7 during infection. To ensure that the phenotype of ibuprofen induced control was not sex and strain dependent, we evaluated bacterial control in female C57BL/6 (Fig 3.4C) and BALB/c (Fig 3.4D) mice as well as male C57BL/6 mice (Fig 3.4E). Similar bacterial
burden trends were observed among the experimental groups suggesting that ibuprofen mediated protection was sex, and strain independent. However, a significant difference was observed only in males between old control and old ibuprofen mice D14 post infection (Fig 34E), suggesting that sex differences might further enhance ibuprofen induced bacterial control.

By D21 post-infection (Fig 3.5A), when antigen-specific IFNγ producing CD4 T cells and bacterial control occurs in M.tb infected mouse lungs, CFU burden were not significantly different among experimental groups. By D35 post-infection (Fig 3.5B), M.tb infected old mice on control diet had an increase bacterial burden compared to young mice, as previously described. Interestingly, a decreasing trend in bacterial burden was still observed in old mice on ibuprofen diet compared to old mice on control diet (Fig 3.5B) suggesting the modulation of inflammation can influence adaptive immune control. Importantly, no differences were observed between young mice on control diet and young mice on ibuprofen diet at any timepoints post M.tb infection, demonstrating that ibuprofen induced bacterial control is strictly an age-associated phenotype. Together, these data suggest that ibuprofen can further enhance bacterial control in M.tb infected old mice but not in M.tb infected young mice.

Ibuprofen enhanced bacterial control in M.tb infected old mice is IFNγ independent

To understand how ibuprofen can further increase bacterial control in M.tb infected old mice, we first investigated a known mechanism of early M.tb control. We have
previously shown using gene disrupted (KO) mice that early enhanced bacterial control in *M.tb* infected old mice compared to *M.tb* infected young mice was dependent on the presence CD8+ cells or the ability to make IFNγ\(^{294,402}\). We therefore sought to determine if the further enhanced *M.tb* control in old mice on ibuprofen was also due to IFNγ production by CD8+ cells. Young and old mice on control or ibuprofen diet were aerosol infected with *M.tb*. CD8+ cells were purified from total lung cells and IFNγ message expression was analyzed. Message expression of IFNγ was significantly increased in old mice on control diet compared to young mice on control diet at D8 (Fig 3.6A) as previously described\(^{296}\), although we observed no significant differences between old mice on control diet or ibuprofen diet. Similarly, no significant differences were observed in any group by D14 post-infection (Fig 3.6B), although an increasing trend was still observed between old and young mice on control diet. To further investigate the relationship between young and old mice on control or ibuprofen diet, we isolated whole lung cells from *M.tb* infected mice for intracellular cytokine staining. As expected, old mice had increased IFNγ positive cells compared to young mice on control diet, basally and upon infection (Fig 3.6C). However ibuprofen did not enhance IFNγ positive cells in old mice and perhaps decreased IFNγ positive cells post-infection compared to old mice on control diet. Taken together, these data suggest that the difference in bacterial control between old mice on ibuprofen supplemented diet and old mice on control diet may not be dependent on CD8+ cells or IFNγ.

Previous studies showed that early resistance was lost when aged CD8+ KO mice were infected with *M.tb*\(^{295}\), demonstrating that the presence of CD8+ cells was important in
the early resistance phenotype. To evaluate the role of CD8+ cells in the ability of ibuprofen to further enhance bacterial control in old mice, we depleted CD8+ cells in vivo using antibodies. Old mice were placed on either control or ibuprofen diet and injected with vehicle control or αCD8 antibodies. Lungs were excised at D14 post-\(M.\text{tb}\) infection for bacterial burden determination. Contrary to our hypothesis, depletion of CD8+ cells did not change the early resistance phenotype in old mice on control diet or old mice on ibuprofen diet (Fig 3.7A). This suggests that CD8+ cells may not contribute to the further enhanced bacterial control in ibuprofen supplemented, \(M.\text{tb}\) infected old mice.

To test whether further \(M.\text{tb}\) resistance induced by ibuprofen in old mice was independent of IFNγ, we neutralized IFNγ in old mice. Old mice were placed on control or ibuprofen diet and were injected with vehicle control or αIFNγ neutralizing antibodies. Lungs of \(M.\text{tb}\) infected mice were excised at D14 post-infection for bacterial burden determination. Although not significant, old mice on control diet injected with anti-IFNγ had increased bacterial burden compared to vehicle control injected old mice, (Fig 3.7B). This observation supports previous published findings suggesting that decreased bacterial burden in old mice is IFNγ dependent. Increased bacterial burden was also observed in old mice on ibuprofen injected with anti-IFNγ antibodies compared to vehicle control injected old mice on ibuprofen (Fig 3.7B). This suggests that IFNγ regulated partial bacterial control in infected old mice on ibuprofen. However, the further enhancement in bacterial control was still evident between old mice and old mice on ibuprofen after anti-IFNγ injection (Fig 3.7B). These data suggest that the difference in bacterial control between old mice on control diet and old mice on ibuprofen diet was IFNγ independent.
Enhanced *M.tb* control in old mice on ibuprofen is dependent on IL-17

Because upregulation of IFNγ is commonly associated with an upregulation in IL-17\(^{405-408}\) and recently, ibuprofen was shown to modulate IL-17 in calves\(^{409}\), we sought to investigate the role of IL-17 in *M.tb* infected old mice. To understand the contribution of IL-17 in old mice infected with *M.tb*, we examined intracellular staining levels of IL-17 positive cells. Similar to IFNγ, old mice on control diet had significantly more IL-17 positive cells compared to young mice on control diet basally and on D8 post-infection (Fig 3.8A). A decreasing trend was observed between old mice on ibuprofen diet and old mice on control diet at basal timepoint which became significant D8 post-infection (Fig 3.8A). CD4+ and CD8+ cells were not the dominant IL-17 positive cells since CD4+ and CD8+ cells combined only accounted for roughly 15% of total IL-17 positive cells (Fig 3.8B). This suggests that ibuprofen supplementation can decrease total IL-17 positive cells within the lungs of *M.tb* infected old mice.

To understand the consequence of increased IL-17 positive cells in *M.tb* infected old mice on control diet, old mice were placed on either control or ibuprofen diet and injected with vehicle control or rIL-17 and aerosol infected with *M.tb*. On D14 post-infection, mice were sacrificed and lungs were excised for bacterial burden determination. *M.tb* burden was increased in both old mice on control and old mice on ibuprofen diet when given rIL-17 compared to vehicle control (Fig 3.8C). Furthermore, when infected old mice were injected with αIL-17 neutralizing antibodies, bacterial burden exhibited a decreasing trend in both control and ibuprofen fed old mice (Fig 3.8D). Together, these
data suggest that IL-17 concentration can modulate *M.tb* bacterial burden in old age. Although early resistance seen in old mice is dependent on IFNγ, the resistance phenotype is also under the influence of IL-17, which is made more apparent when inflammation is decreased.

**Ibuprofen induced bacterial control in old mice is associated with cell death regulation**

Eicosanoids are signaling molecules involved in several biological functions within the body including regulation of inflammation and immune responses \(^{410}\). Furthermore, literature supports the hypothesis that eicosanoids can modulate cell death in *M.tb* infection models \(^{411-413}\). Because ibuprofen is a cyclooxygenase (COX) inhibitor, an enzyme responsible for the generation of several eicosanoids, including prostaglandin E2 (PGE2), we sought to understand how cell death may play a role in the enhanced bacterial control we observed in old mice on ibuprofen diet. Old mice on control or ibuprofen diet were aerosol infected with *M.tb* and sacrificed on D8 post-infection. BAL was obtained from each mouse and cells were stained with annexin V (AV) or 7-AAD. Uninfected animals of the same condition (control/ibuprofen) were used as baseline controls for the apoptosis assay. *M.tb* infection of old mice led to decreased viable cells (AV/7-AAD\(^{-}\)) (**Fig 3.9A-B**), and corresponding increase in cells undergoing apoptosis (AV/7-AAD\(^{+}\)) and exhibiting membrane damage (AV/7-AAD\(^{++}\) and AV/7-AAD\(^{+++}\)) (**Fig**
This cell death was lost when old mice were fed a diet containing ibuprofen, indicating that ibuprofen prevents cell death associated with \textit{M.\textit{tb}} infection in old mice.

To understand how ibuprofen can decrease macrophage cell death in \textit{M.\textit{tb}} infected old mice, we examined the role of PGE2. PGE2 is an eicosanoid derived from COX activation and a known promoter of apoptosis in \textit{M.\textit{tb}} infection\textsuperscript{414,415}. Young and old mice on control or ibuprofen supplemented diet were infected with \textit{M.\textit{tb}} and lung lobes were excised at D8 for PGE2 determination. \textit{M.\textit{tb}} infected old mice on control diet had a trend for increased PGE2 compared to \textit{M.\textit{tb}} infected young mice on control diet (Fig 3.10A). In both \textit{M.\textit{tb}} infected young and old mice, ibuprofen supplemented diet decreased PGE2 concentration within the lungs compared to age-matched mice on control diet. This suggests that ibuprofen can decrease PGE2 concentration \textit{in vivo} post-\textit{M.\textit{tb}} infection.

We further investigated the role of indoleamine 2,3-dioxygenase (IDO), an enzyme that catalyzes the degradation of tryptophan. Increased IDO concentration has been shown to modulate immune responses by inducing macrophage apoptosis\textsuperscript{416}. Young and old mice on control or ibuprofen supplemented diet were infected with \textit{M.\textit{tb}} and lung lobes were excised on D14 post-infection for IDO determination. Although no significant differences were observed among any experimental group due to small sample size, \textit{M.\textit{tb}} infected old mice on ibuprofen diet exhibited a trend for increased IDO concentration (Fig 3.10B). Therefore, the ability of IDO to induce apoptosis did not significantly contribute to macrophage cell death in old mice on control diet post-\textit{M.\textit{tb}} infection since higher IDO
levels were observed in old mice on ibuprofen. Taken together, our data suggest that *M.tb* infected control old mice exhibit increased bacterial burden compared to old mice on ibuprofen that is associated with increased IL-17, PGE2 and cell death (Fig 3.11).

**Discussion**

A chronic low-grade increase in inflammatory mediators during physiological aging (inflammaging) is thought to contribute to increased susceptibility to infectious diseases as well as increased morbidity and mortality during infection in the elderly. We therefore asked two fundamental questions to evaluate the impact of inflammation: 1) is inflammation alone sufficient to induce phenotypes seen in *M.tb* infected old mice and 2) can decreasing inflammation in *M.tb* infected old mice revert bacterial control back to levels seen in infected young mice?

Since several cytokines are known to be increased in inflammaging, and the specific proinflammatory cytokines that might drive *M.tb* susceptibility in old age are unknown, we chose to use LPS because of its ability to induce a variety of proinflammatory cytokines in mice and in human. Because we wanted to mimic a low-grade chronic inflammation seen in old mice, our LPS doses were chosen based on previous published literature examining low-dose LPS injection *in vivo*, demonstrating mild increased serum concentrations of TNFα and IL-6. Although increased inflammatory serum concentrations can be generated after a single low-dose LPS injection, we extended our injection model to 12 days (14 if *M.tb* challenged) to better simulate the chronic
inflammation observed in old mice. We have shown that young mice injected with LPS can induce systemic inflammation similarly observed in old mice. Increased protein concentration trends of TNFα and IL-6 were observed post-LPS injection as well as significantly increased CRP in liver homogenates. In the future, we plan to examine specific inflammatory mediators that might recapitulate LPS driven responses without the use of a broad acting agent.

Although we do not believe that inflammaging is the result of a single mediator, we hypothesize that IL-6 might be a significant contributor of age-associated susceptibility. IL-6 is the best predictor of mortality in healthy elderly individuals\(^{420,421}\), which was independent of confounding risk factors for death including smoking, amount of physical exercise, or body mass index. IL-6 is also the best predictor of mortality in patients with chronic kidney disease\(^{422-424}\), patients with major trauma/multiple injuries\(^{425}\) and patients with coronary diseases\(^{426}\). Furthermore, high circulating IL-6 concentrations are associated with the development of age-associated characteristics including susceptibility to cardiovascular disease development\(^{426}\) and cognitive decline\(^{427}\). However, at this stage it is unclear if age-associated disease and mortality is induced by IL-6 or if alternative causes of disease and morbidity induce high IL-6 concentrations. A recent study published in 2016 demonstrated that the administration of antibodies to neutralize IL-6 in a caecal ligation sepsis model counteracted gastrointestinal disturbances and decreased serum levels of proinflammatory cytokines such as TNFα\(^{428}\) giving support to the idea that IL-6 can induce disease progression.
Of particular interest was the fact that message levels of IL-6 were not significantly increased in the spleen and a decreasing trend for TNFα message levels was observed post-LPS injection in young mice. A study in dogs demonstrated that endotoxin administration did not induce high levels of splenic IL-6 production, instead, the spleen provided a positive feedback loop of nonsplenic IL-6 generation. In this study, endotoxin was administered to control or splenectomized dogs. The authors observed a 75% decrease in total IL-6 plasma concentration when the spleen was removed, however splenic IL-6 only accounted for roughly 2% of whole body IL-6 production in control dogs. The authors thus concluded that increased whole body IL-6 concentrations post-endotoxin administration is mediated but not produced by the spleen. In addition, pulmonary macrophages but not splenic macrophages have been shown to produce high levels of TNFα upon endotoxin challenge in rats. Splenic macrophages have also been shown to aid in the clearance of LPS upon endotoxin administration. Therefore, long-term LPS stimulation might suppress TNFα and IL-6 production in the spleen to maintain homeostasis while taking up LPS for clearance. However, these justifications do not explain the fact that protein concentrations of TNFα and IL-6 were not significantly increased in whole lung homogenates post-LPS injection.

One possible explanation for why TNFα and IL-6 protein concentrations were not significantly different may be a consequence of too many LPS injections. LPS is known to induce endotoxin tolerance where cells isolated from low-dose injected mice given additional LPS ex vivo demonstrate decreased TNFα production compared to NaCl injected mice. Therefore, after several rounds of LPS injection, systemic inflammation
may not have been as robust as natural inflamming, which is not strictly dependent on TLR4 activation. Another possible explanation for why proinflammatory cytokine protein concentrations were not significantly increased is because cytokine levels, including IL-1β and IL-6, are known to peak 2 hours post-initial LPS injection. However, there is little information about cytokine kinetics in response to multiple LPS injections. Therefore, protein concentrations might not have been obtained at the peak level when our mice were sacrificed. Our data showing that CRP in the lung was not significantly increased may also be due to an issue with timing as CRP is thought to peak in blood serum 48 hours after peak serum IL-6 levels. Therefore, without kinetic information of serum IL-6 levels, it is hard to predict the optimal time for CRP measurement. A robust kinetics analysis was not possible due to limitations on old mouse availability.

Despite modest changes in inflammatory mediators in young mice given LPS, we demonstrated that the changes were sufficient to recapitulate the early resistant phenotype observed in M.tb infected old mice. LPS injected young mice infected with M.tb displayed increased bacterial control in both the lung and the spleen compared to NaCl injected young mice at D14 post-infection. These data demonstrate that LPS injection in young mice can induce a low-grade systemic inflammation which is sufficient to drive the early resistance phenotype seen in M.tb infected old mice. Although the exact mechanism for early control in LPS injected young mice remains unclear, increased IFNγ might mediate enhanced bacterial control based on our previously defined early resistance model in M.tb infected old mice. However, chronic low-grade inflammation is
also thought to contribute to the development of behavioral symptoms including depressive-like state, cognitive impairment, fatigue, stress and pain, which have the potential to alter cellular function. Therefore, it is unclear if inflammation alters immune function in LPS injected young mice in a direct or indirect manner. Additionally, no differences were observed at D35 post-infection between NaCl and LPS injected young mice. Since the last LPS injection in our model occurred 2 days post-M.tb infection, the lack of a different response at D35 post-infection might be due to the complete decline in LPS generated inflammation. In addition, in vivo studies of low-grade LPS injection have shown that cellular responses are restored to normal function within 2 weeks post injection, again suggesting the loss of LPS-driven response by D35 post-infection. Alternatively, the lack of a CFU difference at D35 might suggest that at later timepoints, inflammation is not sufficient to alter bacterial control in old age. Instead, other age-associated mediator, such as a dysfunctional intrinsic T cell responses might be responsible for the inability of M.tb infected old mice to control bacterial growth in vivo. Therefore, we demonstrated that inflammation alone is sufficient to generate early M.tb bacterial control but responses at later timepoints will need further investigation.

We next demonstrated that ibuprofen can further enhance bacterial control in M.tb infected old mice, which was contrary to our hypothesis. Enhanced bacterial control in ibuprofen fed, M.tb infected old mice were not dependent on IFNγ or CD8+ cells, instead, protection was associated with decreased IL-17, PGE2 and increased pulmonary macrophage cell survival. Differences between young mice on control diet and old mice
on control diet were evident by D10 post-infection, similar to previously published data. However no differences were seen between old mice on control diet and old mice on ibuprofen diet until D14 post-infection. This suggests that the onset of ibuprofen driven protection occurs later than the old age driven protection. One hypothesis for this delay in bacterial control is that the necessary component driving enhanced ibuprofen protection, such as IL-17, may not be significantly different between the two groups until later during infection. Indeed, no significant differences were observed in IL-17 basally prior to infection between old mice on control or ibuprofen diet. This could potentially explain why in chapter 2, no differences were detected during \textit{in vitro} \textit{M.tb} infection between pulmonary macrophages isolated from old mice on control or ibuprofen diet. Since IL-17 was not significantly different at basal levels in the lungs of old mice when pulmonary macrophages were isolated for infection, addition of IL-17 during \textit{in vitro} experiments might help simulate the environment \textit{in vivo}.

The increase in bacterial control in \textit{M.tb} infected old mice on ibuprofen was sex and strain independent which strongly suggests that ibuprofen induced control is an age-associated phenomenon. However significant differences between control or ibuprofen old mice were only observed in males, suggesting a gender dependent enhancement in ibuprofen induced control. Indeed, in human studies, numerous observations have confirmed that ibuprofen is more effective at reducing pain in men them women. Similarly, it was shown in mouse experiments using 2 different measurements of pain and discomfort (formalin and abdominal constriction test) that ibuprofen was able to induce a stronger analgesic effect in males then females. However, little research
details gender differences in the anti-inflammatory properties of ibuprofen. Since pain is known to alter immune function, and we have shown that inflammation alone is sufficient to induce immune changes, it is therefore hard to extrapolate whether the ability of ibuprofen to further enhance *M. tb* control in old male mice is pain or inflammation dependent. Further experiments using mediators that target inflammation alone would allow a better understanding of ibuprofen induced *M. tb* control in old mice.

We next showed that ibuprofen induced *M. tb* control at early timepoints was independent of IFNγ or CD8+ cells, a previously defined mechanism for *M. tb* control in old mice. Injection of IFNγ neutralizing antibody in infected control old mice reverted CFU back to levels observed in young infected mice. This confirmed previous observations using old IFNγ KO mice suggesting that IFNγ is important for the early resistance observed in *M. tb* infected old mice. However, in contrast to our previous published data, no differences were observed from CD8+ depletion of any old mouse experimental group, including CD8+ depleted and isotype depleted control old mice. This conflicting result may stem from different techniques as our model utilized an antibody mediated depletion method while our previous published data utilized a CD8+ gene-disrupted mouse model. CD8+ gene-disruption from birth might have altered the protective phenotype of *M. tb* infection in old mice. Alternatively, as we did not confirm the efficacy of our CD8+ depletion model, residual CD8+ positive cells could have remained in αCD8+ injected old mice. Incomplete CD8 depletion may mask the role of CD8+ T cells in our experiments.
When ibuprofen fed old mice were given IFNγ neutralizing antibody, bacterial burden was increased compared to isotype injected ibuprofen fed old mice. However, CFU remained significantly decreased in IFNγ neutralized ibuprofen fed old mice compared to infected young mice. In contrast, when old mice on control diet were given IFNγ neutralizing antibody, CFU increased to levels similar to infected young mice. Together, these data suggest that IFNγ mediates the majority of early resistance in old control mice but only partial bacterial control in ibuprofen fed old mice.

A caveat of our in vivo studies was that when *M. tb* infected old mice were subjected to multiple injections we observed that the enhanced bacterial burden between ibuprofen fed and control fed old mice was occasionally lost. This might be due to the stress the injection protocol elicits in old mice since old mice can exhibit increased panic-like behaviors in response to stressful environments or situations compared to young mice, which can alter immune function and bacterial control. Stress responses altering immune function in old age have also been documented in human populations. Specifically, individuals experiencing chronic psychological stress exhibited increased oxidative damage markers which could influence cell death. Of note, however, stressors were controlled within old mouse groups with vehicle/isotype injections, therefore minimizing the influence on our results.

Additionally, we have shown that old mice on control diet had significantly increased IL-17 positive cells in the lung compared to young mice on control diet at both basal levels and 8 days post-*M. tb* infection. IL-17 is an inflammatory cytokine known to induce
chemokine gradients and initiate inflammation, particularly in the lungs\textsuperscript{446-448}. Although IL-17 is believed to play a role throughout mycobacterial infection, it is thought to be dispensable for primary infection in young mice\textsuperscript{449,450}. However, the consequences of increased IL-17 in \textit{M.tb} infection in old age are not known. Here, we have shown that ibuprofen can decrease total IL-17 positive cells in old mice post-\textit{M.tb} infection, which was an age-associated affect since no differences were observed in infected control or ibuprofen fed young mice. In addition, we have shown that excess IL-17 during \textit{M.tb} infection in old mice could be detrimental to bacterial control as evident in our neutralization and recombinant supplementation models. Together, these data support the hypothesis that decreasing IL-17 in infected old mice on ibuprofen is driving the ability of ibuprofen to further enhance \textit{M.tb} control in old age.

We determined that CD4$^+$ and CD8$^+$ cells only accounted for roughly 15\% of total IL-17 positive cells, which suggests that CD4\textsuperscript{-}CD8\textsuperscript{-} double negative cells are the dominant producers of IL-17 in \textit{M.tb} infection in old age. This finding was corroborated in \textit{M.tb} infection in young mice where CD4\textsuperscript{-}CD8\textsuperscript{-} cells, especially $\gamma\delta$ T cells, were shown to be the dominant source of IL-17\textsuperscript{451}. However, $\gamma\delta$ T cells were not the only source of IL-17 production in \textit{M.tb} infected young mice, since cell fractions containing NK cells and NK T cells were also able to produce IL-17\textsuperscript{451}. Although it is unclear if $\gamma\delta$ T cells are the main producer of IL-17 during \textit{M.tb} infection in old age, Turner \textit{et al.} has previously shown that \textit{M.tb} infection of $\gamma\delta$ KO old mice displayed, although not significant, an early decreasing bacterial burden trend in the lung compared to wildtype old mice\textsuperscript{452}, similarly observed in our studies. This suggests decreased IL-17 production in $\gamma\delta$ KO old mice.
might contribute to the decreasing bacterial burden. However, no differences were observed in long-term *M. tb* infection between γδ KO and wildtype old mice, which implies that decreasing IL-17 may not mediate long-term control. On the other hand, IL-17 production in old age has been documented in other cells types including NK T cells during viral infection\(^ {376}\), and it is unknown if IL-17 production can be compensated for by NK T cells in γδ KO old mice during *M. tb* infection. Therefore, it is currently difficult to predict the cell source of IL-17 in *M. tb* infected old age as well as the long-term consequences of IL-17 during infection.

Furthermore, we do not know the mechanism of control induced by IL-17 in old mice on an ibuprofen diet that were infected with *M. tb*. However, because previous publications confirm that IL-17 can modulate cell death in *M. tb* infection\(^ {453}\), we hypothesized that increased IL-17 can increase cell death in *M. tb* infected old mice on control diet resulting in an increased bacterial burden compared to infected old mice on ibuprofen. This hypothesis is further supported by a recent study which revealed that IL-17 can synergize with TNFα to induce cell death by caspase-8 activation\(^ {454}\). We demonstrated that BAL cells isolated from infected old mice on control diet have increased cell death compared to infected old mice on ibuprofen diet. Our cells were stained with annexin V which detects apoptotic cells by the expression of phosphatidylserine on cell surfaces and 7-AAD which is a cell viability stain that has a strong affinity for DNA. However, conclusions regarding the type of cell death that is induced in *M. tb* infected old mice on control diet is limited by our experimental design. Although annexin V single positive staining is thought to indicate apoptosis\(^ {455}\), without further analysis, the mechanism that
induced cellular apoptosis, through caspase dependent (intrinsic or extrinsic pathways) or caspase independent activation remains unknown. This may be an important distinction since published reports have demonstrated that increased IFNγ during high MOI *M.tb* infection *in vitro* can promote increased caspase-independent apoptosis of macrophages which rapidly progressed to necrosis\(^{353,354}\). Furthermore, we cannot clearly distinguish between late stage apoptosis and true necrosis as 7-AAD staining of DNA only indicates a disrupted cell member. Other techniques to elucidate cellular morphology and presence of discriminatory proteins including caspase or mitochondrial activation by western blotting would be necessary to determine the type of cell death that is induced by *M.tb* infection in old mice. Our current hypothesis suggests that increased proinflammatory cytokines, including increased IL-17 and TNFα, in *M.tb* infected old mice on control diet may drive increased apoptosis, as previous studies have shown proinflammatory cytokines, including TNFα, can drive caspase-3 activation leading to increased apoptosis\(^{456,457}\). Additionally, because apoptosis is induced when cytochrome c, a component of the electron transport chain, is released into the cytosol from the mitochondria, and aging is associated with increased cytosolic cytochrome c release, the mitochondria can further amplify cytokine induced apoptosis\(^{458}\) in *M.tb* infected old mice on control diet. These observations suggest that many mechanisms including decreasing TNFα (Chapter 2 non-infected old mice on ibuprofen compared to control old mice) and decreasing IL-17 may contribute to the further enhanced bacterial control in old mice on ibuprofen diet by protecting cells from undergoing cell death.
We have shown that increased cell death in *M. tb* infected old mice on control diet is associated with increased PGE2 expression. PGE2 is a subclass of eicosanoid signaling molecules that exert various controls over the body including inflammation and immunity. Eicosanoids are well documented in their ability to modulate *M. tb* survival by inhibiting TNFα production, modulating cell death, and inhibiting T cell priming. Increased PGE2 expression is generally associated with increased apoptosis, which was observed in our *M. tb* infected old mice on control diet, however increased PGE2 expression is also commonly associated with enhanced bacterial control in *M. tb* infections *in vivo*, which is in contrast to what we have observed between old mice on control diet and old mice on ibuprofen diet. However, because the balance between the eicosanoid lipids including prostaglandins, lipoxins and leukotrienes is extremely important to maintain bacterial control, eicosanoids that were not measured in this study, lipoxin and leukotriene, could be playing a significant role in the regulation of *M. tb* control in old mice. In addition, inflammation is a known driver of eicosanoid production, thus it is possible that *M. tb* infected old mice on control diet induced an extensive amount of eicosanoid lipid mediators, causing dysregulated function while ibuprofen was able to decrease inflammation and decrease overall eicosanoid production to a normal physiological level.

We also showed that increased macrophage cell death in *M. tb* infected old mice was not dependent on increased IDO concentration within the lung. Recently, IDO has been shown to have a variety of immunomodulatory roles including activation of dendritic cells, suppression of T cell proliferation and function as well as the induction of
macrophage apoptosis \(^{416}\). Since the production of IDO is induced by IFN\(\gamma\) and old mice are known to have increased pulmonary IFN\(\gamma\), we hypothesized that IDO production would be significantly increased in \(M.\text{tb}\) infected old mice compared \(M.\text{tb}\) infected young mice. However, contrary to our hypothesis, no differences were observed in IDO concentration between young and old control mice. Furthermore, because ibuprofen has been shown to decrease IDO production \(^{465}\), we hypothesized that ibuprofen treated young and old mice would display reduced IDO expression. Although a trend for decreased IDO was observed in \(M.\text{tb}\) infected young mice on ibuprofen compared to young control mice, the opposite trend was observed in old mice. Unfortunately, at this point it is unclear why old mice displayed similar IDO concentrations compared to young mice, or why ibuprofen fed old mice display increased IDO compared to control old mice. However, we hypothesize that a common mechanism might be instigating both phenotypes. For example, a recent studying by Hosseini-Tabatabaei \textit{et al.} demonstrated that IFN\(\gamma\) failed to induce IDO in a non-obese diabetic mouse model \(^{466}\). Although it is unclear why IFN\(\gamma\) failed to induce IDO, resistance of IDO expression was restricted to the IFN\(\gamma\)-STAT1 signaling pathway since the IFN\(\gamma\)-independent pathway was unaltered. This suggests that in old mice, IDO may be resistant to IFN\(\gamma\) induction resulting in similar IDO expression compared to young mice. In contrast, when old mice were placed on ibuprofen, inhibition of IDO to IFN\(\gamma\) stimulation was lifted resulting in increased IDO expression. Similarly, IL-10 is known to inhibit IFN\(\gamma\) induced IDO expression in neuronal cells \(^{467}\). Since the lungs of naïve old mice display increased IL-10 mRNA expression compared to naïve young mice, IDO production may be inhibited in old age.
Although we did not test for IL-10 expression in ibuprofen treated old mice, we hypothesize that IL-10 expression would decrease in order to maintain environmental homeostasis after ibuprofen induced proinflammatory cytokine decline. This suggests that IDO expression would be increased due to a lack of an inhibitory signal from IL-10 in ibuprofen fed old mice.

Beyond direct immunoregulatory functions, IDO is an enzyme involved in tryptophan catabolism. Interestingly, tryptophan has been shown to be important in \( M.tb \) survival \(^{468}\). A recent paper by Zhang et al. demonstrated that CD4 T cells induce the upregulation of IDO in \( M.tb \) infected macrophages to reduce tryptophan availability. However \( M.tb \) has the ability to synthesis tryptophan under stress conditions prolonging its survival within the host cell \(^{469}\). By using an inhibitor of \( M.tb \) tryptophan synthesis, the authors demonstrated that the immune system was more efficient at eliminating \( M.tb \) as evident by decreased CFU in infected young mice. Since IDO expression is increased in \( M.tb \) infected old mice on ibuprofen compared to infected control old mice, and because the role of IDO is not well characterized in old age, it is possible that increased IDO expression enhanced bacterial control by limiting tryptophan availability in ibuprofen fed old mice. However, more research will be necessary in order to understand the importance of tryptophan and \( M.tb \) infection in old age.

An alternative role for tryptophan is evident in other model systems. Ibuprofen was recently shown to induce the longevity of \( Saccharomyces cerevisiae, Caenorhabditis elegans \) and \( Drosophila melanogaster \)^{470}. This conserved eukaryotic mechanism for
inducing longevity was not dependent on the ability of ibuprofen to reduce inflammation or pain but by inhibiting tryptophan import \(^{470}\). Consistent with the idea of tryptophan being important in aging, several studies have demonstrated that low tryptophan diets can increase the lifespan of rodents, delay tumor onset and improve hair growth and coat conditions \(^{471-473}\). Therefore, although the mechanism by which tryptophan can increase lifespan is not well defined, these studies suggest that our old mice supplemented with an ibuprofen diet might have increased lifespan and altered immune function due to tryptophan regulation and not inflammation or pain control. However, the duration of most research that studies the effects of tryptophan and aging fed rodents a low-tryptophan diet for up to 6-26 months. In comparison, our ibuprofen treatment lasted for no longer than 2 months. Thus, it might be possible that our short experimental timeframe was not sufficient to observe the effects of ibuprofen on tryptophan in old mice.

Lastly, we presented a small piece of data indicating that ibuprofen may continue to enhance \(M.tb\) control at later timpoints in the presence of antigen-specific T cells. The adaptive immune response is known to be delayed in \textit{in vivo} \(M.tb\) infections, where \(M.tb\) antigen-specific T cells are not found in the lungs until 2 weeks post-infection \(^{279}\). This delayed adaptive response suggests that the early bacterial control in old mice on ibuprofen diet compared to control diet is an innate immune mediated phenomenon. By D21 post-infection, no differences were observed among any experimental groups suggesting that the new influx of adaptive immune cells might mitigate the enhanced \(M.tb\) control in old mice. However, by D35 post-infection bacterial burden was significantly higher in old mice on control diet compared to young mice on control diet,
similar to previous published findings. Interestingly, a decreasing trend was observed between old mice on ibuprofen diet and old mice on control diet. Although our results in this chapter were focused on early timepoints, wanting to understand the early innate control of *M.tb* infection in old mice, our current hypothesis suggests that the increased inflammatory environment at D35 causes detrimental tissue damage in *M.tb* infected old mice on control diet. In contrast, the inflammation was kept low in *M.tb* infected old mice on ibuprofen leading to enhanced bacterial control later in infection. Furthermore, increased macrophage necrosis in *M.tb* infected old mice on control diet might propagate bacterial dissemination and disease progression. More research is necessary to understand the adaptive immune changes in *M.tb* infection in old.

Finally, no differences in *M.tb* control were ever observed between infected young mice on control or ibuprofen diet at any timepoint suggesting that 1) ibuprofen does not mediate direct antibacterial effects on *M.tb in vivo* and 2) ibuprofen enhanced *M.tb* control is an age-associated phenotype. Although ibuprofen have been shown to have mycobactericidal activities *in vitro*, the concentration of ibuprofen used *in vitro* was 75mg/L which was the roughly 7 times higher than the concentration of ibuprofen within the serum of mice in our studies (5-10mg/L). This suggests that at physiological concentrations, ibuprofen cannot inhibit mycobacterial growth in young mice *in vivo*.

A recently study estimating drug consumption in the United States determined that usage of NSAIDs including, aspirin, ibuprofen, naproxen and Celebrex increased 59% in elderly individuals 60 years and older between 2005 and 2010. However little is
known about how ibuprofen usage in old age can contribute to susceptibility of infectious diseases. Although our paper suggests that reducing age-associated inflammation may reduce incidence of infectious diseases, more studies are needed in order to examine the full extent of NSAID usage as prophylactic and/or therapeutic treatments in old age.
Figure 3.1: LPS can induce systemic inflammation in young BALB/c mice. Relative expression of IL-1α (A), IL-6 (B) and TNFα (C) within the spleen and IL-1β (D), IL-6 (E), TNFα (F), IL-12 (G), and IFNγ (H) within the lung as determined by RT-qPCR. Mice were injected with NaCl (open bars) or LPS (Dotted bars) for 12 days and whole organ homogenates were collected into TRIzol reagent. cDNA was synthesized using the Omniscript RT Kit and real-time PCR was performed using TaqMan gene expression assays. Data were combined from two independent experiments using five individual young mice in the NaCl group and ten individual young mice in the LPS group per experiment. Student’s $t$-test was used to determine statistical significance.
Figure 3.2: Inflammatory protein responses post-LPS injection in young mice.
Young mice were injected daily for 12 days with NaCl (open bars) or LPS (dotted bars). Whole organ homogenates were collected and stored in the -20 until further analysis. Protein production of TNFα (A), and IL-6 (B) in the lung and C-reactive protein in the liver (C) and lung (D) was determined by ELISA. Data were combined from two independent experiments using five individual young mice in the NaCl group and ten individual young mice in the LPS group per experiment. Student’s t-test was used to determine statistical significance.
Figure 3.3: LPS induces early resistance to infection with *M.tb*. Young mice were injected with NaCl (open bars) or LPS (dotted bars) for 12 days prior to low-dose *M.tb* aerosol infection. Bacteria were enumerated on D14 post-infection the lung (A) and spleen (B) or D35 post-infection in the lung (C) and spleen (D) by culturing whole organ homogenates on 7H11 agar and counting colonies after 21 days of incubation at 37°C. Data were combined from two independent experiments using five individual young mice in NaCl group or five to 10 individual young mice in LPS group (A-B) or from one independent experiment using five individual young mice per group (C-D) per experiment. Student’s *t*-test was used to determine statistical significance.
Figure 3.4: *M.tb* infected old mice supplemented with ibuprofen exhibit further enhanced bacterial control. CFU bacterial burden in *M.tb* infected young control (YC; open bars), young ibuprofen (YI; dash bars), old control (OC; closed bars) or old ibuprofen (OI; grey bars) fed mice. Young and old mice were placed on control or ibuprofen diet for two weeks prior to low-dose *M.tb* aerosol infection. Bacterial burden in the lungs were enumerated in C57BL/6 females on D6 (A), D10 (B), and D14 (C), in BALB/c females on D14 (D) and in C57BL/6 males on D14 (E) post-infection. Lung homogenates were cultured on 7H11 agar and bacterial colonies were counted after 21 days of incubation at 37°C. Data were from one independent experiment with five mice per group (A-B) or combined from two independent experiments with five mice per group per experiment (C-E). Student’s *t*-test was used to determine statistical significance.
Figure 3.5: *M. tb* infected old mice supplemented with ibuprofen exhibit long-term bacterial control. CFU bacterial burden in *M. tb* infected young control (YC; open bars), young ibuprofen (YI; dash bars), old control (OC; closed bars) or old ibuprofen (OI; grey bars) mice. Young and old mice were placed on control or ibuprofen diet for two weeks prior to low-dose *M. tb* aerosol infection. Bacteria burden in the lungs were enumerated in C57BL/6 females on D21 (A) and D35 (B) post-infection. Lungs homogenates were cultured on 7H11 agar and bacterial colonies were counted after 21 days of incubation at 37°C. Data were combined from two independent experiments with five mice per group per experiment. Student’s *t*-test was used to determine statistical significance.
Figure 3.6: *M. tb* infected old mice supplemented with ibuprofen display trends for decreased IFNγ production. IFNγ expression from *M. tb* infected young control (YC; open bars), young ibuprofen (YI; dash bars), old control (OC; closed bars) or old ibuprofen (OI; grey bars) female BALB/c mice as determined by RT-qPCR and intracellular staining. Young and old mice were placed on control or ibuprofen diet for two weeks prior to low-dose *M. tb* aerosol infection. Relative IFNγ message expression in purified CD8+ cells from whole lung on D8 (A) or D14 (B) post-infection by magnetic cell separation and collected into TRIzol reagent. cDNA was synthesized using the Omniscript RT Kit and real-time PCR was performed using TaqMan gene expression assays. For intracellular staining, whole lung cells were isolated from *M. tb* infected mice and incubated with anti-CD3, anti-CD28 and GolgiStop for 4 hours. Cells were washed, fixed, permeabilized and stained for IFNγ. Data were combined from two independent experiments (A, C) or three independent experiments (B) using five individual mice per group per experiment. Student’s *t*-test was used to determine statistical significance.
Figure 3.7: Further enhanced control elicited by ibuprofen in old mice is independent of IFN$\gamma$ or CD8+ cells. Old mice were placed on control or ibuprofen diet and injected intraperitoneally with vehicle control, $\alpha$CD8 (A) or $\alpha$IFN$\gamma$ (B) antibodies for two weeks prior to low-dose $M$. $tb$ aerosol infection. Bacteria burden in the lungs were enumerated in BALB/c females on D14 post-infection. Lung homogenates were cultured on 7H11 agar and bacterial colonies were counted after 21 days of incubation at 37$^\circ$C. Data were combined from one independent experiment with five mice per group (A) or two independent experiments with five mice per group per experiment (B). Student’s $t$-test was used to determine statistical significance.
Figure 3.8: Further enhanced bacterial control in *M. tb* infected old mice on ibuprofen is associated with decreased IL-17. Young and old BALB/c female mice were placed on control or ibuprofen diet for two weeks prior to low-dose *M. tb* aerosol infection. IL-17 expression from *M. tb* infected young control (YC; open bars), young ibuprofen (YI; dash bars), old control (OC; closed bars) or old ibuprofen (OI; grey bars) female BALB/c mice as determined intracellular staining (A-B). Whole lung cells were isolated from *M. tb* infected mice and incubated with anti-CD3, anti-CD28 and GolgiStop for 4 hours. Cells were washed, fixed, permeabilized and stained for total IL-17 positive cells at specific timepoints (A) or percentage of IL-17 positive cells on D8 post-infection (B). For CFU bacterial burden, old mice were placed on control or ibuprofen diet and injected intraperitoneally with vehicle control, rIL-17 (C) or αIL-17 antibodies (D) for two weeks prior to low-dose *M. tb* aerosol infection. Bacterial burden in the lungs were enumerated on D14 post-infection. Lung homogenates were cultured on 7H11 agar and bacterial colonies were counted after 21 days of incubation at 37°C. Data were combined from two independent experiments with five mice per group (A-C) or one independent experiment with five mice per group per experiment (D). Student’s *t*-test was used to determine statistical significance.
Figure 3.9: Ibuprofen can modulate pulmonary macrophage cell death in *M. tb* infected old mice. Old BALB/c female mice were placed on control or ibuprofen diet for two weeks and either left uninfected (dark gray bars) or infected with low-dose *M. tb* (red bars). Pulmonary macrophages were isolated by bronchoalveolar lavage, counted and stained with annexin V (AV) and 7-AAD. Representative images of apoptosis assay (A) quantified as AV/7-AAD$^{-/-}$ (B), AV/7-AAD$^{+/+}$ (C) and AV/7-AAD$^{-/-}$ and AV/7-AAD$^{+/+}$ combined (D). Quantitative data were from one independent experiment using two to three individual mice in each group. Student’s *t*-test was used to determine statistical significance.
Figure 3.10: Increased macrophage cell death in *M.tb* infected old mice on control diet is associated with increased PGE2 but not IDO. Young and old mice were placed on control or ibuprofen diet for two weeks prior to low-dose *M.tb* aerosol infection. Protein concentrations of PGE2 (A) at D8 post-infection and IDO (B) at D14 post-infection in young control (YC; open bars), young ibuprofen (YI; dash bars), old control (OC; closed bars) or old ibuprofen (OI; grey bars) mice. Lung homogenates were isolated at specific timepoints and protein concentrations were analyzed by ELISA. Data were from one independent experiment using five individual mice per group. Student’s *t*-test was used to determine statistical significance.
Figure 3.11: Early regulation of cell death differentiates bacterial control in old mice on control or ibuprofen supplemented diet. *M. tb* (red oval) infected old mice display increased IFN\(\gamma\) production and enhanced intracellular killing *in vivo* compared to infected young mice during early timepoints. Compared to young mice, infected old mice on control diet also display increased total IL-17 positive cells and increased PGE2 concentrations which are associated with increased macrophage cell death. In contrast, infected old mice on ibuprofen diet exhibit similar total IL-17 positive cells and PGE2 concentrations compared to young mice which is associated with cellular survival. The balance of IFN\(\gamma\), IL-17 and PGE2 is therefore important in determining the outcome of bacterial growth during early and possibly late infection.
Chapter 4: Significance of findings

In this work, we have demonstrated that old mice can experience the effects of inflammaging and that cells isolated from the inflammatory lung environment of old mice display altered immune phenotype and function. Secondly, we demonstrated that in young mice, inflammation alone is sufficient to generate many phenotypes similarly observed in old mice. Lastly, we have shown that contrary to our original hypothesis ibuprofen can further enhance *M. tb* bacterial control which may lead to long term protection benefits in old age. Together, these findings contribute to our understanding of immune changes in old age and enhance our knowledge of inflammation induced changes in immune control.

There is no question that the global aging trend is shifting. The elderly population is on the rise while the youth population is declining. This significant change in the aging population is complicated by the fact that elderly individuals are more susceptible to chronic and infectious diseases leading to increased morbidity and mortality. Indeed, recent data by the CDC demonstrate that elderly individuals 65 years and older have the highest rate in overall poor health, culminating in the highest rate of mortality in 13 out of the 15 leading causes of death. These include mortalities due to heart disease, cancer,
diabetes, and lower respiratory diseases (excluding suicide and liver disease)\textsuperscript{476}. In order to improve healthspan as well as lifespan in elderly individuals, it is crucial for scientists to discover and understand age-associated changes that may result in increased susceptibility to chronic conditions.

\textit{Using the mouse as a model for aging research}

Although the most ideal way to study aging would be in a human population, this is complicated by many factors including ethical issues and our naturally long life span. To circumvent this, our group and those of many others turn to the mouse model of aging. We realize that although there are limitations in studying age-associated phenomenon in mice, there are also many benefits that make this an appealing model. Numerous studies identifying and characterizing mice have given researchers a wealth of knowledge on the health background of different strains allowing for proper assessment of aging and age-associated symptoms. Furthermore, rodent models of aging allow for the regulation of environmental and genetic factors that help minimize confounding variabilities within the experimental setup. Although the average maximum lifespan is significantly different between mice and humans, the respective rates at which they age or their “life phases”, including childhood, mature adulthood and old age are surprisingly similar. Furthermore, mice share similar physiology to humans\textsuperscript{477} as well as similar immunologic responses to pathogens\textsuperscript{478} and age-associated diseases such as cardiovascular disease and cancer\textsuperscript{479}. In the case of studying \textit{M.tb} infection, different mouse strains successfully replicate key
elements of *M.tb* pathogenesis. Perhaps most importantly, mice have a relatively short lifespan compared to humans allowing for lifelong studies which would be more complex to achieve in long living animals.

In some of our experiments, we included data from 2 different strains of mice and also mice of the opposite sex. This experimental design was an attempt to differentiate between an age-associated phenomenon and a strain/sex dependent phenotype. This was an important distinction since commonly used inbred mice display strain differences in age-associated phenotypes. While the average lifespan of C57BL/6 mice is approximately 24.9 months for females and 26.1 months for males, the average lifespan of BALB/c mice is approximately 21.9 months for females and 20 months for males. This suggests that while female and male C57BL/6 mice used in our research were at the beginning of their “old age life phase”, female BALB/c mice were reaching closer to the middle of their old age life phase. Since our experiments occurred in either early (C57BL/6) or middle (BALB/c) old age, our experiments are not thought to be confounded by extreme age frailty which is defined by physical weakness, deregulation of tissue homeostasis and increased vulnerability to adverse health outcomes. Furthermore, because C57BL/6 mice have low susceptibility to tumor growth and BALB/c mice have low tumor incidence until late in life, and since all mice with detectable tumors were removed from our experiments, we believe our experimental observations are not confounded by spontaneous tumor generation, commonly observed late in life, which could alter immunological responses to infection. This suggests that the bacterial control observed in both C57BL/6 and BALB/c old mice is the result of an
early aging-associated phenotype. Although studying *M. tb* control in late old age was beyond the scope of this project, it would be interesting to examine how modulating inflammation may alter bacterial control in the presence of comorbidities such as frailty and tumor, since these are common factors ailing the extreme old age group.

Strain dependent differences are also observed in immune responses to infection. Although both C57BL/6 and BALB/c are considered relatively resistant to *M. tb* infection, compared to other mouse strains such as CBA/J and DBA/2, differences are observed when examining specific cellular responses. BALB/c mice are generally classified as Th2 biased, and better able to produce robust amount of cytokines such as IL-4 and IL-13. This is in direct contrast to C57BL/6 mice which are generally classified as Th1 biased being better able to produce IL-12 and IFNγ. In the context of *M. tb* infection, Jung *et al.* demonstrated that *M. tb* infected BALB/c mice generated significantly lower total numbers of IFNγ producing T cells in the lungs compared to the C57BL/6 strain. Furthermore, CD4 T cells isolated from infected mice capable of secreting IFNγ in response to the presence of either early secretory antigen 6 (ESAT6) peptide or *M. tb* ex vivo were significantly reduced in BALB/c mice compared to C57BL/6 mice. Similarly, another study by Paula *et al.* confirmed high IFNγ production as well as high IL-17 and low IL-10 production within the lung homogenates of C57BL/6 mice compared to BALB/c mice. Finally, CD4+Foxp3+ regulatory T cells (Treg), known to suppress immune function, were increased in the lungs of BALB/c mice compared to C57BL/6 mice. Together these data suggests that C57BL/6 mice and BALB/c mice may use some different mechanisms or magnitude of response to
control *M.tb* infection. Despite these differences, both strains display similar CFU bacterial burden in young and old mice when challenged with *M.tb*.

Although the exact mechanisms that facilitate *M.tb* control between different mouse strains is currently unclear, we hypothesize that the balance of immune function leads to similar mortality outcomes between C57BL/6 and BALB/c. For example, increased IFNγ within C57BL/6 mice, which is thought to increase macrophage bactericidal activities, may lead to increased cell death, and tissue destruction. In contrast, highly suppressive phenotypes (increased Treg and increased IL-10) within the BALB/c mice, thought to inhibit bactericidal inflammation, might provide a platform for tissue protection and optimum bacterial control. However, it is also feasible that different mechanisms for *M.tb* control between C57BL/6 and BALB/c have not yet been fully investigated, such as differences in alveolar macrophage phagocytosis and infection induced cell death, which may greatly impact *M.tb* control. Therefore, consistent findings between different strains heightened the age-associated impact of our discoveries.

**Inflammation as the driver for age-associated susceptibility to infectious diseases**

Since aging is associated with an increase in inflammatory cytokines including IL-1β, IL-6 and TNFα, and elderly people are more susceptible to *M.tb* infection, we hypothesized that inflammation may induce TB susceptibility in old age. To test this hypothesis, we first defined inflammaging in old age and then modulated inflammation by two different
methods including 1) inducing inflammation in young mice and 2) decreasing inflammation in old mice. By modulating inflammation in these systems, we were able to study the influence of inflammation on *M. tb* bacterial control.

**Inflammation and its impact on susceptibility to infectious diseases in old age**

Although inflammaging is well characterized in elderly humans, the existence of inflammaging is not well defined in mice. Therefore, to understand how inflammation may alter immune control in old age, we sought to determine if inflammaging is a conserved phenotype in old mice and within the lung. In chapter 2, we were able to confirm the existence of inflammaging in old mice as demonstrated by increased IL-1β, IL-6 and TNFα as well as increased IFNγ. Furthermore, we were able to characterize altered cellular phenotype and function in macrophages isolated from the lungs of old mice compared to cells from young mice. When young and old mice on control diet were infected with *M. tb in vivo*, we observed an early transient resistance followed by the loss of bacterial control where old mice succumb to greater bacterial burden, as we have previously described. Reduced bacterial burden in old mice was associated with an increase in IFNγ and IL-17 positive cells compared to *M. tb* infected young mice. This suggests that increased IFNγ and/or IL-17 in *M. tb* infected old mice may be beneficial in early but detrimental to long-term bacterial control.
Indeed, in the context of *M.tuberculosis* infection, several recent articles have expanded upon the role of IFNγ. Lee *et al.* demonstrated that upon high *M.tuberculosis* MOI *in vitro*, IFNγ can induce increased caspase-independent apoptosis of BMDMs which rapidly progressed to necrosis. Furthermore, Sakai *et al.*, established that increasing production of IFNγ by CD4 T cells in the lungs of *M.tuberculosis* infected young mice led to early mortality. On the other hand, beyond the ability of IFNγ to activate macrophage function, IFNγ has been shown to be an important inhibitor of excessive neutrophil recruitment. Extensive neutrophil accumulation during *M.tuberculosis* infection is associated with increased bacterial burden, pathology and mortality of its host. Therefore, although IFNγ is necessary and beneficial, increased production of IFNγ could be detrimental to *M.tuberculosis* infection outcomes.

Although IL-17 is generally thought to be dispensable in *M.tuberculosis* infected young mice, exacerbated IL-17 production in old age may be unfavorable in long-term control. This is supported in a study using herpes simplex virus (HSV) in both young and old mice. Systemic infections of old mice with HSV led to increased IL-17 production and mortality due to increased liver damage. In contrast, young mice infected with HSV had low levels of IL-17 production, minimal liver damage and were able to survive the infection. Taken together, we hypothesize that cytokine responses during infection in old age may be intensified due to an already inflammatory environment, leading to unfavorable consequences.

Our hypothesize is further supported by previous data demonstrating that inadequate but also excessive levels of proinflammatory cytokines, such as TNFα and IL-1β, can be
detrimental to *M.tuberculosis* bacterial control during infection. Using *Mycobacterium marinum* (*Mm*) infected zebrafish, Tobin *et al.* demonstrated that infection of TNF KO zebrafish exhibited increased bacterial growth within macrophages leading to cell lysis and bacterial escape into the extracellular milieu compared to infected WT zebrafish. In contrast, *Mm* infected zebrafish given recombinant TNF demonstrated a transient increased bacterial control, which was then followed by increased bacterial burden within the macrophages leading to macrophage lysis compared to infected WT zebrafish. In mouse models of *M.tuberculosis* infection, Mishra *et al.* demonstrated that excessive IL-1β led to significant immunopathology characterized by extensive tissue necrosis, neutrophil infiltration and significant tissue wasting. However, Mayer-Barber *et al.* demonstrated that IL-1β during *M.tuberculosis* infection was necessary to prevent IFNβ induced disease exacerbation by inhibiting nitric oxide, and IL-12 production while increasing IL-10 production. Together, these data indicate that a balance between inflammatory induced bactericidal benefits and inflammatory induced host damage is necessary for proper control of *M.tuberculosis* infection. Furthermore, this supports the concept that if inflammatory cytokines are augmented post-*M.tuberculosis* infection in old mice due to alterations in inflammatory environment and/or cell function, then *M.tuberculosis* control would be impaired in old age.

A lack of control of proinflammatory cytokines leading to aggravated disease in old age has also been documented in other infections including *Streptococcus pneumoniae* and influenza. When young and old mice were infected with *S. pneumoniae*, only the old mice experienced severe pathology including vascular congestion, alveolar edema and
excessive neutrophil infiltration leading to early mortality. Susceptibility of aged mice to 
*Streptococcus pneumoniae* infection was correlated with increased proinflammatory cytokines, 
including TNF. When young mice were implanted with osmotic pumps delivering 
systemic TNF prior to *S. pneumoniae* infection, disease progression was comparable to 
those observed in old mice. Similarly, when examining mobility impairments which 
can occur post influenza infection, old mice exhibited an inflammatory response that was 
prolonged and elevated, leading to increased muscle atrophy and proteolysis compared to 
infected young mice. Thus, with our observations and published reports from other 
researchers, further validity has been given to the hypothesis that excessive inflammation 
in old age can drive increased susceptibility to infectious diseases.

**Modulation of inflammation in the young host and its impact on *M.tb* infection**

In order to further test our hypothesis, we induced an inflammatory environment in young 
mice in order to determine how inflammation alone can alter *M.tb* susceptibility. We 
hypothesized that inducing inflammation in young mice would result in similar *M.tb* 
control compared to old mice. Inflammation in young mice was generated in our model 
by the use of LPS. Since it was unclear which inflammatory component may drive 
inflammaging, LPS was chosen because of its ability to generate several proinflammatory 
cytokines in mice. Remarkably, and despite being a purified TLR4 agonist, a modest 
but ongoing LPS stimulus in young mice led to systemic inflammation similarly observed 
in naïve old mice. Furthermore, when LPS injected young mice were infected with *M.tb*,

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bacterial control at early timepoints were similar compared to infected old mice. At later timepoints, LPS injected young mice failed to recapitulate the *M.tb* control observed in old mice. While the bacterial control between LPS injected young mice and naturally aged old mice was only observed during early infection (D14 and not D35), our hypothesis states that inflammation alone can generate long-term increased susceptibility and increased mortality to infectious diseases. To test this hypothesis, we would need to design a model that can induce long-term and low-grade inflammation such as constant low-dose LPS, or other inflammatory mediators like IL-6, given through osmotic pumps to eliminate stress from repeated injections. Extended CFU and survival experiments would need to be conducted in order to understand the effects of inflammation over time. However, the lack of a difference in bacterial burden at D35 might also suggest that while inflammation alone can contribute to early resistance phenotypes, at later timepoints other age-associated factors such as impaired adaptive immunity may be regulating bacterial control. Therefore, we demonstrate that the induction of inflammation using a single agonist was sufficient to alter early outcome during *M.tb* infection. Although more studies will be necessary to characterize the specific mediators of inflammaging, our study indicates the robustness of the early inflammation phenotype in old mice.

**Modulation of inflammation in old mice and its impact on *M.tb* infection**

We next tested our hypothesis by reducing inflammation in old mice in order to study the impact of inflammation during infection in old age. When old mice were placed on an
ibuprofen supplemented diet, macrophages isolated from old mice reverted back to phenotypes similarly observed in young mice. This suggests that reducing inflammation in old mice could restore cellular function. However, similar restoration was not observed in our work in vivo. Previously, we have shown that old mice infected with M. tb display a transient bacterial resistance phenotype that is observed at early timepoints compared to M. tb infected young mice. Based on this observation, we had hypothesized that when old mice are placed on an ibuprofen supplemented diet, bacterial control would revert to levels similarly seen in young mice. Contrary to what we hypothesized, we saw a further enhancement in bacterial control in old mice on ibuprofen supplemented diet compared to old mice on control diet.

Although we did not observe similar bacterial burden between old mice on ibuprofen diet and young mice on control diet during early infection, we do not believe that this contradicted our overarching hypothesis implicating inflammation as a driver of disease susceptibility in old age. This is made apparent when looking at bacterial burden later during infection. At D35 post-infection bacterial burden is highest in old mice on control diet compared to both young mice on control diet and old mice on ibuprofen. This suggests that if M. tb infection was lengthened in our experimental model, we might observe decreased bacterial burden and pathology as well as extended lifespan in old mice fed an ibuprofen diet. Instead, we chose to focus on the mechanism behind ibuprofen induced bacterial control at early timepoints. This was an important decision since ibuprofen induced control was only observed in M. tb infected old mice and not M. tb infected young mice, which suggest that the ability of ibuprofen to modulate
bacterial control is an age-dependent mechanism. Since *M.tb* control is lost during long-term infection in old mice, understanding the mechanism behind ibuprofen enhanced bacterial control could lead to improved therapeutic opportunities for *M.tb* infected elderly individuals independent of ibuprofen usage. Ibuprofen can lead to immunosuppression which could be detrimental to *M.tb* control, as seen in HIV and transplant patients[^507]^[508]. Furthermore, ibuprofen usage is associated with gastrointestinal complications[^509] suggesting that long-term ibuprofen might have unfavorable immune and tissue specific consequences.

Decreased bacterial burden in old mice fed an ibuprofen diet compared to old mice fed control diet was correlated with decreased IL-17 and cell death. Previously, we discussed how IL-17 has been implicated in augmented mortality in old age during HSV infection by increasing neutrophil recruitment and hepatocyte necrosis. However, IL-17 is also known to regulate cell death during *M.tb* infection *in vitro*[^506]. In our *in vivo* model, *M.tb* infected old mice on control diet exhibited increased IL-17 which was correlated with pulmonary macrophages undergoing increased cell death as evident by increased proportion of cells staining positive for annexin V and 7-AAD. This suggests that macrophages were experiencing apoptotic cell death as well as membrane damaged cell death in the presence of *M.tb*. In contrast, *M.tb* infected old mice on ibuprofen exhibited decreased IL-17 which was correlated with decreased proportions of pulmonary macrophages staining for cell death markers. However, at this time, we do not have conclusive evidence connecting IL-17 as the inducer of cell death post-*M.tb* infection. Further studies examining cellular damage in the presence or absence of IL-17 in old mice...
mice, either by the addition of recombinant IL-17 or by IL-17 neutralization, will be necessary in order to understand the ability of IL-17 to modulate cell death in vivo.

Increased cell death leading to increased tissue damage is a recurring theme in disease progression of old hosts \(^{376,505}\) as well as young hosts experiencing exacerbated inflammatory responses \(^{363,412,504,510}\). Interestingly, in the previously mentioned publication where increased IL-1β induced cell death and tissue damage during M.tb infection in vivo, the authors used a mutant M.tb which replicates only in the presence of streptomycin. Thus, they were able to maintain bacterial load at a constant level demonstrating that tissue damage was independent of bacterial burden since similar CFU was observed between WT and excessive IL-1β producing mice \(^{504}\). This data gives rise to the possibility that inflammation induced by the host, and not by increased bacterial burden, can generate immunopathology that would be detrimental to the host. Therefore, in our study, we will need to further address histological data in addition to our CFU data in order to understand how inflammation can alter pathogenesis and progression of disease in old mice on control or ibuprofen diet.

Inflammatory diseases in human hosts can alter susceptibility to infection

Unmistakably, evidence for the impact of inflammation exists in human adult populations, such as individuals with chronic conditions that drive inflammatory responses, including obesity and HIV. Numerous studies have demonstrated that obesity
and excess nutrient intake can cause a low-grade chronic inflammation, with increased CRP, IL-6 and TNFα\textsuperscript{511,512}, similarly observed in inflammaging. More importantly, obesity has now been linked to chronic conditions where the number one risk factor is aging. These chronic conditions include cardiovascular diseases\textsuperscript{513} cancer\textsuperscript{514} and Alzheimer’s\textsuperscript{515,516}. Furthermore, recent retrospective analysis of the 2009 H1N1 influenza pandemic has generated support linking obesity and susceptibility to infection\textsuperscript{517}. Specifically, in California, over half of all adult patients >20 years old hospitalized with H1N1 were obese (BMI >30). In addition, of all the adult patients who succumbed to the infection, 61% of those individuals were obese. Similar observations implicating inflammation as the driver of disease susceptibility have been made in adults living with HIV. Unfortunately, the production of most proinflammatory mediators including IL-6 and CRP remain high in HIV treated individuals\textsuperscript{518} resulting in a chronic inflammatory state. Recently, emerging data suggest that adult HIV+ patients experience age-associated disorders such as cardiovascular diseases, metabolic syndromes and bone abnormalities as well as decreased lifespan\textsuperscript{519,520}. These data suggest that chronic conditions that can enhance proinflammatory mediators may propagate age-associate phenotypes leading to premature susceptibility of chronic and infectious diseases.

In this work, we have shown that increasing inflammation in young adults by LPS injection can lead to age-associated bacterial control. In contrast, reducing inflammation in old age by ibuprofen can enhance \textit{M. tb} bacterial control at early and possibility late timepoints post-infection. In addition, studies within the adult human population with increased inflammation demonstrate a correlation between inflammation and
susceptibility to age-associated diseases. Therefore, based on our observations and reports within the human population, we propose that excessive inflammation in young and old age can alter immune control leading to increased susceptibility to chronic and infectious diseases.

**Inflammation alone can drive cellular tolerance in old age**

LPS was utilized in our experiments to generate inflammatory mediators in young mice. Interestingly, LPS has been shown to induce a phenomenon known as endotoxin tolerance. Injection of animal models with high dose LPS leads to the activation of immune cells resulting in the production of proinflammatory cytokines. However, high-dose LPS injection can cause prolonged inflammatory responses leading to eventual tissue destruction, septic shock and even death. Endotoxin tolerance on the other hand, occurs after the introduction or “priming” of an initial low-dose LPS. When animals are primed with low-dose LPS, they are protected from the detrimental consequences of a subsequent restimulation of a lethal high-dose LPS. The protection from lethality is thought to be due to the downregulation of inflammatory cytokines caused by LPS priming, leading to reduced tissue damage and organ destruction.

Although the exact mechanisms for inducing endotoxin tolerance are not completely understood, alterations in macrophage function are thought to play a crucial role. Interestingly, similar observations have been observed in macrophages isolated from
naïve old mice compared to LPS tolerized macrophages from young mice. As previously discussed, naïve macrophages from old mice and humans exhibit altered immune responses (Chapter 1). One of the most well documented dysfunctions of macrophages isolated from aged hosts is a decrease in TNFα and IL-6 production when stimulated with LPS in vitro. Decreased TNFα and IL-6 production is also a consistently observed phenotype in macrophages from young hosts that have been primed and restimulated with LPS. Other similarities have also been observed including decreased MHCII expression, and decreased TLR4 expression. Since macrophages isolated from LPS primed animal models exhibit similar phenotypes compared to macrophages isolated from naïve old mice and because continual LPS injection in our young mouse model (which could induce tolerance) demonstrate similar M.t.b bacterial control compared to M.t.b infected old mice, we propose that immune cell dysfunction in old age may be due to immune tolerance and cells that are refractory to further stimulation.

In support of this hypothesis, numerous researchers suggest that tolerant phenotypes (decreased TNFα, IL-6 and IL-1β production) are not a LPS specific phenomenon. Priming and restimulation of cells with TLR2 exhibit a tolerance phenotype with decreased TNFα gene expression and protein production. Most importantly, tolerant phenotypes can be generated with the use of proinflammatory cytokines, independent of any TLR engagement. In animal studies, daily doses of IL-1β injection in young mice decreased colony-stimulating factor (CSF) production and mortality in response to endotoxin challenge. Similarly, treatment of rats with
recombinant TNFα for 8 consecutive days rendered the rats hyporesponsive to lethal doses of TNFα or endotoxin. In human studies, PBMCs incubated with IL-1β or IL-1α alone without pathogenic antigen induced significant decreases in TNFα production upon endotoxin challenge compared to vehicle control. In addition, PBMCs isolated from patients experience high circulating IL-6 and IL-1β in response to trauma exhibited decreased TNFα, IL-1β and IL-1α production after challenge with staphylococcal toxic shock syndrome toxin-1. This implies that regardless of the source of inflammation in old age, increased circulating proinflammatory levels in the absence of pathogenic antigen can induce a cellular tolerance phenotype that is refractory to further stimulation.

Consequently, defects observed in innate immune cells isolated from elderly hosts could partially be due to inflammation induced immune tolerance. This phenomenon could also explain the conflicting data from studies examining aging immune function. For example, immune responses from elderly patients would be dependent on the inflammatory environment in which cells were isolated. Therefore, cell function from individuals with decreased manifestation of inflammaging may appear unaltered and similar to young controls. Unfortunately, because cellular tolerance was first discovered by the use of endotoxin, most studies examining the effects of inflammatory induced tolerance still utilize LPS as the secondary challenge. Therefore, to further understand how inflammatory mediators such IL-1β and TNFα can induce tolerance, cellular responses will need to be better characterized in other pathogenic models such as influenza or M.tb infection. Nevertheless, we propose that future studies examining cellular defects in old
age should take into consideration the systemic, low grade inflammation which may be present in aging hosts.

**Concluding remarks**

Throughout this collective work, we have examined aspects of immunity in response to inflammation in both young and old mice. We have demonstrated that old mice experience inflammaging and decreasing inflammation in old age may lead to enhanced long-term *M.tb* bacterial control. We have also shown that inflammation induced in young mice can recapitulate early old age-associated *M.tb* bacterial control. Based on our experimental findings, we believe that inflammation in old age can alter immune function and increase susceptibility of elderly individuals to chronic and infectious diseases. If this idea holds true, it is imperative that we understand ways to prevent and treat inflammaging. Although we used a non-steroidal anti-inflammatory drug to decrease inflammation in our research model, and although other pharmacological interventions to decrease inflammation in old age have been studied, we do not advocate the use of any drugs for prophylactic prevention (especially without a physician’s recommendation). Ibuprofen provided a fast and efficient way to decrease inflammation in our old mice, however, long-term drug treatments to decrease inflammation are known to generate side effects such as gastrointestinal distress and cardiovascular complications. Therefore, lifestyle changes including dietary modification and exercise may prove an effective long-term solution to limiting inflammation.
and decreasing age-associated chronic conditions. Whether by prevention, control and maintenance or treatment of inflammation in old age, we hope that our work can serve as a groundwork towards a better understanding of the effects of inflammation in old age leading to longer but most importantly, healthier lives.
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