Assessment of Lymph Node Stromal Cells as an Underlying Factor in Age Related Immune Impairment to Influenza Infection

April Masters
University of Connecticut - Storrs, amasters@uchc.edu

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

Recommended Citation
Masters, April, "Assessment of Lymph Node Stromal Cells as an Underlying Factor in Age Related Immune Impairment to Influenza Infection" (2017). Doctoral Dissertations. 1698.
https://opencommons.uconn.edu/dissertations/1698
Assessment of Lymph Node Stromal Cells as an Underlying Factor in Age Related Immune Impairment to Influenza Infection

April Rose Masters, PhD
University of Connecticut, 2018

ABSTRACT
Influenza is an often lethal infection for elderly populations. Here we determine how aging effects humoral, CD4+ T cell, splenic stromal cell and lymph node stromal cell response to influenza infection. T follicular helper (TFH) cell responses are essential for generation of protective humoral immunity during influenza infection. Aged mice have smaller germinal centers, with fewer germinal center B cells resulting in decreased influenza specific IgG. Influenza specific TFH cells are generated in similar numbers in young and aged animals during infection, but TFH cells from aged mice fail to reach full maturity, have reduced expression of costimulatory molecules and elevated production of IL-10 and IFNγ, which potentially impairs interaction with cognate B cells. Also, more influenza specific T cells in aged mice have a regulatory phenotype and adoptive transfer studies with young T cells demonstrated that TGF-β1 in the aged splenic environment may drive increased regulatory T cell accumulation. Another component of the splenic environment that is altered with age are the stromal cell subset, fibroblastic reticular cells (FRC). FRCs are located in the T cell zone of the splenic white pulp and produce homeostatic chemokines which direct cells into the T cell zone. We found that aged spleens have fewer FRCs and lower homeostatic chemokine concentration than young, which may delay recruitment of T cells. Unlike spleens, young and aged lymph nodes had similar numbers of FRCs at homeostasis, but aged lymph node FRCs took longer to expand in number due to decreased proliferation after infection. Aged lymph nodes also had reduced homeostatic
chemokine concentrations. From adoptive transfer studies, we went on to determine that young influenza specific CD8+ T cells have reduced proliferation in aged compared to young lymph nodes after infection. The mechanisms involved are not clear since there are no age-related differences in lymph node FRC suppressive capacity. Collectively these studies expand our understanding of how aging impacts the immune response to influenza infection.
Assessment of Lymph Node Stromal Cells as an Underlying Factor in Age Related Immune Impairment to Influenza Infection

April Rose Masters

B.S., University at Buffalo, The State University of New York, 2011
M.S., University at Buffalo, The State University of New York, 2013

A Dissertation
Submitted in Partial Fulfillment of the
Requirements for the Degree of
Doctor of Philosophy
at the
University of Connecticut
2018
APPROVAL PAGE

Doctor of Philosophy Dissertation

Assessment of Lymph Node Stromal Cells as an Underlying Factor in Age Related Immune Impairment to Influenza Infection

Presented by
April Rose Masters, B.S., M.S.

Major Advisor ________________________________________________________________
Laura Haynes

Associate Advisor ___________________________________________________________
Lynn Puddington

Associate Advisor ___________________________________________________________
Kamal M. Khanna

University of Connecticut, 2018
ACKNOWLEDGMENTS

My mentor Laura Haynes deserves the first and foremost acknowledgement. I will be forever grateful for her mentorship and support during my graduate research. She let me think independently, explore new ideas and created a fun and encouraging environment to do science. I would like to thank my committee members Lynn Puddington and Kamal Khanna for their guidance, support and constructive criticism. Their outside perspectives were crucial to shaping my project into the story it became.

My past and present lab mates deserve special acknowledgments. Erica, Jenna, Iman, Jake, Spencer, Qui, Judy, Sandy and all of the other members of the Center on Aging have been a wonderful team to work with. Countless times they dropped everything they were doing to help me with an experiment or gave me the months batch of aged mice. Thank you for putting up with all my questions and listening to me present over and over again at lab and PO1 meetings.

My friends have truly made graduate school an unforgettable experience. I will be forever grateful to have such an excellent group of people in my life.

I acknowledge my sister, for never letting me forget that life is fun. I can I always rely on her for advice, a laugh, and a memorable adventure.

I owe everything to my parents. They raised me to be determined, strong minded, and patient; to never quit on my dreams, to fight, to work hard, to give it everything I have, and to live the life that I wanted. There aren't words to sufficiently express the love, gratitude and respect that I have for them. Their support, wisdom and belief in me, helped me every day of my Ph.D.

Without the support and love of all of these people, I would have never made it through the failures and could not have enjoyed the successes of graduate school.
# TABEL OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE PAGE</td>
<td>i</td>
</tr>
<tr>
<td>COPYRIGHT</td>
<td>ii</td>
</tr>
<tr>
<td>APPROVAL PAGE</td>
<td>iii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>x</td>
</tr>
<tr>
<td>CHAPTER ONE</td>
<td>1</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>An aging world</td>
<td>1</td>
</tr>
<tr>
<td>Influenza and the elderly</td>
<td>1</td>
</tr>
<tr>
<td>Influenza virus</td>
<td>2</td>
</tr>
<tr>
<td>Age related changes in primary lymphoid organs</td>
<td>3</td>
</tr>
<tr>
<td>Impaired humoral immune response to influenza infection with age</td>
<td>4</td>
</tr>
<tr>
<td>T cell responses to influenza infection</td>
<td>5</td>
</tr>
<tr>
<td>Impact of aging on CD4+ T cell responses</td>
<td>6</td>
</tr>
<tr>
<td>Secondary lymphoid stromal cells: an underlying contributor to immunosenescence?</td>
<td>8</td>
</tr>
<tr>
<td>Age-related alterations of lymph node stromal cells</td>
<td>10</td>
</tr>
<tr>
<td>Age-related alterations of splenic stromal cells</td>
<td>15</td>
</tr>
<tr>
<td>Age-related changes in follicular dendritic cells</td>
<td>17</td>
</tr>
<tr>
<td>Goals of thesis</td>
<td>18</td>
</tr>
<tr>
<td>References</td>
<td>20</td>
</tr>
<tr>
<td>CHAPTER 2</td>
<td>38</td>
</tr>
</tbody>
</table>
Age-related impairment of humoral response to influenza is associated with changes in antigen specific T follicular helper cell responses

Abstract

Introduction

Materials and methods

Results

Discussion

Acknowledgements

References

CHAPTER 3

Attrition of fibroblastic reticular cell number and function in aged spleens

Abstract

Introduction

Materials and methods

Results

Discussion

Acknowledgements

References

CHAPTER 4

Assessment of lymph node stromal cells as an underlying factor in age related immune impairment to influenza infection

Abstract

Introduction

Materials and methods

Results
**LIST OF FIGURES**

1. Lymph node architecture ........................................................................................................ 11
2. Splenic architecture .................................................................................................................. 16
3. Reduced antibody production and GC development during influenza infection in aged mice .............................................................................................................................................. 48
4. Aged CD4+ T cells differentiate into pre-T<sub>FH</sub> cells .................................................................................. 51
5. Increased number of T follicular helper cells and T follicular regulatory cells in aged mice compared to young mice .............................................................................................................................................. 52
6. Aged CD4+ T cells fail to progress to fully differentiated T<sub>FH</sub> cells .......................................................... 54
7. Decreased germinal center T<sub>FH</sub> cell response to influenza infection in aged mice ..... 56
8. Differential expression of PD-1 on young and aged T follicular helper cells .................. 58
9. NP-specific CD4+ T cells produce cytokines indicative of regulatory T cells ........... 60
10. T regulatory and T follicular regulatory cells accumulate in aged mice after influenza infection .............................................................................................................................................. 62
11. More young CD4+ T cells transferred into aged host express Foxp3 compared to young hosts .............................................................................................................................................. 64
12. Chapter 2 Graphical summary .............................................................................................. 69
13. Reduction of fibroblastic reticular cell number in aged spleens ..................................... 81
14. Smaller T cell zone FRC areas in aged spleens .................................................................... 82
15. Reduced homeostatic chemokines in aged spleens correlate with impaired recruitment of young T cells .............................................................................................................................................. 84
Figure 16. CCL21 localizes to T cell zone FRCs in young and aged spleens.........................86

Figure 17. Aged mice have decrease survival and increased weight loss after sub lethal infection with influenza..........................................................104

Figure 18. Reproducible, high validity stromal cell digestion reveals altered stromal cell expansion kinetics in aged mediastinal lymph nodes after influenza infection.........................106

Figure 19. Decreased proliferation, but not increased death, contributes to reduced stromal cell numbers in MLNs after influenza infection.........................................................108

Figure 20. Disrupted lymph node architecture in aged mice after influenza infection.........109

Figure 21. Aging does not alter T cell zone FRC morphology.............................................111

Figure 22. Decreased homing of young T cells into aged lymph nodes correlates with decreased homeostatic chemokines and altered HEV structure.................................114

Figure 23. Reduced proliferation and activation of young influenza specific CD8+ T cells in aged lymph nodes after infection.................................................................116

Figure 24. Aged FRCs maintain their ability to suppress T cell proliferation.........................118
LIST OF TABLES

Table 1. Antibodies used for phenotyping T cells, B cells and cytokine production by flow cytometry for TFH..........................43

Table 2. Antibodies used for confocal microscopy of TFH........................................44

Table 3. Antibodies used for flow cytometry of stromal cells......................................100

Table 4. Antibodies used for immunofluorescence of stromal cells.............................101
CHAPTER 1
INTRODUCTION

An Aging World

Simplistically, aging can be defined as a time-dependent functional decline that influences most living organisms\(^1\). Despite this simple definition, aging is a complex, multifactorial process that is poorly understood. Why we age, when we start aging, and why aging is biologically important, are three central and almost philosophical questions that the field is working to address. Regardless of these uncertainties, it is clear that the human species is aging. The World Health Organization estimates that the global population over the age of 60 will double to a staggering two billion people by the year 2050 and the population over the age of 80 will quadruple to 395 million people in the same time period. An unprecedented demographic shift is also predicted to occur in this time period. The population over the age of 60 will outnumber the population under the age of five for the first time in human history\(^2\). Aging is the biggest risk factor for the development of numerous chronic diseases such as cancer, heart disease, stroke, dementia, and osteoarthritis\(^3\). This increase in the size of the elderly population and their predisposition to multiple diseases will place immense financial burdens on health care systems which are currently ill adept to treat such populations\(^4\).

Influenza and the elderly

Along with chronic diseases, aging predisposes the elderly to increased susceptibility to infectious agents. Influenza infection is a major problem for the elderly, ranking in the top 15 causes of death for persons over the age of 75 in the United States\(^5\). It is estimated that 90% of influenza related deaths are in patients above the age of 65\(^6\,^7\), resulting in an annual death toll averaging around 5,500 patients in the U.S.A. alone\(^8\). Not surprisingly, the population over the age of 65 has the highest rate of hospitalizations associated with influenza. The CDC estimates that 321.1 per
100,000 patients over the age of 65 were hospitalized during the 2015/2016 influenza season, a striking eight times higher incidence than the 18-49 age group (39.9 / 100,000).9

Despite high rates of seasonal influenza vaccination10, efficacy remains low in the elderly populations11, emphasizing the importance of developing new therapeutics to protect this at risk population. In order to develop new treatments for the elderly, it is imperative to understand what is going wrong in the aged immune response to influenza infection, which is the central focus of this thesis.

**Influenza virus**

Influenza virus is the pathogenic agent responsible for influenza infection. Influenza infection can manifest as mild to severe illness with symptoms often including fever, chills, fatigue, body aches, runny nose, and cough12. As discussed above, complications often arise during infection in the elderly, in many cases leading to death. Influenza viruses are negative sense, single stranded RNA viruses belonging to the family Orthomyxoviridae. There are three main types of influenza viruses A, B, and C. Influenza A is the most common influenza virus associated with human infections13. Influenza A virus is a roughly spherical enveloped virus, ranging from 80-100nm in diameter14. It has eight segments of RNA which encode 11 proteins15. The viral envelope contains hemagglutinin (HA) which binds to sialic acid on epithelial cells of the upper and lower respiratory track to mediate endocytosis into the host cells16. Neuraminidase (NA) is an envelope glycoprotein, which is important for the release of the viral particles from the host cell via cleavage of sialic acid17. The last envelope glycoprotein is matrix protein M2 (M2) which is an ion channel functioning in maintenance of viral pH18. The internal portion of the viral lipid envelope is coated by matrix protein 1 (M1), which provides rigidity and strength and functions in the export of viral RNA with nuclear export protein (NEP)19. In the viral core, RNA is complexed with structural
nucleoprotein (NP), and polymerase subunits: polymerase PB1, polymerase PB2, Acid polymerase (PA)\textsuperscript{20}. The last protein encoded by influenza A is Non-structural protein 1 (NS1), which is an interferon antagonist\textsuperscript{21,22}. Many of these proteins are recognized by different parts of the immune system, which will be discussed subsequently. Aging has a dramatic impact on immune system composition, function, and response to influenza infection, which is discussed in detail below.

**Age related changes in primary lymphoid organs**

Both primary lymphoid organs, the thymus and the bone marrow, experience age related alterations. The thymus is a primary lymphoid organ important for the development and maturation of T cells\textsuperscript{23}. With increasing age, the thymus undergoes a process known as involution. The thymic epithelial space, composed of the medulla and cortex, where T cell maturation occurs, decreases in cellularity and increases in adiposity during involution\textsuperscript{24}. This process was thought to begin at puberty, but it is now clear that involution begins much earlier, starting at one year of age in humans\textsuperscript{25}. Thymic involution results in the decreased output of naïve T cells into the periphery\textsuperscript{26–28}. Thymic involution also impacts the development of T cells, results in decreased TCR diversity in aged CD8+ T cells\textsuperscript{29}. The aged bone marrow also contributes to changes in T cells with age.

In the aged bone marrow, hematopoietic stem cells (HSC) increase in number, but are functionally impaired and have decreased regenerative potential\textsuperscript{30–34}. HSCs also shift toward the production of common myeloid progenitor cells away from common lymphoid progenitors, further decreasing the production of cells of the lymphoid lineage\textsuperscript{30}. Age related alterations in primary lymphoid organs are upstream to the problems that arise in the adaptive immune cells.
Impaired humoral immune response to influenza infection with age

Humoral immunity is an important component of the adaptive immune response to influenza\textsuperscript{35}. B cell deficient, µMT mice, are 100 times more susceptible to influenza than wild type mice\textsuperscript{35}. Antibody responses to HA, NA and NP provide protection against the virus. Anti-HA antibodies neutralize the virus, by preventing entry into the host cell\textsuperscript{36,37}. SCID mice, which lack functional T cells and B cells, are highly susceptible to influenza infection, but neutralizing monoclonal IgG antibodies directed against HA alone can provide protection from an otherwise lethal influenza infection\textsuperscript{38}. Anti-HA and anti-NA antibodies enhance virus uptake via Fc mediated phagocytosis and promote killing of virally infected cells through antibody dependent cell mediated cytotoxicity (ADCC)\textsuperscript{39}. Antibodies directed against the internal influenza protein NP have also been shown to provide protection against influenza, through a mechanism that remains to be elucidated\textsuperscript{40–42}. Of note, antibody against influenza virus does not need to be neutralizing to be protective as in the case of anti-NP\textsuperscript{42,43}.

Changes in B cells contribute to the increased susceptibility of the elderly to influenza. B cells experience age related declines, starting with changes in the bone marrow. In the aged bone marrow there are fewer precursor B cells, including pre-B, which contribute to decreased output of naive B cells into the periphery\textsuperscript{44–46}. Despite the decreased output of B cells, the number of follicular B cells remains consistent with age as a result of decreased turnover of B cells in the aged environment\textsuperscript{47,48}. B cells also exhibit functional impairments with age including declining B cell receptor (BCR) diversity in mice\textsuperscript{49–52} and some elderly subjects\textsuperscript{53}. Furthermore, aged B cells experience impaired class switch recombination, in part due to decreased expression of the transcription factor E47 and the enzyme activation induced cytidine deaminase (AID)\textsuperscript{54,55}. Finally, production of high affinity, class switched antibodies to newly encountered pathogens like influenza is reduced with age\textsuperscript{56–58}. Class switching and affinity maturation occur in germinal
centers within secondary lymphoid organs\textsuperscript{59,60} and, not surprisingly, with age germinal center formation is reduced and the germinal centers that do develop are small and disorganized\textsuperscript{61}. These changes to antibody and germinal center responses are in part due to the described B cell intrinsic defects along with age related changes in CD4+ T cell help.

**T cell responses to influenza infection**

T cells are important for protection against influenza infection. The most immunodominant epitopes in influenza for both CD4+ and CD8+ T cells can be found in the NP molecule\textsuperscript{62–64}. Influenza specific CD8+ T cells alone, but not CD4+ T cells, can promote recovery of B cell deficient mice from influenza infection\textsuperscript{35}. CD8+ T cells kill virally infected cells by exocytosis of granules containing granzymes and perforin\textsuperscript{65,66}. CD4+ T cells in combination with antibody can also protect B cell deficient mice from influenza\textsuperscript{67}. Studies with CD4+ T cell depleted mice have shown that CD4+ T cells are not required for the clearance of influenza virus\textsuperscript{68,69}. CD4+ T cells are thought to contribute to the immune response to influenza infection by providing “help” to both B cells and CD8+ T cells. CD4+ T cells in particular are classically considered helpers, although we have recently reported the first case of IL-21 producing CD8+ “helper” T cells\textsuperscript{70}. CD4+ T cells help sustain CD8+ T cell responses by production of Type 1 cytokines IFN-\(\gamma\), IL-2 and TNF-\(\alpha\)\textsuperscript{71}. CD4+ T cells also “help” B cells by aiding in the production of IgG antibodies against influenza created in germinal centers\textsuperscript{72}.

Germinal centers are highly organized structures in B cell follicles where high affinity antibody responses are generated\textsuperscript{59}. GC formation is dependent on the differentiation of naïve CD4+ T cells into mature germinal center T follicular helper cells (T\(_{FH}\)) and their resulting functions\textsuperscript{60,73–75}. The maturation of naïve CD4+ T cells into mature germinal center T\(_{FH}\) is a well characterized spatiotemporally regulated process. The first step of T\(_{FH}\) development occurs after contact with...
dendritic cells in a B cell independent manner\textsuperscript{76}. This step results in the formation of pre-\(T_{\text{FH}}\) which express the B cell follicle homing receptor CXCR5, transcription factor Bcl6, ICOS, PD-1 and start to produce IL-4 and IL-21\textsuperscript{77}. Upregulation of CXCR5 and down regulation of CCR7 and PSGL1 promote pre-\(T_{\text{FH}}\) migration from the border of the T cell zone and B cell zone into the B cell follicle in response to follicular CXCL13\textsuperscript{78–81}. This stage depends on follicular B cell engagement of ICOS-L with ICOS on pre-\(T_{\text{FH}}\), which induces pseudopod formation and migration of pre-\(T_{\text{FH}}\) into the B cell follicle\textsuperscript{82}. The exact signals that induce the maturation of pre-\(T_{\text{FH}}\) to germinal center \(T_{\text{FH}}\) are not yet well established, but SAP\textsuperscript{83} and TCR signal strength appear to be important\textsuperscript{84,85}. Germinal center \(T_{\text{FH}}\) function in a variety of ways including production of cytokines such as IL-4 and IL-21, which aid in class switch recombination and promote B cell/plasma cell differentiation and survival\textsuperscript{86–88}. Germinal center \(T_{\text{FH}}\) also express molecules important for promoting B cell functions such as OX40, Ly108 and SLAM89. Germinal center \(T_{\text{FH}}\) are often identified by their expression of \(T_{\text{FH}}\) markers PD-1, CXCR5 and GL7, a sialylated glycan\textsuperscript{90}.

Germinal center responses are controlled by a recently defined subset of CD4+ T cells, called T follicular regulatory cells\textsuperscript{91–93}. T follicular regulatory cells (\(T_{\text{FR}}\)) are derived from thymic regulatory T cells and are identified by their expression of CD4, PD-1, CXCR5, Bcl6, and Foxp3\textsuperscript{92}. One way \(T_{\text{FR}}\) inhibit \(T_{\text{FH}}\) and B cell function is suppression of responses via PD-1\textsuperscript{94}. Sage \textit{et al} recently characterized how \(T_{\text{FR}}\) cells alter metabolism in \(T_{\text{FH}}\) and B cells to control their functionality\textsuperscript{95}. Yet it remains unclear how aging impacts \(T_{\text{FH}}\) and \(T_{\text{FR}}\) development. This will be addressed in chapter two of this thesis.

**Impact of aging on CD4+ T cell responses**

With age there is a shift in the CD4+ T cell pool away from the naïve population toward cells of the memory phenotype\textsuperscript{96–99}. Like B cells, the upstream changes in primary lymphoid organs
decrease the output of naïve T cells into the periphery\textsuperscript{100,101}. Changes in homeostasis, including decreased expression of pro-apoptotic factor Bim, increase the lifespan of naïve CD4+ T cells in the periphery\textsuperscript{102,103}. While newly generated CD4+ T cells from aged mice function as well as young CD4+ T cells, defects in function arise with increasing time in the periphery, suggesting that the aged environment has a negative impact on T cell function\textsuperscript{100,103,104}. Importantly, CD4+ T cells experience many intrinsic defects that arise with age and time in the aged circulation. Aged naïve CD4+ T cells exhibit reduced proliferation and production of IL-2 in response to peptide stimulation\textsuperscript{105}. Aged CD4+ T cells also experience defective immune synapse formation\textsuperscript{106,107}, reduced TCR signaling, including reduction in ZAP-70\textsuperscript{108} and Raf-1 activation\textsuperscript{109}. Unlike B cells, which have reduced BCR diversity\textsuperscript{49}, CD4 + T cells do not appear to have reduced TCR repertoire diversity with age\textsuperscript{110}. Influenza NP specific CD4+ T cell precursor frequency is similar in young and aged mice\textsuperscript{110}. In contrast, CD8+ T cells have altered V\beta usage and reduced TCR diversity, which correlates with impaired response to influenza infection\textsuperscript{29,111}. There are also shifts in CD8+ T cell immunodominance, due to holes in the T cell repertoire, resulting in fewer NP specific CD8+ T cells in aged mice\textsuperscript{29,111}. Finally, during influenza infection, the appearance of both CD4+ and CD8+ influenza specific NP T cells in aged compared to young lungs is delayed\textsuperscript{29,110}. This may be due to differences in precursor frequency along with changes in priming and may contribute to delayed viral clearance in aged mice after influenza\textsuperscript{41,112,113}.

Impaired T cell and B cell responses with age may be linked to changes in Foxp3+ regulatory T cells (Tregs). Aged mice have been shown to have increased numbers of Tregs in secondary lymphoid organs but not in the peripheral blood\textsuperscript{114–118}. This increase in Tregs could lead to delayed immune responses found in aged mice and humans. It is unclear how aging impacts Treg function, but several studies have demonstrated that aged T regs maintain their ability to suppress T cell
proliferation in vitro\textsuperscript{119,120}. How aging impacts the Treg response to influenza infection is explored in chapter two of this thesis.

Impaired humoral responses and decreased germinal center (GC) formation are well established age related immune function changes\textsuperscript{61,121}. We have also shown that young CD4+ T cells transferred into aged CD4 KO mice can overcome B cell mediated defects\textsuperscript{122}, suggesting that age related alterations in CD4+ T cells contribute to B cell problems with age. Our previous studies suggest that the aged splenic environment inhibits the differentiation of young CD4+ T cells into T\textsubscript{FH}\textsuperscript{123}. Yet it remains to be determined how aging impacts T\textsubscript{FH} development and function. Chapter two of this thesis addresses this question by characterizing how aging impacts the antigen specific T\textsubscript{FH} response to influenza infection. Our hypothesis predicts that age related changes in T\textsubscript{FH} development and function may be contributing factors to the deceased humoral response in age mice to influenza infection. This chapter also describes how aging impacts the development of T\textsubscript{FR} and how these changes may contribute to impaired humoral responses with age. In chapters three and four we explore the impact of the aged secondary lymphoid organ environment on impaired immunity to influenza infection, with a focus on stromal cell biology.

**Secondary lymphoid stromal cells: an underlying contributor to immunosenescence?**

Stromal cells are non-hematopoietic cells in secondary lymphoid organs (lymph nodes, spleen) that were once considered purely structural in nature\textsuperscript{124}. During the past decade this simplistic view has been overturned by an insurgence of research revealing the integral role of stroma in maintaining and controlling immune cell function. While much of aging immunology research has been focused heavily upon determining cell intrinsic defects in adaptive and innate immune cells, the contribution of secondary lymphoid stromal cells to age-related defects in immunity are just beginning to become unraveled\textsuperscript{123,125,126}. 
Adoptive transfer studies have been the key to dissecting the impact of the aged “environment” on CD4+ and CD8+ T cell responses. By transferring young OTII CD4+ T cells intravenously (IV) into young or aged hosts immunized with OVA and alum adjuvant, Lefevbre et al. showed that young T cells have delayed entry (18 hours after transfer), proliferation, expansion and differentiation (into T_{FH}) in aged spleens\textsuperscript{123}. Richner et al. showed that young naïve CD4+ T cells have impaired entry one hour after IV transfer into the lymph nodes and spleens of West Nile virus infected aged mice when compared to young\textsuperscript{127}. Beckuland et al also found that young T cells transferred IV into young or aged naive hosts had reduced homing into aged lymph nodes four hours after transfer compared to young lymph nodes\textsuperscript{128}.

Other studies have shown that the aged splenic environment appears to be inhibitory to CD8+ T cell responses. Jiang et al. transferred young influenza specific Clone 4 CD8+ T cells into young or aged hosts that had been intravenously infected with influenza virus, and found reduced expansion and decreased interferon gamma production in aged versus young spleens\textsuperscript{129}. Li et al. described that after intravenous \textit{Listeria-OVA} infection, young OTI CD8+ T cells transferred into young and aged host had reduced expansion in aged compared to young spleen\textsuperscript{130}. These studies have begun to elucidate a scenario in which the aged “environment” of secondary lymphoid organs hinders the development of robust T cell responses. Yet which factors of the aged spleens and lymph nodes are contributing to reduced recruitment and proliferation are unclear and will be address in chapters three and four of this thesis, with the focus on stromal cell biology. Our hypothesis predicts that changes in secondary lymphoid organ stromal cell function many contribute to impaired T cell immunity found in aged mice to influenza infection. Chapter three will elucidate how aging impacts splenic stromal cells and chapter four explores how aging impacts lymph node stromal cells.
**Age-related alterations of lymph node stromal cells**

Lymph nodes are highly organized structures important for the development of adaptive and innate immune responses. In lymph nodes, B cells are segregated to peripheral follicles and T cells remain in the central T cell zone, also known as paracortex. The medullary sinus is a site where activated T cells exit the lymph node through lymphatic vessels (Fig. 1A). The majority of secondary lymphoid organ stromal cell research has focused upon the lymph node stromal cells. Recent reviews by Fletcher et al.\textsuperscript{131} and Change et al. \textsuperscript{132} describe in detail the biology of the various lymph node stromal cell niches. Simplistically, lymph node stroma can be divided into four subsets; lymphatic endothelial cells (LECs), blood endothelial cells (BECs), fibroblastic reticular cells (FRCs) and cells negative for the markers of these subsets, called double-negative cells (DNCs)\textsuperscript{131} (Fig. 1B).
A technical aside of studying stromal cells, is that they are incredibly difficult to digest out of secondary lymphoid organs. We have found that a modified version of Fletcher et al.’s digestion protocol gave the highest viability and yield of all stromal cell subsets from both lymph node and spleen\textsuperscript{133}. If the digestion is performed improperly, there will be high rates of cell death. It has been reported that hematopoietic cells in the process of dying can down regulate CD45 and fall into the stromal cell gate for the DNC population\textsuperscript{133}. Normally DNCs range from 5-10% of the stromal cell population in lymph nodes\textsuperscript{133}. When the frequency of the DNC population is high in lymph nodes, this is indicative of poor digestion technique. Three recent studies have attempted to quantify the number of stromal cells in aged lymph nodes using digestion protocols and flow
cytometry, but appear to be caveated by questionable digestions\textsuperscript{128,134,135}. In all of these studies greater than 50% of the lymph node stromal cells are DNCs, bringing into question the validity of their quantification. Double-negative cells are a poorly defined subset of lymph node stromal cells, and they are believed to be contractile FRC-like pericytes\textsuperscript{136}. As little is known about these cells, it is not surprising that it is unknown how aging changes their function. A recent publication by Davies \textit{et al.} claimed that naïve aged cervical lymph nodes had fewer total stromal cells than young lymph nodes, due to decreases in the numbers of DNCs\textsuperscript{135}. Turner \textit{et al.} found similar decreases in the number of DNCs in pooled cervical, axillary, brachial and inguinal naïve lymph nodes from aged compared to young mice\textsuperscript{134}. The digestions in both of these studies were highly questionable and need to be repeated before we can understand how aging impacts DNC numbers in lymph nodes.

Lymphatic endothelial cells are a stromal cell subset that line the lymphatic vessels\textsuperscript{137}. Lymphatic vessels are conduits important for maintenance of fluid homeostasis which transport lymph, soluble antigens and immune cells from tissues to draining lymph nodes\textsuperscript{138}. Aged lymphatic collectors have increased leakiness and a decreased ability to support active lymph flow\textsuperscript{139–141}, which results in decreased capacity to transport bacteria\textsuperscript{141}. Functional attrition of aged lymphatics is due in part to increased oxidative stress and protein carbonylation\textsuperscript{141}. Defects in cellular and antigenic transport caused by age-related changes in lymphatic collectors may be a contributing factor behind the delayed initiation to immune responses found in elderly people. How aging impacts lymph node lymphatic endothelial cells at homeostasis is unclear, although Turner \textit{et al.} and Davies \textit{et al.} found similar number of LECs in young and aged naïve lymph nodes\textsuperscript{134,135}. How LECs respond in aged mice after immune challenge remains to be determined, and is a focus of chapter four of this thesis.
Blood endothelial cells in the lymph node can be separated into capillaries and cuboidal-shaped high endothelial venules (HEVs). BECs facilitate entry of naive T and B cells into the lymph node, through a well described multi-step process, called trans endothelial migration. How aging impacts HEVs or capillaries in the lymph node is still unclear. Richner et al. showed that aged naive CD4\(^+\) T cells transferred into young mice infected with WNV have delayed entry into the lymph node, and it was observed that aged CD4\(^+\) T cells have altered migration through HEVs compared to young T cells, suggesting T cell intrinsic defects. However, this study did not directly examine the changes occurring in aged HEVs, which may contribute to the delayed entry of young cells into aged lymph nodes. Turner et al. digested pooled cervical, axillary, inguinal and brachial lymph nodes and found lower frequencies of BECs, but similar number in aged compared to young lymph nodes at homeostasis. This study also stated that the localization of BECs in aged inguinal lymph nodes was more diffuse than young inguinal lymph nodes, but did not quantify this observation. A study by Becklund et al. claimed from imaging data of frozen sections that young and aged lymph nodes have similar numbers of HEVs based on CD31 staining, but also did not quantify their images to support this statement. Although when visualized in frozen section HEVs appear to be plump, cuboidal circles, their true structure is vastly more complex. In lymph nodes the vasculature forms a tree, with HEVs localizing to specific branch points. Either two photon microscopy or tissue clearing must be used to completely visualize the complexity of the HEV network in lymph nodes. Aging of the vascular system is a well-studied phenomenon characterized by mechanical and structural changes to the vascular including arteriolar stiffening. It is likely that lymph node blood vessels would experience age-related changes which could result in delayed immune responses found with increasing age.

Fibroblastic reticular cells are a diverse subset of stromal cells in the lymph node. Follicular dendritic cells (FDC) are one type of FRC. The impact of aging on FDCs is discussed separately.
below. The most thoroughly studied variety of FRCs are those of the lymph node T cell zone. T cell zone FRCs maintain the architectural organization of the T cell zone and B cell follicles. FRC-produced chemokines CCL19 and CCL21 interact with their receptor, CCR7, on T cells and dendritic cells controlling the localization of these immune cells to the T cell zone of the lymph node. One recent study showed that at steady state, CCL21 concentration was similar in young and aged popliteal lymph nodes, but after infection with West Nile virus aged lymph nodes had lower CCL21 concentrations when compared to young lymph nodes. It is important to note that this study did not normalize the CCL21 levels to total protein or lymph node weigh, putting into question their results. Disruption of B cell follicles in aged lymph nodes may suggest alterations in T cell zone FRCs, but this has not been studied thoroughly. It is unclear whether there are fewer T cell zone FRCs in the aged lymph node or if the aged FRCs are functionally impaired. Becklund et al. claimed that there are fewer FRCs in aged compared to young lymph nodes at homeostasis but did not perform statistics to validate this claim. Turner et al. and Davies et al. found that the number of FRCs in aged compared to young naive lymph nodes were not significantly different.

FRCs proliferate and increase in number after antigenic challenge through a poorly understood mechanism. This aids in the expansion of the lymph node during immune responses. It remains to be determined how aging impacts FRC expansion after immune challenge, which is also addressed in chapter 4 of this thesis. Functional changes in FRCs with age may have a major impact on the initiation and control of adaptive immune responses in aged individuals. Decreased CCL21 concentrations may reduce the recruitment and localization of activated dendritic cells and naive T cells into draining lymph nodes, which could dramatically diminish the magnitude of the immune response. FRCs are also a major source of IL-7 and CCL19, both of which are important for survival of naive T cells. Aging reduces the number of naive T cells dramatically, due in
part to thymic involution, and results in changes to T cell homeostasis\textsuperscript{152}. FRCs may contribute to T cell homeostatic difficulties if IL-7 and CCL19 production is altered. FRCs also have the ability to directly inhibit T cell proliferation via production of nitric oxide (NO) when in contact with activated T cells, IFN-\(\gamma\) and TNF\(\alpha\). The FRC produced NO inhibits T cell proliferation along with PD-L1 which is upregulated on FRCs\textsuperscript{153,154}. It is possible that aged FRCs have increased inhibitory capacity and may contribute to impaired T cell immunity, by blocking T cell proliferation in aged lymph nodes. This hypothesis is addressed in chapter 4 of this thesis.

A study by Denton \textit{et al.} demonstrated that FRCs are required for the development of immune responses to influenza infection. This study used a mouse model in which lymph node FRCs could be conditionally depleted. When FRCs were depleted before mice were infected with influenza, this reduced influenza specific PA CD8\(^+\) T cell, T\(_{FH}\), GC B cells, and plasma cell responses. This demonstrated the importance of FRCs in the initiation of the immune response to influenza. Interestingly if FRCs were deleted six days after the mice were infected with influenza, there was no change in adaptive immune response formed\textsuperscript{155}. This study provided evidence that FRCs are required for the initiation of the immune response to influenza, but may be dispensable after the response has been initiated. How aging impacts lymph node FRCs at homeostasis and after influenza infection is a major focus of chapter 4 of this thesis.

**Age-related alterations of splenic stromal cells**

The spleen is a secondary lymphoid organ located in the upper right quadrant of the abdomen that filters blood, and is a critical component in the defense against blood-borne pathogens such as encapsulated bacteria\textsuperscript{156,157}. The spleen is compartmentalized into red and white pulp, as shown in Fig. 2. The white pulp consists of B cell follicles which surround a T cell periarteriolar sheath. The marginal zone surrounds the B cell follicle. This is where the central arteriole empties
and immune cells enter the spleen\textsuperscript{156}. Bridging channels are FRC-lined conduits that allow for entry of immune cells into the splenic T cell zone from the marginal sinus\textsuperscript{158}. A frequently overlooked component of immune system efficacy is locality\textsuperscript{159}. Immune responses are dependent upon the interaction of rare cells with one another, and the intricate organization of secondary lymphoid organs is designed to increase the probability of these interactions occurring\textsuperscript{159}. With age, there is considerable attrition of splenic white pulp organization. The splenic marginal zone (B cells\textsuperscript{160} and macrophages\textsuperscript{125,161}) and follicular dendritic cells\textsuperscript{162} show significant disruption with age, and there is also a merging of the B cell follicles and the T cell areas\textsuperscript{125,160,163}. Stromal cells are a non-haematopoietic component of secondary lymphoid tissues. In the spleen, the markers podoplanin (PDPN) and CD31 can be used to identify three stromal subsets: fibroblastic reticular cells (PDPN\textsuperscript{+}CD31\textsuperscript{−}), blood endothelial cells (CD31\textsuperscript{+}PDPN\textsuperscript{−}) and double-negative cells (PDPN\textsuperscript{−}CD31\textsuperscript{−}), which are mainly red pulp fibroblasts\textsuperscript{164,165}. Unlike the lymph node, spleens do not contain lymphatic endothelial cells, due to the absence of lymphatic vessels.

**Figure 2. Splenic architecture.** The T cell zone of the splenic white pulp is supported by podoplanin (magenta)-positive fibroblastic reticular cells (FRCs). B cell follicles surround the T cell zone and are devoid of FRCs, but contain follicular dendritic cells. The red pulp consists of ER-TR7\textsuperscript{+} red pulp fibroblasts (green). Bridging channels, lined with FRCs, connect the T cell zone of the white pulp to the marginal zone and red pulp of the spleen. Image was acquired using confocal microscopy. Scale bar = 100 μm. Imaged is a C57BL/6 mouse spleen.
Splenic FRCs (gp38^+CD31^-ERTR7^+) play a variety of roles in the immune response, including providing a conduit for lymphocytes, dendritic cells\textsuperscript{158,166} and antigen\textsuperscript{167} trafficking, production of homeostatic chemokines important for T and dendritic cell localization to the T cell area (CCL19, CCL21)\textsuperscript{145}, production of IL-7\textsuperscript{168}, maintenance of the B cell homeostasis and follicular organization\textsuperscript{145}. A recent report by Aw et al. used microscopy to examine how aging altered splenic FRC morphology\textsuperscript{125}. In this study, aged mice had an increased area of splenic FRCs which correlated with the merging of the T cell zones and B cell follicles\textsuperscript{125}. Splenic FRC production of homeostatic chemokines CCL19 and CCL21 have been shown to decrease in aged mice after antigenic challenge, which contributes to improper migration of T cells into the T cell zone\textsuperscript{163}. Yet it remains to be determined if the concentrations of homeostatic chemokine are different at homeostasis in young and aged mice differ. One report suggests that aged splenic stroma \textit{in vitro} have increased production of IL-6, but this study used a relatively crude stromal cell isolation technique and needs to be repeated\textsuperscript{169}. Studies of human spleens have noted increased collagen composition in spleens of elderly people\textsuperscript{170} and attrition of elastic fibres in splenic capsules\textsuperscript{171}. Further studies are required to determine how aging alters red pulp fibroblasts, and splenic arteries. Senescence may also have a profound impact upon age-related splenic stromal cell dysfunction, but this has yet to be determined. Wang et al. quantified senescent cells in the spleens of aged mice using γH2Ax staining and found that senescence increases with age\textsuperscript{172}. Further analysis of other senescence markers and careful identification of which splenic cells are senescent needs to be performed. We are just beginning to understand how aging impacts splenic stromal cells.

**Age-related changes in follicular dendritic cells**

Follicular dendritic cells (FDCs) are a subset of FRCs that defines the structure of B cell follicles in secondary lymphoid organs\textsuperscript{173}. Functionally, FDCs facilitate B cell-mediated responses by
maintaining the germinal center and facilitating the production of high-affinity antibodies\textsuperscript{174}. Aging is associated with a decline in antibody-mediated responses which can, in part, be attributed to B cell intrinsic defects\textsuperscript{175} and functional attrition of T follicular helper cell responses\textsuperscript{94,127,163}. Age-related changes in FDC function may also contribute to the decline of humoral response. One way that FDCs maintain the organization of the B cell follicle is through production of the chemokine CXCL13\textsuperscript{176}. Conflicting reports exist about how aging changes CXCL13 production. Splenic production of CXCL13 in aged BALB/c mice was shown to be increased compared to young mice at steady state\textsuperscript{126}, whereas 18 hours after antigenic challenge it was determined that CXCL13 localization in the spleen was diffuse and spread into the T cell areas of aged C57BL/6 mice\textsuperscript{123}. Quantification of CXCL13 in young and aged lymph nodes at steady state showed no significant difference, but after infection with West Nile virus aged mice had lower CXCL13 levels\textsuperscript{127}. Aged FDCs also have defects in their ability to trap and present immune complexes to B cells\textsuperscript{177}. Decreased expression of FCγRII, CD21L and FDC-M2 on FDCs after antigenic challenge may contribute to these defects\textsuperscript{177–179}. Defects in aged FDCs may be a major contributor to age-related defects in the humoral response.

**Goals of thesis**

The primary goal of this dissertation is to expand our understanding of how aging impacts various components of the immune response to influenza infection, with focus on stromal cell biology. Our hypothesis predicts that changes in secondary lymphoid organ stromal cells are an underlying and overlooked factor contributing to impaired adaptive immunity to influenza infection in aged mice.

In chapter two “Age-related impairment of humoral response to influenza is associated with changes in antigen specific T follicular helper cell responses” we elucidate how aging impacts
several components of the adaptive immune response to influenza infection. We determine how $T_{FH}$ and $T_{FR}$ development and function change with age and how these changes may alter humoral immunity to influenza infection. We also assess the contribution of the aged splenic environment in the promotion of impaired immune response to influenza infection.

In chapter three “Attrition of fibroblastic reticular cell number and function in aged spleens” we directly characterize how aging impacts splenic fibroblastic reticular cell numbers, morphology and function.

In chapter four “Assessment of lymph node stromal cells as an underlying factor in age related immune impairment to influenza infection” we characterize how aging impacts lymph node stromal cells at homeostasis and after infection and address our hypothesis that changes in lymph node stromal cells may be an underlying factor contributing to decreased adaptive immunity with age. We also assess the contribution of fibroblastic reticular cells to the impaired response of young influenza specific CD8+ T cells transferred into aged versus young mice.

This body of work progresses the field of aging immunology by expanding our understanding of how aging alters T follicular helper cells, T follicular regulatory cell, splenic stromal cells and lymph node stromal cells at homeostasis and in response to influenza infection. These studies are of vital importance for the field of aging immunology because they provide new insight into targets to modulate to enhance the immune response to influenza infection in hope of protecting the elderly from the harmful effects of influenza virus.
REFERENCES


43. Mozdzanowska, K., Furchner, M., Washko, G., Mozdzanowski, J. & Gerhard, W. A pulmonary influenza virus infection in SCID mice can be cured by treatment with hemagglutinin-


CHAPTER 2

Age-related impairment of humoral response to influenza is associated with changes in antigen specific T follicular helper cell responses

ABSTRACT

T follicular helper (T\textsubscript{FH}) cell responses are essential for generation of protective humoral immunity during influenza infection. Aging has a profound impact on CD4\textsuperscript{+} T cell function and humoral immunity, yet the impact of aging on antigen specific T\textsubscript{FH} responses remains unclear. Influenza specific T\textsubscript{FH} cells are generated in similar numbers in young and aged animals during infection, but T\textsubscript{FH} cells from aged mice exhibit significant differences, including reduced expression of ICOS and elevated production of IL-10 and IFN\textgamma, which potentially impairs interaction with cognate B cells. Also, more influenza specific T cells in aged mice have a regulatory phenotype, which could contribute to the impaired T\textsubscript{FH} function. Adoptive transfer studies with young T cells demonstrated that TGF-\beta1 in the aged environment can drive increased regulatory T cell accumulation. Aging and the aged environment thus impact antigen specific T\textsubscript{FH} cell function and formation, which contribute to reduced protective humoral responses.

INTRODUCTION

Aging is associated with a dramatic decline in immunity including production of high affinity neutralizing antibodies\textsuperscript{1}, which leads to increased susceptibility to clinically relevant pathogens such as influenza. High affinity antibodies are generated by B cells selected in germinal centers (GC) and GC formation is dependent upon the proper function of T follicular helper (T\textsubscript{FH}) cells\textsuperscript{2-5}. Following initial activation, naïve CD4\textsuperscript{+} T cells progress through several well defined steps to become functional T\textsubscript{FH} cells in GCs \textsuperscript{6,7}. First, pre-T\textsubscript{FH} cells up-regulate the B cell follicle homing chemokine receptor CXCR5 and the transcriptional repressor B cell lymphoma 6 (Bcl6). These
cells also produce IL-4 and IL-21, key cytokines of B cell activation. This first stage is the result of T-dendritic cell (DC) interactions and is independent of B cells. The activated T cells then progress toward a fully differentiated T_FH cell state characterized by the expression of numerous markers including the inhibitory receptor programmed death (PD)-1, the co-stimulatory molecules ICOS and OX40, along with the adaptor protein signaling lymphocytic activation molecule (SLAM)-associated protein (SAP). These molecules facilitate efficient T-B interactions, which are essential for GC formation. Deficiencies in ICOS or SAP result in reduced T_FH cell generation and/or reduced GC formation. These interactions with B cells induce the further differentiation of T_FH cells into GC T_FH cells, which are identifiable through the sustained expression of GL7 Akiba, et al. Importantly, how aging impacts the stages of T_FH cell generation and differentiation is yet to be determined.

The presence of functional defects in aged CD4^+ T cells is now well-established. We demonstrated that aged CD4^+ T cells exhibit impaired upregulation of CD154, suggesting that reduced B cell activation and humoral responses observed in aged mice could result from improper T-B interactions. Differentiation of young CD4^+ T cells into T_FH effectors cells was also significantly reduced when these cells were transferred into aged hosts, suggesting that the aged environment is unable to sustain T_FH cell differentiation. These observations were made in adoptive transfer models using T cell receptor (TCR) transgenic CD4^+ T cells. Whether endogenous antigen-specific aged CD4^+ T cells have the ability to acquire a T_FH cell phenotype and/or whether endogenous aged T_FH cells have functional defects during infection remains unclear. A recent study using an OVA immunization model showed that aged mice have more T_FH than young mice but aged T_FH were less functional using in vitro assays. This study did not track antigen specific responses in vivo, and did not thoroughly define the T_FH maturation status. Moreover, the capacity of aged mice to mount an antigen-specific CD4^+ T cell response equivalent
to that of young mice and whether it correlates with an antigen-specific B cell response remains to be investigated. Indeed, these are important points and only when they are fully addressed can we begin to understand the age-related defects in humoral immunity and, more importantly, how they can be overcome.

Here, we have employed reagents that allow us to follow the responses of endogenous influenza nucleoprotein (NP)-specific CD4+ T and B cells in young and aged mice. Studying endogenous antigen specific responses to infection is critical to understanding the full scope of immunological defects that occur with age. This has allowed us to determine specific defects in responding T_{FH} cells in aged mice and their impact on humoral responses.

**MATERIALS AND METHODS**

**Mice.**

Young (2-4 months) and aged (18-24 months) C57Bl/6 mice were bred and housed at the Trudeau Institute or University of Connecticut Health Center animal facilities. Alternatively, aged C57BL/6 were obtained from the National Institute of Aging (NIA) aging colony bred and housed at Charles River Laboratories. Young mice were also obtained from Jackson Laboratories. All mice were kept in sterilized, individually ventilated, HEPA-filtered cages under specific pathogen-free conditions. The mice were anesthetized with isoflurane and infected intranasally with 600 PFU of the mouse-adapted influenza A H1N1 strain Puerto Rico/34/8 (PR8). The mice were sacrificed by CO₂ asphyxiation. Age-matched non-infected mice were used as controls. The Trudeau Institute or University of Connecticut Health Center Animal Care and Use Committees approved all experimental procedures using animals and all procedures were carried out in accordance with their guidelines.
**Determination of antibody titers.**

Young and aged mice and were anesthetized with isoflurane and blood was harvested by cardiac puncture on days 0, 7, 14 and 21 post infection. The blood was allowed to coagulate for 2 hours at room temperature, centrifuged (×10,000 rpm) 10 min at room temperature, and the serum was collected and kept at -20°C until analysis was performed. PR8-specific IgG was determined by ELISA using HRP-labeled anti-isotype antibodies (Southern Biotech Inc.). Antibody titers were determined by the last serum dilution with an optical density above background. Neutralizing antibody titers were determined using an in vitro neutralization assay. In brief, serum dilutions were incubated with 400 PFU of PR8 for one hour at 37°C. The mixtures were added on Madin-Darby Canine Kidney cell monolayers and centrifuged (×3,000 rpm) 70 min at room temperature. The cells were next washed and incubated 20-24 hours at 33°C in Zero-Serum Refeed Medium-PSGA (Diagnostic Hybrids) containing 4 µg/ml trypsin (Sigma). The virus was next detected using a biotin-labeled anti-influenza-A antibody (Millipore). The neutralizing antibody titers were determined as the last serum dilution with fewer viral plaques than the normal mouse serum control.

**Flow cytometry.**

To identify the NP-specific CD4+ T cells, single cell suspensions of splenocytes were first stained for one hour at room temperature with MHC class II NP Tetramer (PE/APC, Trudeau Institute Molecular Biology Core Facility). Further phenotyping was performed by staining with directly fluorochrome conjugated antibodies (Table 1). For intracellular transcription factor detection, cells were fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer’s instructions. NP-specific GC B cells were detected by staining with PE labeled B cell tetramer (Trudeau Institute Molecular Biology Core44) for 30 min on ice. For intra-cellular cytokine staining, samples were enriched for CD4 cells by MACS® using
the CD4 enrichment kit II (Miltenyi Biotech) according to the manufacturer’s instruction. The cells were then resuspended at 5-10×10^6 cells/ml in RPMI (Cellgro) containing 0.18 μM β-mercaptoethanol, 4 mM glutamine, antibiotic-antimycotic solution (Cellgro), 10 mM HEPES, 10% fetal bovine serum and 4 μl/6ml GolgiStop™ (BD). The cell suspensions were stimulated for 5 hours with 1 μg/ml phorbol 12-myristate 13-acetate and 1 μg/ml ionomycin at 37°C. The cells were then washed, fixed, and permeabilized using the BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit (BD) according to the manufacturer’s instructions. Control samples containing the different “fluorescence minus one” antibody cocktails were used to determine the appropriate gating strategy for each cytokine. All flow cytometry samples were analyzed on a Canto II flow cytometer or on a LSR II flow cytometer using the FACSDiva software (BD). Data analyses were then performed using the FlowJo software (Tree Star).
Table 1. Antibodies used for phenotyping T cells, B cells and cytokine production by flow cytometry.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Fluorochrome</th>
<th>Clone</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4</td>
<td>APC-eFluor®780 eFluor®450</td>
<td>RM4-5</td>
<td>eBioscience</td>
</tr>
<tr>
<td>GL7</td>
<td>FITC</td>
<td>GL7</td>
<td>BD</td>
</tr>
<tr>
<td>CXCR5</td>
<td>PE-Dazzle™594 BV421</td>
<td>L138D7</td>
<td>BioLegend</td>
</tr>
<tr>
<td>PD-1</td>
<td>PE-Cy7</td>
<td>RMP1-30</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD44</td>
<td>BV510</td>
<td>IM7</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD45</td>
<td>AF700</td>
<td>Dan11mag</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD45.1</td>
<td>BV510 BV421</td>
<td>A20</td>
<td>BioLegend</td>
</tr>
<tr>
<td>ICOS</td>
<td>AF488</td>
<td>C398.4A</td>
<td>BioLegend</td>
</tr>
<tr>
<td>Ly108</td>
<td>PE</td>
<td>13G3-19D</td>
<td>eBioscience</td>
</tr>
<tr>
<td>OX40</td>
<td>PE</td>
<td>OX-86</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD150</td>
<td>PE</td>
<td>Q38-480</td>
<td>BD</td>
</tr>
<tr>
<td>CD19</td>
<td>BV650 PE-Cy7</td>
<td>6D5</td>
<td>BioLegend</td>
</tr>
<tr>
<td>Bcl6</td>
<td>PerCP-eFluor®710</td>
<td>BCL-DWN</td>
<td>eBioscience</td>
</tr>
<tr>
<td>Foxp3</td>
<td>eFluor®450</td>
<td>FJK-16s</td>
<td>eBioscience</td>
</tr>
<tr>
<td>Peanut lectin agglutinin</td>
<td>FITC</td>
<td></td>
<td>Sigma</td>
</tr>
<tr>
<td>CD38</td>
<td>Pacific Blue</td>
<td>90</td>
<td>BioLegend</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>FITC</td>
<td>XMG1.2</td>
<td>BD</td>
</tr>
<tr>
<td>IL-4</td>
<td>PerCPCy5.5</td>
<td>11B11</td>
<td>BD</td>
</tr>
<tr>
<td>IL-2</td>
<td>APC-Cy7</td>
<td>JES6-5H4</td>
<td>BD</td>
</tr>
<tr>
<td>IL-10</td>
<td>V450</td>
<td>JES5-16E3</td>
<td>BD</td>
</tr>
<tr>
<td>IL-21R Fc chimera (IL-21 Primary)</td>
<td>Unconjugated</td>
<td></td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>Goat anti-human IgG Fcy Frag Specific (IL-21 Secondary)</td>
<td>PE</td>
<td></td>
<td>Jackson ImmunoResearch</td>
</tr>
<tr>
<td>Propidium iodide (Live/Dead Indicator)</td>
<td></td>
<td></td>
<td>Sigma</td>
</tr>
<tr>
<td>Carboxylic Acid, Succinimidyl Ester (Live/Dead Indicator)</td>
<td>AlexaFluor®350</td>
<td></td>
<td>Life Technologies</td>
</tr>
<tr>
<td>CD16/CD32 (FC Block)</td>
<td>Unconjugated</td>
<td>93</td>
<td>eBioscience</td>
</tr>
</tbody>
</table>

Immunofluorescence.

Spleens were fixed overnight in medium containing 0.05 M phosphate buffer, 0.1 M l-lysine, pH 7.4, 2 mg/ml NaIO4, and 10 mg/ml paraformaldehyde and were dehydrated with 30% sucrose in phosphate buffer. Tissues were frozen in Tissue-Tek O.C.T. compound (Sakura Finetek) and
sectioned using a CM1850 cryostat (Leica). 20-μm frozen sections were permeabilized with 1% Triton X-100 (Sigma) for ten minutes at room temperature, blocked for 30 minutes with Background Buster (Innovex Biosciences), stained over night at 4°C in a humidified chamber with an antibody cocktail (Table 2), then mounted with Immu-Mount (Thermo Sceintific). Immunofluorescence confocal microscopy was performed with the Zeiss 780 laser scanning microscope (Carl Zeiss; air objective 20× Plan-APOCHROMAT with NA 0.5) using multichannel frame scans. The ZEN 2012 software (Carl Zeiss) was used for image acquisition. For half spleen images, the ZEN 2012 tile scan function was used to stitch individual 20x images together. Quantification of GC area, GC T<sub>FH</sub> cells and image processing was performed using Imaris 8.1 software (BITPLANE).

Table 2. Antibodies used for confocal microscopy of TFH.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Fluorochrome</th>
<th>Clone</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>B220</td>
<td>BV510</td>
<td>RA36B2</td>
<td>BioLegend</td>
</tr>
<tr>
<td>GL7</td>
<td>AF488</td>
<td>GL7</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD4</td>
<td>BV421</td>
<td>GK1.5</td>
<td>BD</td>
</tr>
<tr>
<td>Bcl6</td>
<td>AF647</td>
<td>K112-91</td>
<td>BD</td>
</tr>
</tbody>
</table>

Adoptive transfer.

The splenocytes were prepared from young non-infected CD45.1<sup>+</sup> C57/Bi6 mice (Jackson Laboratories) and enriched for CD4<sup>+</sup> cells by MACS® enrichment kit II (Miltenyi Biotech) according to the manufacturer’s instruction. Purity of this population was confirmed by flow cytometry (≥85%). The isolated young CD45.1<sup>+</sup>CD4<sup>+</sup> T cells were tail vein injected into young or aged CD45.2<sup>+</sup> C57BL/6 hosts at 3.6x10⁷ cells/200μl/ mouse. One day later the mice were infected with 600 PFU PR8 influenza and subsequently sacrificed 14 days later for flow cytometric analysis.
**Active TGF-β 1 ELISA.**

Prior to flow analysis, the spleens from young and aged mice that received adoptive transfer of young CD45.1<sup>+</sup>CD4<sup>+</sup> T cells, where crushed through 100μM wire mesh with 400μL of PBS. The suspension was then centrifuged (x300 rpm) for 2 minutes and supernatant was collected and stored at -80°C until analysis was performed. TGF-β1 was measured in the spleen supernatants using the LEGEND MAX™ Free Active TGF-β1 ELISA kit (BioLegend). Samples were run in duplicate according to manufactures instructions. Spleen weights did not differ significantly in young and aged mice (data not shown).

*In vitro generation of regulatory T cells.*

A sterile single cell suspension was prepared from spleens and lymph nodes pooled from four young mice. Erythrocytes were lysed using ACK lysis buffer (Gibco by Life Technologies). CD4<sup>+</sup> T cells were isolated by negative selection using Miltenyi’s CD4 cell isolation kit according to manufactures instructions. The CD4<sup>+</sup> population was then stained with PE conjugated anti-CD25 (BD). After washing, the cells were then incubated with anti-PE microbeads (Miltenyi). CD25<sup>+</sup> cells were removed by positive selection using the LS column (Miltenyi). Purity of the CD25-CD4<sup>+</sup> T cells was confirmed by flow cytometry. The purified cells were suspended at 2x10<sup>6</sup> cells/ml in X-Vivo15 serum free medium (Sartorius Stedim Biotech) and cultured in 24 well plates with Dynabeads® Mouse T-Activator CD3/CD28 (Life Technologies) at a bead to cell ratio of 1:1. TGF-β1 (R&D Research Systems) was either added at 5000pg/ml (recommended concentration for generation of regulatory T cells (Fantini, et al<sup>35</sup>) 100 pg/ml (concentration found in aged spleens), or 0 pg/ml (negative control). All conditions were plated in triplicate and cultured for 5 days at 37°C 5%CO<sub>2</sub>.
**Statistical analyses.**

All experiments were performed at least twice, and each group contained 3-7 animals. Statistical significance was determined by Student’s t test, One-way or Two-way ANOVA, Repeated Measures ANOVA, or Pearson’s correlation as specified in the figure legends. Statistical analyses were performed with Prism 5 or Prism 6 software (GraphPad Software inc.) or SPSS Software (IBM). Differences were considered significant at p<0.05.

**RESULTS**

**Impaired humoral response in aged mice following influenza infection**

One hallmark of aging is the inability to mount a strong humoral response. Aged mice infected with a sublethal dose of influenza A H1N1 PR8 generated significantly lower titers of influenza-specific IgG (Fig. 3A) and neutralizing antibodies (Fig. 3B) compared to young. Generation of high-affinity antibodies is dependent upon formation of GCs, which can be impacted by age-related functional declines\(^20\). Thus, we sought to investigate the contribution of GCs to diminished formation of influenza-specific antibody titers in aged mice in this model.

GC B cells were identified as CD19+ PNA\(^{hi}\) CD38\(^{lo}\) cells (boxes in Fig. 3C). The kinetics of total (Fig. 3C and D) and NP-specific (Fig. 3I and J) GC B cell generation were similar for both young and aged mice, with the proportion and number peaking at 14 days post-infection (dpi). However, the amplitude of the response was significantly lower in aged compared to young mice. At 14 dpi, approximately 1% of total B cells acquired a GC B cell phenotype in aged mice compared to a proportion of over 7% in young mice (Fig. 3C). This translated to a significantly lower number of total GC B cells produced in the spleens of aged compared to young mice (Fig. 3D).
Upon examination of the splenic GCs using confocal microscopy, there were clear morphological differences in young and aged mice (Fig. 3E). At the peak of the GC response (14 dpi), aged mice exhibited significantly fewer (Fig. 3F) and smaller GCs (Fig. 3G) compared to young mice. Importantly, there was a strong correlation between the number of splenic GCs and weight recovery at 14 dpi (Fig. 3H). Young and aged mice that generated more splenic germinal centers had increased weight recovery after influenza infection (Fig. 3H), indicating that rapid recovery from influenza infection is correlated with robust GC formation. The percentage of GC B cells that were specific for NP was slightly, but not significantly, lower in aged (22.7 ± 7.2%) compared to young mice (37.7 ± 3.9%, p=0.104) (Fig. 3I) at 14 dpi, but the total number of NP+ GC B cells in aged mice was approximately ten-fold lower than in young mice (Fig. 3J).

Despite age related declines in B cell function$^{21}$, young polyclonal CD4$^+$ T cells transferred into aged hosts can overcome the defects in GC B cell numbers normally found in aged mice at 14dpi. When aged mice were supplemented with young CD4$^+$ T cells prior to infection (aged + CD4 group), the number of total (Fig. 3K) and NP specific (Fig. 3L) GC B cells reaches that of the endogenous response of young mice (young group) at 14 dpi, emphasizing the importance of efficient CD4$^+$ T cell help in the development of a robust B cell response. These results are in concordance with our previous finding that young TCR transgenic CD4$^+$ T cells can overcome B cell defects in aged CD4 KO mice$^{17}$. Thus, the remainder of this study was aimed at determining how age-related defects in the CD4$^+$ T cell response contribute to the impaired humoral response in aged mice.
Figure 3. Reduced antibody production and GC development during influenza infection in aged mice. (A) Influenza-specific IgG titers, and (B) influenza-neutralizing antibody titers in young and aged mice infected with 600 PFU of PR8 at 0, 7, 14 and 21 dpi. Data are the mean ± SEM of 4-5 mice/group from a representative experiment of 4 independent experiments. (C) Kinetics of CD38/PNA expression following influenza infection after gating on live CD19+ lymphocytes. The gates delineate the GC B cell population (PNA hi, CD38 lo) with numbers adjacent representing frequency of GC B cells. The data are 5 mice/group from a representative experiment of 3 performed independently. (D) Total number of splenic GC B cells determined using the gating strategy shown in (C). Data are the mean ± SEM of 8-13 mice/group from three independent experiments. (E) Representative half spleen images from young and aged mice 14 dpi, GL7 (green) B220 (magenta) with scale bar at 500μm. GC numbers per half spleen (F) and GC area (G) quantified by confocal microscopy. (H) Correlation of splenic GC number and weight recovery.
at 14 dpi. Data are the mean ± SEM from 3 combined experiments with 3-4 mice per group. (I) Frequency of NP-specific GC B cells (gated as in (C)). Histograms show data of 4-5 mice/group from one representative experiment of two performed independently. (J) Total splenic NP-specific GC B cells determined using the combined gating strategies shown in (C) and (I). Data are the mean ± SEM of 8-13 mice/group from three independent experiments. Number of total (K) or NP-specific (L) GC B cells at 14 dpi in young or aged mice that received/did not receive 3.6x10^7 young CD45.1^+CD4^+ T cells one day prior to infection with PR8, gated on CD45.1- GC B cells as in (C and I). Data are the mean ± SEM of 4-7 mice per group from a representative experiment of 2 performed independently. Statistical significance was determined by (A,B,D,J) two-way ANOVA (Bonferroni’s post tests), (F), (G), Student’s t test, (H) Pearson’s correlation and (K,L) one-way ANOVA (Tukey HSD post tests). *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001.

**Aged influenza specific CD4^+ T cells differentiate into pre-T_{FH} cells**

To determine how the antigen specific CD4^+ T cell response is affected by aging, we used a MHC Class II influenza nucleoprotein (NP)-specific tetramer to track cells in young and aged mice following infection. Figure 4A shows a representative gating strategy for phenotyping young (top row) and aged (bottom row) NP-specific CD4^+ T cells 14 dpi. The kinetics and number of NP-specific CD4^+ T cells accumulating in the spleen of young and aged mice following influenza infection were equivalent (Fig. 4B). Analysis of NP-specific CD4^+ T cells showed that most (~70%) displayed a T_{FH} cell phenotype (CXCR5^{hi} PD-1^{hi}) at 14 dpi (Fig. 4A, red boxes), but also at 7 and 21 dpi (data not shown). The numbers of NP-specific T_{FH} cells were also equivalent in both young and aged mice at all time-points tested (Fig. 4C). Also of note, a higher percentage of NP Tet^{neg} CD44^{lo} CD4^+ T cells in aged mice express PD-1 compared to young mice (Fig. 4A blue box), a feature characteristic of aging cells^{22,23}. Total T_{FH} responses did not follow the trend of the antigen specific response, with aged mice having a higher frequency and number of T_{FH} than young mice before infection, seven days post infection and twenty one days post infection (Fig. 5). Young mice responded to influenza infection with an expansion of T_{FH} that peaked at day 14 post infection (Fig. 5). Aged mice had relatively stable T_{FH} numbers and frequency through the infection (Fig. 5), demonstrating that total response is not representative of the antigen specific response occurring in aged mice.
To confirm the identity of the CXCR5\textsuperscript{hi} PD-1\textsuperscript{hi} NP-specific CD4+ T cells as T\textsubscript{FH} cells, we assessed their expression of Bcl6, the central driver of T\textsubscript{FH} cell differentiation\textsuperscript{180}. Bcl6 expression in both young and aged NP-specific T\textsubscript{FH} cells was compared to the CXCR5\textsuperscript{lo} PD-1\textsuperscript{lo} CD4+ population (Fig. 4D). The Bcl6 mean fluorescence intensities (MFI) of young and aged NP-specific T\textsubscript{FH} cells were not significantly different (Fig. 4E). To further confirm that both young and aged NP-specific CD4+ T cells were differentiating into T\textsubscript{FH} cells, we assessed their ability to produce IL-4 and IL-21, cytokines produced by T\textsubscript{FH} cells that are important for GC generation. Due to the limitation in the number of markers that could be used at once for flow cytometry analyses, and since the great majority of the Ag-specific CD4 T cells displayed T\textsubscript{FH} characteristics (Fig 4A), we considered that the splenic Ag-specific CD4 T cells were T or “T\textsubscript{FH}-committed” cells for the remainder of these studies. In agreement with the differentiation of CD4+ T cells to a T\textsubscript{FH} cell phenotype, both young and aged NP-specific CD4 T cells produced IL-4 (Fig. 4F) and IL-21 (Fig. 4G) in similar proportions. Thus, our data demonstrate that age does not impair the ability of CD4+ T cells to differentiate into pre-T\textsubscript{FH} cells (CXCR5+ Bcl6+ CD4+ T cells)\textsuperscript{6}. Since our previous data using aged TCR transgenic cells showed that aged CD4+ T cells exhibit defective helper function\textsuperscript{17}, we hypothesized that defects in the later stages of T\textsubscript{FH} cell differentiation may be impaired in aged NP-specific CD4+ T cells.
Figure 4. Aged CD4\(^+\) T cells differentiate into pre-T\(_{FH}\) cells. (A) CXCR5/PD-1 expression of young (top row) and aged (bottom row) Tet\(^+\) CD44\(^{hi}\) (red boxes) and Tet\(^{-}\) CD44\(^{lo}\) (blue boxes) cells after gating on live CD4\(^+\) T lymphocytes at 14 dpi. Plots show concatenated data of 5 mice/group from one representative experiment of three performed independently. (B) Total number of splenic NP-specific CD4\(^+\) T cells determined by flow cytometry using the gating strategy shown in (A). Data are the mean ± SEM of 8-13 mice/group pooled from three independent experiments. (C) Total number of splenic Tet\(^+\) CXCR5\(^{hi}\) PD-1\(^{hi}\) in young and aged mice determined by flow cytometry using the gating strategy shown in (A). Data are the mean ± SEM of 8-13 mice/group pooled from three independent experiments. (D) Bcl6 expression by young and aged NP-specific CXCR5\(^{hi}\) PD-1\(^{hi}\) CD4\(^+\) T cells at 14 dpi. Negative expression (dashed line) was determined using young CXCR5\(^{lo}\) PD-1\(^{lo}\) CD4\(^+\) T cells. The histogram represents concatenated data of 5 mice/group from one representative experiment of three performed independently. (E) Bcl6 MFI shown in (D). Frequency of young and aged NP Tet\(^+\) CD4\(^+\) T cells producing (F) IL-4, and (G) IL-21 following PMA and ionomycin stimulation at 7 dpi. The data show the mean ± SEM of 5 mice/group from one representative experiment of two performed independently. Statistical significance was determined by Student’s t test.
Figure 5. Increased number of T follicular helper cells and T follicular regulatory cells in aged mice compared to young mice. Young and aged mice were infected with 600 pfu PR8 influenza virus. (A) Representative flow cytometry plots of T follicular helper cells from non-infected, day 7, day 14 and day 21 post infection were gated on alive, CD19−, CD4+ cells that expressed CXCR5 and PD-1. Total number of T follicular helper cells through the course of influenza infection (B). (C Left) The frequency of T follicular regulatory cells at 14 days post infection, gated as in figure A, with the addition of Foxp3. (C Right) Total number of T follicular regulatory cells in young and aged mice through the course of influenza infection. Data shown are one representative experiment with mean ± SEM of 5-8 mice/group from 3 independent experiments. Statistical significance was determined by two-way ANOVA followed by Bonferroni’s post tests. *, p<0.05; **, p<0.01.

Aged influenza specific CD4+ T cells have impaired differentiation to a mature T<sub>FH</sub> cell phenotype

Following the pre-T<sub>FH</sub> stage, cells upregulate the expression of surface markers involved in T-B interactions, and migrate to the T-B cell border where their initial interaction with B cells occurs. To determine if aged NP-specific T cells express the appropriate cell surface molecules to interact efficiently with B cells, we evaluated expression of ICOS, Ly108, OX40 and CD150 on young and
aged NP-specific T cells (Fig. 6). ICOS and Ly108 surface expression on aged NP-specific CD4+ T cells were significantly lower than on young CD4+ T cells. Conversely, the expression of OX40 on aged NP-specific CD4+ T cells was significantly higher. CD150 expression was similar on both young and aged cells.

These data suggest that aged NP-specific CD4+ T cells may have impaired ability to interact with B cells due to their failed ability to appropriately up-regulate ICOS and Ly108. Since ICOS and Ly108 play an important role in T-B interactions and GC formation\textsuperscript{10,25}, lower expression of these molecules may contribute to the functional defects of aged CD4+ T cells which in turn may contribute to the reduced humoral responses observed in aged mice.
Figure 6. Aged CD4+ T cells fail to progress to fully differentiated T_{FH} cells. (Left) Histograms showing ICOS, Ly108, OX40 and CD150 expression by young and aged NP Tet+ CD4+ T cells at 7 dpi. Negative or baseline expression (dashed line) was determined using the young Tet$^{-}\text{neg}$ CD44$^{\text{lo}}$CD4 T cell population. (Right) MFI of the histograms shown on left. The histograms (Left) show concatenated data, and the graphs (Right) show the mean ± SEM, of 5 mice/group from one representative experiment of two performed independently. Statistical significance was determined by Student’s t test.**, p<0.01 ****, p<0.0001.

The last stage of T_{FH} cell differentiation corresponds to entry into the GC. This is characterized by the expression of GL7, a marker expressed by T_{FH}-committed CD4+ T cells and associated with the mature GC T_{FH} cell phenotype. Considering that the NP+ pre-T_{FH} have altered receptor expression that may impair their interaction with cognate B cells, we expected that the progression of the aged NP-specific CD4+ T cells to the final GC T cell phenotype may also be impaired. We therefore evaluated whether aged T_{FH} cells were progressing normally to the GC T_{FH} cell phenotype by evaluating GL7 expression on these cells. While 34.5% of the young NP-specific CD4+ T cells up-regulated GL7 at 7 dpi, only 19.7% of the aged cells were GL7 positive at that time-point (Fig. 7A). This resulted in a significantly higher number of GC NP-specific CD4+ T cells
in young compared to aged mice which were maintained at 14 dpi (Fig. 7B). To further explore the incomplete differentiation of aged antigen specific T\textsubscript{FH} cells to a GC T\textsubscript{FH} cell phenotype, we analyzed the expression of CXCR5, an important molecule for the migration of pre-T\textsubscript{FH} cells into the GC\textsuperscript{3}. The aged NP-specific T\textsubscript{FH} cells exhibited lower expression of CXCR5 compared to their young counterparts (Fig. 7C), corresponding to a pre-T\textsubscript{FH} cell phenotype\textsuperscript{14}.

Our data thus far indicate that aged influenza specific CD4\textsuperscript{+} T cells differentiate normally to a pre-T\textsubscript{FH} cell phenotype, but less progress to a mature GC phenotype compared to young mice. To visualize this immunological reaction \textit{in situ} we used multi-color confocal microscopy. Due to the limitations of imaging with MHC class II tetramers, we focused on visualizing the total response to influenza infection in young and aged spleens. There was increased GC disorganization, noted by the scattered GL7\textsuperscript{+} areas (Fig. 7D, left panels). The merged images demonstrate that aged mice had notable disruption of their splenic white pulp architecture when compared to young, denoted by the merging of the T and B cell areas (Fig. 7D, right panels). The considerable disruption of the microarchitecture observed in GCs from aged mice could also contribute to the reduced production of a protective humoral response. Next we sought to determine if T\textsubscript{FH} localize to the germinal center comparably in aged and young mice. T\textsubscript{FH} cells were identified by co-localization of Bcl6 and CD4 (Fig. 7E, insert from Fig. 7D middle panel). To quantify GC T\textsubscript{FH} cells, the cells expressing CD4 and Bcl6 in the GL7\textsuperscript{+} areas were counted. Aged mice had fewer T\textsubscript{FH} cells per GC compared to young mice at 14 dpi (Fig. 7F) in concordance with our antigen specific T\textsubscript{FH} flow cytometry data (Fig. 7B). However, since the size of each GC is smaller in area in aged mice when compared to GC in young mice (Fig. 3G), the number of T\textsubscript{FH} per 1000 \( \mu \text{m}^2 \) of GC is not different between young and aged mice (Fig. 7G). From this data we can conclude that the decreased number of T\textsubscript{FH} in the germinal center is a function of the decreased germinal center size, not density.
Figure 7. Decreased germinal center T<sub>FH</sub> cell response to influenza infection in aged mice

(A) GL7 expression on young and aged NP Tet<sup>+</sup> CD4<sup>+</sup> T cells. Negative expression (dashed line) was determined using young NP Tet<sup>-neg</sup> CD44<sup>lo</sup> CD4<sup>+</sup> cells. The histograms show concatenated data of 4-5 mice/group from one representative experiment of 3 performed independently. Numbers in the upper right corners are the frequency of the young (black) and aged (grey) cells in the GL7<sup>+</sup> gate. (B) Total number of splenic NP Tet<sup>+</sup> GL7<sup>+</sup> cells determined by flow cytometry using the gating strategy shown in (A). Data represents the mean ± SEM of 14-18 mice/group from 3 independent experiments. (C) CXCR5 MFI of NP Tet<sup>+</sup> T<sub>FH</sub> cells from young and aged mice at 7 and 14 dpi (using gating strategy from Fig. 2A). Data represents the mean ± SEM of 3-7 mice/group from 3 independent experiments. (D) Representative images of spleens from young (top row) and aged (bottom row) mice 14 dpi stained with GL7 (green) to denote GCs, CD4 (yellow), Bcl6 (red) and B220 (magenta) with scale bars at 80μm. Inserts in the middle panel are magnified in figure (E). (E) T<sub>FH</sub> cells were characterized by co-localization of Bcl6 and CD4 (white). (F) T<sub>FH</sub> cells per GC were enumerated by counting the T<sub>FH</sub> cells in the GL7 area using Imaris software. (G) T<sub>FH</sub> cells per 1000μm<sup>2</sup> GC area were calculated using the GC area (determined by creating an isosurface of GL7 with Imaris) and the number of T<sub>FH</sub> cells per GC. Data are mean ± SEM pooled from three independent experiments with images from 17 mice containing 1-4 GC per image (D, E, F). Statistical significance was determined by Student’s t test. *, p<0.05; **, p<0.01, ***, p<0.001.
Taken together, these data support the hypothesis that the activation and differentiation of the aged NP-specific CD4+ T cells in response to influenza infection is impaired and results in lower numbers of GC T<sub>FH</sub> cells that may contribute to a deficient GC response. We then interrogated the expression of PD-1 on young and aged CD4 T cells during influenza infection since increased PD-1 expression on aged T<sub>FH</sub> was described recently by Sage et al<sup>19</sup>, who suggested that increased PD-1 expression could be responsible for the reduced function of aged T<sub>FH</sub>. The total aged CD4 T cell population (Fig. 8 A,B,C) as well as the total aged T<sub>FH</sub> population (Fig. 8D,E) do indeed express higher levels of PD-1 when compared to young. Importantly, and in contrast to the report by Sage et al, there is no difference in PD-1 expression on NP-specific T<sub>FH</sub> from young and aged mice at 7 dpi (Fig. 8F) and by 14 dpi, young T<sub>FH</sub> express higher levels when compared to aged (Fig. 8G). Thus, in our influenza model, increased expression of PD-1 by aged Ag-specific T<sub>FH</sub> cannot account for their reduced function.
Figure 8. Differential expression of PD-1 on young and aged T follicular helper cells. Young and aged mice were infected with 600 pfu PR8 influenza virus and the splenic response was determined at 7 and 14 days post infection. PD-1 MFI of total CD4+ T cells (gated on lymphocytes, single cells, alive, CD4+) of A) naïve mice, B) 7 days post infection and C) 14 days post infection. The PD-1 MFI of T follicular helper cells gated as described in Fig 5. was determined in D) 7 days post infection and E)14 days post infection. The expression of PD-1 on NP Tetramer+ T follicular helper cells (as gated in Fig. 2) was determined F) 7 days post infection and G)14 days post infection. Data shown are one representative experiment with mean ± SEM of 3-8 mice/group from 2 independent experiments. Statistical significance was determined by the Mann-Whitney Test.*, p<0.05,**, p<0.01.
NP-specific CD4⁺ T cells from aged mice have a regulatory-like phenotype

Since the progression of CD4⁺ T cells into fully differentiated GC T<sub>FH</sub> cells is impaired in aged mice, we hypothesized that their B cell helper activity would be affected. In the next series of experiments, we evaluated the IL-2, IL-10 and IFNγ production by young and aged NP-specific CD4⁺ T cells by flow cytometry. Consistent with previous reports<sup>26</sup>, the proportion of aged NP-specific CD4⁺ T cells producing IL-2 was significantly lower than that of young cells (Fig. 9A). Interestingly, a higher frequency of aged cells produced IL-10 (Fig. 9B) and IFNγ (Fig. 9C) than young cells. The dysregulated cytokine production by aged NP-specific CD4⁺ T cells could indicate an inappropriate effector differentiation program that might impact helper activity of these cells, and therefore, the formation of GCs.
Figure 9. NP-specific CD4+ T cells produce cytokines indicative of regulatory T cells. Intracellular cytokine staining of young and aged NP Tet+ CD4+ T cells harvested 7 dpi and stimulated with PMA/ionomycin. The graphs show the mean ± SEM of the frequency of NP-specific CD4+ T cells producing: (A) IL-2, (B) IL-10, and (C) IFNγ. The data are from 5 mice/group of one representative experiment of two performed independently. Statistical significance was determined by Student’s t test. ***, p<0.001; ****, p<0.0001.

Reduced IL-2 production combined with increased IL-10 production is reminiscent of a regulatory T cell phenotype and it is reported that the frequency of regulatory T cells increases with aging. Upon examination of total Foxp3+ CD4+ T Cells our data also showed increased frequency of regulatory T cells in aged mice at all time points test (Fig. 10A,B). Total number of regulatory T cells was only significantly higher in aged mice at steady state and seven days post influenza infection (Fig. 10C). To determine if more aged NP-specific CD4+ T cells exhibited regulatory T
cell properties, we evaluated Foxp3 expression in young and aged influenza infected mice. At 7 dpi, the number of NP-specific CD4+ T cells expressing Foxp3 was not significantly different in young and aged groups (Fig. 10D). By 14 dpi however, the number of NP-specific CD4+ T cells expressing Foxp3 was significantly higher in aged compared to young mice (Fig. 10E). Recently, a subset of regulatory T cells that specifically control GC B cell and GC T\textsubscript{FH} cell responses, T follicular regulatory (T\textsubscript{FR}) cells, was characterized. T\textsubscript{FR} cells are identified by expression of CD4, PD-1, CXCR5, Foxp3 and Bcl6\textsuperscript{31-33}. At 7 dpi there was no difference in NP-specific T\textsubscript{FR} cell formation (Fig. 10F), but at the peak of the GC response (14 dpi) aged mice had increased numbers of NP-specific T\textsubscript{FR} cells (Fig. 10G). Total T\textsubscript{FR} cell number was also increased aged mice compared to young mice at all time points post infection (Fig. 5). The increase in NP-specific T\textsubscript{FR} cells and regulatory T cells may contribute to the poor GC T\textsubscript{FH} cell and humoral response observed in aged mice after influenza infection. In order to further understand how aging impacts regulatory cells, we next explored the contribution of the aged environment to their formation.
Figure 10. T regulatory and T follicular regulatory cells accumulate in aged mice after influenza infection (A) Representative histograms of total CD4+ T cells expressing Foxp3 in young and aged mice. Frequency (B) and number (C) of total CD4+ T cells that express Foxp3 in spleens from young and aged mice before influenza infection, 7dpi and 14dpi. Number of NP-specific CD4+ T cells expressing Foxp3 in the spleen of young and aged mice at (D) 7 dpi and (E) 14 dpi. Number of NP-specific CD4+ CXCR5hi PD-1hi Bcl6+ Foxp3+ cells in spleens of young and aged mice at (F) 7 dpi and (G) 14 dpi. Data are the mean ± SEM of 3-10 mice per group from a representative experiment of 3 performed independently. Statistical significance was determined by Two way ANOVA with LSD post hoc (B,C) or Student’s t test (D,E,F,G). *, p<0.05, p<0.001.

Impact of the aged environment on regulatory T cells

To assess whether the accumulation of regulatory T cells in aged mice was cell intrinsic or if the aged environment contributed to their accumulation, we performed an adoptive transfer experiment. Young and aged mice received young polyclonal CD45.1+ CD4+ T cells one day prior to influenza infection. We employed a polyclonal model for this experiment since T FR cells are thymically derived and are not present in TCR transgenic populations such as the OT-II model31. The phenotype of the young CD4+ transferred T cells was analyzed by flow cytometry 14dpi. A greater frequency of donor T cells in aged mice expressed Foxp3 when compared to donor cells in young mice (Fig. 11A). This was complemented by the increased regulatory to effector ratio of young CD4+ transferred cells in aged hosts (Fig. 11B). Examination of donor CD4+ T cells
expressing a T<sub>FR</sub> phenotype (Bcl6<sup>+</sup> and Foxp3<sup>+</sup>) showed no difference in frequency in young or aged hosts (Fig. 11C). Although the factors that promote T<sub>FR</sub> cell development are still being elucidated, TGF-β1 is a known inducer/stabilizer of Foxp3 expression<sup>34</sup>. There was a significantly higher level of active TGF-β1 in the spleens of aged mice compared to young mice at 14 dpi (Fig. 11D), which may contribute to accumulation of regulatory T cells in aged hosts. To determine whether the concentration of active TGF-β1 in the aged spleens was sufficient to promote regulatory T cell development, we performed an in vitro assay where young CD4<sup>+</sup>CD25<sup>-</sup> T cells were cultured with either a standard concentration (5000 pg/ml) of TGF-β1 to polarize T cells to regulatory cells<sup>35</sup>, the concentration of TGF-β1 in aged spleens (100pg/ml) or no TGF-β1. TGF-β1 at 100pg/ml converted CD4<sup>+</sup>T cells to regulatory T cells at a similar frequency (7%) to the in vivo transfer experiments (Fig. 11E), suggesting that TGF-β1 in the aged splenic environment can drive formation of regulatory T cells. These data suggest that there are both CD4<sup>+</sup> T cell intrinsic and extrinsic factors that contribute to the regulatory environment of aged mice, which may negatively contribute to T<sub>FH</sub> cell function and, in turn, dampen the humoral response in aged mice during influenza infection.
Figure 11. More young CD4+ T cells transferred into aged host express Foxp3 compared to young hosts. Purified young CD45.1+CD4+ polyclonal T cells were transferred via tail vein injection into young or aged hosts at 3.6x10^7 cells per mouse. One day post transfer, recipient mice were infected with 600PFU PR8 influenza and subsequently sacrificed for analysis 14 days later. (A) Foxp3 expression of CD4+ transferred cells in young or aged hosts. (B) Regulatory to effector ratio of CD4+ transferred cells in young or aged host, calculated by the percent of Foxp3+ donor CD4+ cells. (C) CD4+, CXCR5hi, PD-1hi, Bcl6+ transferred cells expressing Foxp3 in young or aged host. (D) Active TGFβ-1 levels in spleen supernatants from the mice who received transferred cells described above. Data are the mean ± SEM of 4-7 mice per group from a representative experiment of 3 performed independently. Statistical significance was determined by Student’s t test (A,B,C,D). *, p<0.05.***, p<0.001. (E) CD4+CD25- T Cells from young mice were cultured with 5000 pg/ml (standard concentration for making iTregs 35), 100 pg/ml (concentration in aged spleens) or 0pg/ml TGF-β1. Combined results from three independent experiments. Statistical significance was determined using repeated measures ANOVA with Sidak post hoc test. *p<0.05.
DISCUSSION

Aging is associated with a decline in immune system function, including reduced humoral responses. Here we show that low influenza-specific antibody titers found in aged mice following influenza infection are associated with decreased numbers of total and NP-specific GC B cells and decreased GC size, number and responses compared to young mice. We have also shown that defects in total and NP-specific GC B cell numbers can be overcome by the addition of young CD4⁺ T cells. This suggests that B cell intrinsic defects are likely insufficient to explain the extent of the GC impairment and that other factors, such as age-related changed in TFH cells, may contribute to these defects.

In previous studies, we have shown that aged CD4⁺ T cells acquire intrinsic defects that impair their helper functions. These conclusions were drawn following the observation that aged TCR transgenic CD4⁺ T cells transferred into young CD4 KO mice support a weaker GC response when compared to young CD4⁺ T cells. This age-associated functional defect was correlated with impaired CD154 expression. Moreover, we have demonstrated that the aged micro-environment also contributes to reduced helper function of CD4⁺ T cells using an adoptive transfer model. In these experiments, young CD4⁺ T cells displayed delayed activation and expansion, as well as reduced differentiation to a TFH cell phenotype, when transferred into aged hosts when compared to young hosts. This correlated with the impaired accumulation of the young donor cells in the T cell areas of the spleen in aged mice, a phenomenon likely related to the reduced expression of CCL19 and CCL21 in this organ. These experiments allowed us to determine the contribution of the age-associated defects intrinsic and extrinsic to the CD4⁺ T cell compartment in the impaired GC response observed in aged mice.
Results presented herein provide evidence that endogenous, aged antigen-specific CD4+ T cells exhibit a defect in their ability to fully differentiate to a GC T_{FH} cell phenotype in response to influenza infection. While the same number of NP-specific CD4+ T cells acquired pre-T_{FH} characteristics (CXCR5+ PD-1+ Bcl6+) in young and aged mice, aged T_{FH} cells express lower levels of ICOS and Ly108 and fewer expressed GL7, a marker for GC T_{FH} cells. Importantly, this defect in T_{FH} cell differentiation is associated with impaired generation of NP-specific GC B cells. GL7 is a molecule readily expressed by activated NP-specific CD4+ T cells and maintenance of its expression in GC T_{FH} cells depends on the establishment of cognate T-B interactions. Thus, we believe that the low frequency of aged NP-specific CD4+ T cells expressing GL7 is indicative of inadequate T-B cell interactions.

The hypothesis that aging negatively impacts T-B cell interactions is supported by several observations. First, the reduced number of NP-specific B cells in the spleen of aged mice likely contributes to the impaired GL7 expression. Second, this could be the result of impaired recruitment of T cells to B cell follicles. Our current findings are in agreement with our previous work showing that the architecture of B cell follicles in aged mice spleens was disorganized. Moreover, the amount of CCR7 ligands CCL19 and CCL21 were significantly lower, while the CXCR5 ligand CXCL13 was more abundant in aged compared to young mouse spleens. These signals may contribute to smaller GCs found in aged mice that have fewer T_{FH} cells than their young counterparts. Because of the imbalance of chemotactic signals and the disorganization of the splenic white pulp, the ability of aged T_{FH} cells to find and interact with their cognate B cells is likely compromised in aged mice.

Furthermore, we observed that expression of the co-stimulatory molecule ICOS was significantly reduced on aged compared to young NP-specific CD4+ T cells. A recent study by Xu and
colleagues showed that ICOS participates in CD4+ T cell localization within B cell follicles in an ICOS ligand-independent manner15. A lower expression of ICOS on \( T_{FH} \) cells could reduce their ability to migrate within the follicles and interact with B cells. In concordance with these results, we also showed that NP-specific \( T_{FH} \) cells have lower expression of CXCR5, another important molecule for homing of \( T_{FH} \) cells to B cell follicles3. ICOS has a central role in multiple steps of the GC reaction, and ICOS deficient CD4+ T cells are unable to differentiate to a \( T_{FH} \) cell phenotype10. The equivalent generation of NP-specific \( T_{FH} \) cells in both young and aged mice, albeit with the lower expression of ICOS by the aged cells, suggests that this level of ICOS expression is sufficient for initial \( T_{FH} \) cell differentiation, but not for full differentiation to a GC \( T_{FH} \) cell phenotype.

ICOS signaling also contributes to the GC response by inducing the expression of other co-stimulatory molecules such as CD40L38-40 and influencing cytokine production by T cells38-40 and GC B cells41. Ly108 has also been identified as a major regulator of T-B cell interactions25 through both positive and negative signals42. The reduced expression of both ICOS and Ly108 by the aged NP-specific CD4+ T cells could therefore have a major impact on the stability of the T-B cell interactions and the subsequent development of GCs.

Finally, we observed that a greater number of NP-specific CD4+ T cells express the transcription factor Foxp3 in aged compared to young mice, with fewer aged cells producing IL-2 and more cells producing IL-10. Increased regulatory T cell frequency and/or number in aged mice have been reported by multiple groups27-30 and this increase has also been linked to reduced immune function27-30. Interestingly, the proportion of GL7+ GC \( T_{FH} \) cells is increased in mice deficient in regulatory T cells 43. \( T_{FR} \) cells are important suppressors of GC B cell and \( T_{FH} \) cell responses that are derived from thymic regulatory T cells31-33. The increase in Foxp3+ regulatory T cells found in aged mice could contribute to the increased number of total and antigen specific \( T_{FR} \) cells found in aged mice. Sage et al. in a recent report demonstrated that young and aged \( T_{FR} \) cells have
equal suppressive capacity. Therefore, T_{FR} cell from aged mice could contribute to reduced GC B cell and GC T_{FH} cell generation after infection. We have also demonstrated that the aged environment is conducive to regulatory T cell development, in part due to increased levels of active TGF-β1, a known inducer of Foxp3 expression\(^{34}\). Future studies will address the cellular source of TGF-β in aged mice.

In conclusion, we show that an appropriate number of NP-specific CD4\(^+\) T cells develop to pre-T_{FH} cell stage in aged mice. However, these cells are unable to fully progress to the GC T_{FH} phenotype (GL7\(^+\) T_{FH} cells)\(^{14}\), and sustain the development of an efficient GC response when compared to the young NP-specific T_{FH} cells. Thus, we propose that inadequate T-B cell interactions, as well as increased regulatory T cell number in aged mice contribute to age-related impairments in humoral responses to influenza infection (Fig. 12).
ACKNOWLEDGEMENTS

We gratefully acknowledge Amanda Buck, Ron LaCourse, Zhijuan Qiu, Erica Lorenzo, Jenna Bartley, Sandra Jastrzebski, Judith Kalinowski, Spencer Keilich for their technical support and help with the manuscript preparation. We would also like to acknowledge Evan Jellison and the UCONN Health Flow Cytometry core along with Susan Krueger and the UCONN Health Cell
Analysis and Modeling Microscopy Facility for their technical assistance and resources. The authors declare no competing financial interests. This study was supported by National Institute of Aging Program Grant #AG021600 and the UCONN Center on Aging. J.S. Lefebvre is the recipient of a postdoctoral fellowship provided jointly by the Fonds de recherche du Québec-Santé/Réseau québécois de recherche sur le vieillissement/National Institute of Aging.

REFERENCES


Eaton, S. M., Burns, E. M., Kusser, K., Randall, T. D. & Haynes, L. Age-related defects in CD4 T cell cognate helper function lead to reductions in humoral responses


26 Haynes, L., Linton, P. J., Eaton, S. M., Tonkonogy, S. L. & Swain, S. L. Interleukin 2, but not other common gamma chain-binding cytokines, can reverse the defect in generation


CHAPTER 3
Attrition of fibroblastic reticular cell number and function in aged spleens

ABSTRACT
Aging has a profound impact on multiple facets of the immune system culminating in aberrant functionality. The architectural disorganization of splenic white pulp is a hallmark of the aging spleen, yet the factors underlying these structural changes are unclear. Fibroblastic reticular cells (FRCs) are one stromal cell subset in the spleen important for maintenance of architectural organization, yet it remains to be determined how aging impacts these cells. In this study, we sought to determine how aging impacts splenic FRC numbers, morphology and function. Using a mouse model of aging, we found that aged naïve spleens have fewer FRCs than young spleens. This reduction in FRC number correlated with reduced CCL19 and CCL21 concentrations in aged spleens, which may contribute to impaired homing of T cells. CCL21 in both young and aged spleens localized with FRCs. Aged FRCs extended marginally into B cell follicles, and may contribute to the blending of the T cell zone and B cell follicles in aged spleens. The described age-related changes in FRCs number and function may be an underlying factor contributing to impaired immune system function with age.

INTRODUCTION
Organization is a frequently overlooked component of an effective immune response\(^1\). The likelihood of low frequency antigen-specific T cells\(^2\)–\(^4\) interacting with the antigen presenting cell displaying their cognate antigen is amplified by multiple layers of organization, including secondary lymphoid organs. The spleen is one such organ located in the upper right portion of the abdomen\(^5\). The spleen is attached to the vasculature\(^6\) and is an important defense against blood-borne pathogens like encapsulated bacteria\(^7\),\(^8\). Blood enters into the spleen through blunt
ended central arterioles which empty into the marginal sinus surrounding the splenic white pulp. From the marginal sinus, immune cells can use bridging channels to enter into the T cell zone. The T cell zone is surrounded by highly segregated B cell follicles, in which germinal centers can develop and promote the production of high affinity class-switched antibody responses.

Non-hematopoietic stromal cells provide the framework and directional cues to optimize the organization of immune cells in the splenic white pulp. Fibroblastic reticular cells (FRCs) are the most abundant variety of stromal cell in the splenic white pulp. These cells form a lattice-like conduit network in the bridging channels and T cell zone upon which antigen, lymphocytes and dendritic cells traffic. FRCs produce homeostatic chemokines, CCL19 and CCL21 which bind to CCR7 expressing cells to direct them into the T cell zone. FRCs are identified by their expression of the mucin podoplanin (PDPN), lack of expression of the vasculature marker CD31, and lack of hematopoietic lineage maker CD45. A specialized subset for FRCs called follicular dendritic cells (FDCs) are located in the B cell follicles. FDCs produce the chemokine CXCL13 which interacts with CXCR5 on B cells and T follicular helper cells to direct them to the B cell follicle. FDCs also trap antigen and provide cues to help B cells in the production of high affinity antibodies.

It is well established that aging leads to disorganized splenic architecture, characterized by merging of cells in the B cell follicles and T cell zone. Yet it is less clear how aging impacts the stromal cells underlying this organization. While several studies have described age related attrition of FDC function and morphology, it largely remains to be determined how aging impacts splenic FRC. In this study, we sought to characterize how aging impacts the number, morphology and function of splenic FRCs to further our understanding of the aging immune system.
Our results show that aged spleens have fewer FRCs and a decreased T cell zone FRC area. Concomitant with the reduction of FRCs number, homeostatic chemokine levels are reduced in aged spleens correlating with impaired recruitment of young T cells. Interestingly, CCL21 largely co-localizes with FRCs and appears to extend slightly into the B cell follicles in aged but not young spleens. Reduction of FRC number and homeostatic chemokine concentrations may contribute to altered splenic architecture and impaired T cell homing found in aged spleens.

MATERIALS AND METHODS

Mice
Aged (18-20 months) male C57BL/6J mice were obtained from the National Institute of Aging (NIA) aging colony at Charles River Laboratories. Young male C57BL/6J mice (2-4 months) were obtained from Jackson Laboratories. All mice were kept under specific pathogen free conditions in sterilized, individually ventilated, HEPA-filtered cages. Mice were sacrificed by CO₂ asphyxiation. The Institutional Animal Care and Use Committee at UConn Health reviewed and approved all experimental procedures using animals.

ELISA
Spleens were harvested, flash frozen and stored at -80°C. Spleens were homogenized in 1ml of TPER (Thermofisher) with 5μM EDTA (Invitrogen) and 1x HALT protease and phosphatase inhibitor cocktail (Thermofisher). Homogenates were centrifuged at 1000g for 10 minutes and supernatants were aliquoted and frozen at -80°C until analysis. CCL21 was measured using mouse CCL21/6kine DuoSet ELISA (R&D Systems). CCL19 was measured using mouse CCL19/MIP-3 beta DuoSet ELISA (R&D Systems). Chemokine concentrations were normalized to total protein concentration determined using Pierce BCA Protein Assay (Thermofisher).
**T cell Transfer**

CD8+ T cells were isolated from pooled spleens and lymph nodes of CD45.1+ F5 RAG KO mice using MojoSort™ Mouse CD8 T cell isolation kit (Biolegend) according to the manufacture’s instructions. Before transfer, the purity of the isolated CD8+ T cells was confirmed at 85% or above. 2.3x10^6 CD8+ T cells in 200µl PBS were tail vein injected into each mouse, which were sacrificed exactly 30 minutes after transfer for splenic removal.

**Immunofluorescence**

Spleens were fixed overnight in medium containing 0.05 M phosphate buffer, 0.1 M l-lysine, pH 7.4, 2 mg/ml NaIO4, and 10 mg/ml paraformaldehyde then dehydrated with 30% sucrose in phosphate buffer. Spleens were frozen in Tissue-Tek O.C.T. compound (Sakura Finetek) and 20µm sections were cut using a CM1850 cryostat (Leica). Sections were blocked for 30 minutes with 2% goat serum, stained overnight at 4°C in a humidified chamber with primary antibody cocktail (anti- PDPN clone 8.1.1 unconjugated (Origene) 1:300 dilution, anti-B220 PE-Dazzle AF594 clone RA3-6BP (Biolegend) 1:100 dilution, anti-CD31 AF647 clone MEC13.3 (Biolegend) 1:100 dilution, anti-CD8α BV421 clone 53.6.7 (Biolegend) 1:100 dilution in 2% goat serum, washed in PBS then stained for 2 hours at room temperature with secondary antibodies (goat anti-hamster AF488 (Invitrogen) 1:400 dilution). For detection of CCL21, sections were blocked as described and stained overnight with biotinylated anti-CCL21 antibody (R&D) (1:20 dilution) in 2% goat serum with 0.3% Triton X-100 (Sigma). After PBS washes, CCL21 signal was amplified using Alexa Fluor™ 594 Tyramide SuperBoost™ Kit, streptavidin (ThermoFisher Scientific) according to manufacturer’s instructions. After staining, slides were mounted with Immu-Mount (Thermo Scientific) and cover-slipped. Images were acquired using the Zeiss 780 laser scanning microscope (Carl Zeiss; air objective 20x Plan-Apochromat with NA 0.5 or water objective 10x C-Apochromat NA 0.45) using multichannel frame scans. The ZEN Black software (Carl Zeiss) was used for image acquisition. For full spleen images, the ZEN Black tile scan function was used to
stitch individual 10x images together. PDPN area was quantified by creating an isosurface of the staining of 20x images and image processing was performed using Imaris 8.1 software (BITPLANE).

**Splenic Stromal Cell Digestion/Flow Cytometry**

To quantify the number of stromal cells in the spleen by flow cytometry, spleens were cut into 2.5mm pieces then enzymatically and mechanically digested as previously described\(^\text{37}\). The chopped spleens were placed in 2 ml of enzyme mix composed of RPMI with 0.2mg/ml of Collagenase P (Roche), 0.8mg/ml Dispase II (Roche) and 0.1mg/ml DNAse 1 (Sigma) and incubated for 20 minutes in a 37°C water bath (inverting every 5 minutes). The chopped spleens were gently pipetted up and down three times using a 1 ml pipette tip. The fragments were allowed to settle for 30 seconds and the supernatant containing the cells released by digestion were collected into a tube containing 10 ml of ice cold FACS buffer made from 2% FCS, 5mM EDTA and PBS and centrifuged for 4 minutes at 300g and 4°C. Two ml of freshly prepared enzyme mix was added to the pellet and incubated for an additional 10 minutes in the 37°C water bath. The spleens were then vigorously pipetted for 30 seconds using a 1 ml pipette tip. Fragments were again allowed to settle for 30 seconds and the digested cells in the supernatant were added to 10 ml of FACS buffer and centrifuged. Two ml of new enzyme mix was added to the pellet and then incubated in the 37°C water bath. Every five minutes until the spleens were completely digested (no visible aggregates), the cells were vigorously pipetted for 30 seconds. On average, the aged spleens took about 5 to 10 minutes longer to digest than the young spleens. Red blood cells were lysed for five mins at RT in ACK lysis buffer (ThermoFisher Scientific). After digestion cell number and viability were quantified using the Cellometer Auto2000 (Nexcelom Biosciences) with AOPI. Five million viable splenocytes were stained for flow cytometric quantification of splenic stromal cell populations. Cells were stained in an antibody cocktail of 100µl in FACS buffer (anti-
CD16/CD32 clone 93 Fc block unconjugated (ThermoFisher), anti-PDPN Alexa Fluor 594 clone 8.1.1 (Biolegend), anti-CD45 PerCPcy5.5 clone 30F-11 (Biolegend), anti-CD31 APC clone MEC13.3 (Biolegend), Carboxylic Acid Succinimidyyl Ester AlexaFluor 350 (Life Technologies). Fluorescence minus one controls were created for each antibody and used to set gates for analysis. Without fixation, samples were acquired immediately on an LSRII flow cytometer (BD). Flow cytometry data analysis was performed using Flowjo software (Tree Star).

**Statistical Analysis**

Data was tested for normality using the F test. For normally distributed data an unpaired two tailed T test was used to determine significance. For data with unequal variances, analysis was performed using the Mann-Whitney Test. Significance was set at p<0.05. Statistical analysis was performed using GraphPad Prisim 6 software.

**RESULTS**

**Reduction of FRC numbers in aged naïve spleens**

Despite the well characterized disruption of splenic white pulp architecture with age, it is unclear how aging impacts splenic stromal cell numbers and function. We first sought to quantify the number of stromal cells in both young and aged spleens. Using a rigorous mechanical and enzymatic digestion, stromal cells were isolated out of spleens and quantified using flow cytometry. No significant differences were found in either the total number of cells (Fig. 13A) or CD45- stromal cells (Fig. 13C) in young and aged spleens. Interestingly, aged spleens had significantly fewer FRCs (Fig. 13D) as gated in Figure 13B. Although flow cytometry is a powerful tool, digestion of stromal cells from spleens is an imperfect method and may underrepresent the true number of cells present. To further validate the reduction of FRC numbers in aged spleens quantitative confocal microscopy was employed.
Figure 13. Reduction of fibroblastic reticular cell number in aged spleens. Spleens were mechanically and enzymatically digested to liberate stromal cells for flow cytometric quantification. A. Total number of nucleated cells in young and aged spleens. B. For quantification of total splenic stromal cells by flow, samples were gated on alive, single cells that were CD45- (left). FRCs were further gated as PDPN+ CD31- (right). C. Number of CD45- cells in young and aged spleens. D. Number of FRCs in young and aged spleens. Statistical significance was determined by two tailed T test A,D or Mann-Whitney Test C. *p<0.05. Error bars =±SEM. Data are combined from three independent experiments n=7-8 mice per group.

T cell zone FRCs were identified by their expression of PDPN (Fig. 14A left and their localization under CD8+ T cells and surrounding B cells (Fig. 14A right). A digital isosurface rendering of the PDPN staining was created using Imaris Imaging software (Fig. 14A middle). Quantification of the PDPN isosurface revealed that the area of aged splenic FRCs in the white pulp were smaller than their young counterparts (Fig. 14B), confirming flow cytometric quantification. Full spleen images (Fig. 14C) provide further support to the reduction of FRC area in aged spleens.
Figure 14. Smaller T cell zone FRC areas in aged spleens. A. Image analysis pipeline for splenic FRC quantification. Representative images from Young (top) and aged (bottom) splenic white pulp. FRCs were identified by expression of PDPN (left). A digital rendering of the PDPN staining was created using Imaris isosurface function (middle). Combined image of CD8α (yellow), B220 (blue), CD31 (red), PDPN (green) (right). Scale bar= 50µm. B. PDPN area quantification was accomplished by averaging the area of four different white pulp 20x z stack images (1µm step size, four steps) per mouse (6 mice per group). C. Longitudinal images of entire spleens from naïve young (top) and aged (bottom) mice. Scale bar =200µm. Data are representative of n=6 mice per group. Statistical significance was determined by two tailed T-test **p<0.01. Error bars =+SEM.
**Attrition of aged splenic FRC function**

FRC produced homeostatic chemokines CCL19 and CCL21 are important cues directing the localization and entry of immune cells into the T cell zone of the splenic white pulp\(^{18-20}\). It has been previously reported that young CD4+ T cells have reduced recruitment into aged spleens compared to young spleens after antigen challenge\(^{27,38}\). In order to determine the impact of aging on CD8+ T cell recruitment, we transferred young T cells into young and aged hosts and examined homing after 30 minutes, a time point where exit was unlikely\(^7\) (Fig. 15A, B). We found a similar reduction in the frequency (Fig. 15C) and number (Fig. 15D) of young CD8+ T cells transferred into aged mice when compared to young mice, but it remains unclear how aging impacts the concentrations and localization of homeostatic chemokines, important for recruitment of T cells into the spleen. Quantification of CCL21 (Fig. 15E) and CCL19 (Fig. 15F) protein levels from homogenized spleens revealed a reduction in the concentrations of both chemokines in aged spleens compared to young. Thus, the reduced homeostatic chemokine concentrations in aged spleens may contribute to the impaired recruitment of young CD8+ T cells from the vasculature into aged spleens.
Figure 15. Reduced homeostatic chemokines in aged spleens correlate with impaired recruitment of young T cells. The ability of aged and young spleens to recruit young T cells was tested using the experimental design outlined in A. B. Transferred young T cells were identified in young or aged spleens by gating on lymphocytes, single cells, viable cells, CD45+, and CD8α+CD45.1+. C. Frequency and D. number of young transferred CD8+ T cells in young or aged spleens. E. CCL21 and F. CCL19 levels were quantified in homogenized naïve spleens and normalized to total protein. B, C, D Data are one representative experiment of three performed independently, n=4-5 mice per group. E, F Data are pooled from two independent experiments, n=18-20 mice per group. Statistical significance was determined by two tailed T-test *p<0.05, ****p< 0.0001. Error bars =±SEM.
We have previously showed that in aged spleens after challenge with OVA protein and alum adjuvant, CCL21 localization is altered; spreading from the T cell zone into the B cell follicles\textsuperscript{27}. Since antigenic challenge has been shown to dramatically alter homeostatic chemokine concentration and localization\textsuperscript{39}, we next sought to determine where CCL21 localized in young and aged spleens at homeostasis. Due to low concentrations, CCL19 was not detectable by microscopy in young or aged spleens, even with the use of amplification reagents (data not shown). CCL21 in both young and aged spleens localized with PDPN+ T cell zone FRCs (Fig 16. A,C). Aged FRCs appeared to extend marginally into the B cell follicles, where there was less overlap in young spleens (Fig. 16 A,C). Of note, despite blocking some nonspecific punctate background staining for CCL21 was found in the splenic red pulp surrounding the B cell follicles (Fig. 16B). These data suggest that aging reduces FRC produced homeostatic chemokine concentration, which localize near FRCs, and may contribute to the reduced recruitment of young T cells into aged spleens.
Figure 16. CCL21 localizes to T cell zone FRCs in young and aged spleens. A. Representative images of young (top) and aged (bottom) splenic white pulp, showing the localization of CCL21 to the T cell zone, where FRCs localize. B. Negative controls for PDPN and CCL21 staining. Insert in A is magnified in C. and co-localization of CCL21 and PDPN is shown in white (far right). Representative images from two independent experiments with n=3 mice per group. Scale bar=50µm

DISCUSSION

Aging is a multifactorial process that has profound effects on immune system function. Despite the extensive characterization of the aging immune system, many facets have yet to be rigorously cataloged and described. Several reports have implied that the “environment” of aged secondary
lymphoid organs hinders the development of robust T cell responses\textsuperscript{31,38,40,41}. We have previously reported that young ovalbumin-specific CD4+ T cells transferred into aged hosts have delayed proliferation, reduced expansion and impaired differentiation into T follicular helper cells compared to those transferred into young hosts, following OVA immunization\textsuperscript{27}. In the aged hosts, the donor cells had impaired localization to the splenic T cell zones of the white pulp 18 hours after immunization. This was in part due to altered localization of CCL21\textsuperscript{27}. Although this report hinted at FRC dysfunction, it was not directly examined. We also found that aged splenic environment is rich in active TGF-\(\beta\) and promotes the differentiation of young CD4+ T cells into of regulatory T cells, which may contribute to impaired T cell and B cell immunity\textsuperscript{31}.

Studies have shown similar findings for young CD8+ T cells transferred into aged mice. Young influenza specific Clone 4 CD8+ T cells, when transferred into young or aged hosts that had been intravenously infected with influenza virus, had reduced expansion and decreased interferon gamma production in aged spleens\textsuperscript{42}. Another study found that after intravenous \textit{Listeria} infection, young CD8+ T cells transferred into aged mice had reduced expansion in the spleen. This study linked poor CD8+ T cell responses to reduced CD8\(\alpha\) dendritic cell responses, and did not consider the implications of the disorganized splenic architecture or stromal cell dysfunction\textsuperscript{40},

In this current study, we sought to directly examine how aging impacts one component of the splenic microenvironment, stromal cells. The disorganized architecture of the splenic white pulp lead us to hypothesize that aging may influence FRC number and function, due to their important role orchestration of splenic architecture\textsuperscript{15,18}.

We found that aged spleens had fewer FRCs, which results in smaller FRC area in the T cell zone of the splenic white pulp. We also described how aged spleens have decreased concentrations of homeostatic chemokines CCL19 and CCL21 which is corroborated by previous studies\textsuperscript{27}. We
found that CCL21 localizes near FRCs which extend marginally into the B cell follicles of the aged splenic white pulp and may contribute to architectural disruption. CCL21 localization in aged spleens at homeostasis did not appear to be as dramatically altered as previously reported after antigenic challenge, which may correlate with more dramatic changes in architecture after challenge. We also found that decreased homeostatic chemokine concentrations also correlated with reduced homing of young T cells into aged compared to young spleens. Along with reductions in CCL19 and CCL21 concentrations, other alterations in aged spleens, like changes to splenic arteries may contribute to T cell homing defects, and remain to be examined.

FRCs are functionally important for the organization of the splenic white pulp, but are most likely only one component contributing to the age related changes in the spleen. Changes in chemokine receptor expression on immune cells and their ability to respond to chemotactic gradients has also been observed and may add to altered architecture. The mechanism behind why there are fewer FRCs in aged spleens also remains to be determined. One possible explanation may be that there are interruptions to important maintenance signals via lymphotoxin beta receptor. If aging impacts lymphotoxin beta-receptor expression on stromal cells or lymphotoxin beta expression on immune cells remains to be determined.

Apart from their role in architectural organization, recent studies have elucidated roles for fibroblastic reticular cell in the control, survival and initiation of T cell responses. It remains to be determined if aging impacts any of these functional attributes of FRCs. Whether lymph node stromal cells experience similar changes to splenic stromal cells also remains unclear. On a broader scale, it also remains to be determined if splenic and lymph node fibroblastic reticular cells are of similar origin and composition. Erosion of secondary lymphoid organ stromal cell function may have far-reaching effects on multiple facets of the aged immune
system and, despite the advances make in this manuscript, we are still in the infancy of understanding how aging impacts these cells.

ACKNOWLEDGMENTS

We would like to acknowledge Jenna Bartley, Erica Lorenzo, Jacob Hopkins, Spencer Keilich, Jacob Hopkins, Judith Kalinowski, and Sandra Jastrzebski for their technical assistance with experiments. We would also like to acknowledge Linda Cauley for supplying the F5 RAG KO mice.

REFERENCES


CHAPTER 4

Assessment of lymph node stromal cells as an underlying factor in age related immune impairment to influenza infection

ABSTRACT

It is well established that aging negatively impacts immunity, resulting in inefficient responses to vaccinations and infections, such as influenza. Fibroblastic reticular cells (FRCs) are the major stromal cell subset in lymph nodes (LN) and play an intricate role in the orchestration and control of adaptive immune responses. Although stromal cells have a major impact on immune responses, the impact of aging on LN stromal cells has yet to be determined. We sought to determine how aging impacts FRC number, phenotype and morphology at steady state and after influenza infection. Quantitative analysis of lymph node stromal cells by flow cytometry revealed that there are no significant differences in the number stromal cells in young and aged lymph nodes at steady state, but after influenza infection aged FRCs have delayed expansion. Reduced proliferation of aged FRCs, but not increased death, results in this decreased expansion. Aged LNs also exhibited reduced concentrations of FRC produced homeostatic chemokines, correlating with reducing homing of naïve T cells. Image analysis of lymph nodes revealed that young and aged T cell zone FRCs have similar morphology at both steady state and after infection. In addition, aged FRCs did not appear to be a contributing factor in the reduced proliferation of young T cells transferred into aged LNs after influenza infection. These results are the first evidence that aging alters lymph node stromal cells ability to respond to challenge and functionality. Furthermore, these changes in aged FRCs may be an underlying contributor to the impaired immune response to influenza infection found in the elderly.
INTRODUCTION

Influenza is a highly problematic and often lethal infection for elderly populations\textsuperscript{1,2}. Decline of immune system function, collectively termed “immunosenescence”, contributes heavily to the increased susceptibility of the elderly to influenza\textsuperscript{3}. Intrinsic defects in adaptive immune cells have been well characterized, with strong focus on cells of the T cell lineage\textsuperscript{4,5}. Both CD4 and CD8+ T cell responses to influenza infection are altered with age. Aged mice have delayed influenza nucleoprotein (NP) specific CD4+ T cells responses in their lungs compared to young mice\textsuperscript{6}. Concomitantly, influenza NP specific CD8+ cell responses are reduced in the lungs of aged mice after influenza infection\textsuperscript{7}. This correlates with the delayed clearance of virus from aged lungs\textsuperscript{8,9}. It is possible that changes in secondary lymphoid organs may exacerbate problems with T cell immunity and age.

Adoptive transfer studies have been the key to dissecting the impact of the aged environment on T cell mediated responses. In these studies, young T cells with full functional capacity are transferred into either young or aged hosts, which are subsequently challenged and the response of the transferred T cells is analyzed. This approach tests the influence of T cell independent factors in the secondary lymphoid organ microenvironment on the development of antigen-specific T cell responses. These studies have provided striking insight into the deleterious impact of the aged secondary lymphoid organ environment on T cell responses. Several studies have shown that young T cells have delayed entry, priming and reduced function in aged compared to young lymph nodes and spleens after challenge\textsuperscript{10–12}. Yet it remains to be determined which factors in the aged secondary lymphoid organs hinders young T cell responses.

Stromal cells in secondary lymphoid organs such as lymph nodes, are non-hematopoietic cells of mesenchymal origin\textsuperscript{13}. These cells were once thought to solely function as the structural skeleton
of lymph nodes, mediating architectural organization, but are now appreciated to be involved in many aspects of both innate and adaptive immune responses\textsuperscript{14–16}. In lymph nodes there are four major subsets of stromal cells, fibroblastic reticular cells (FRCs), lymphatic endothelial cells (LEC)s, blood endothelial cells (BEC)s and double negative cells (DNCs). LECs and BECs line lymphatic and blood vessels respectively, functioning to facilitate transport of fluid, cells and antigen into and out of lymph nodes\textsuperscript{17,18}. DNCs are a rare and poorly understood subset of lymph node stromal cells that are thought to be contractile FRC like pericytes\textsuperscript{19}. FRCs are the major stromal cell subset of the lymph node and play an intimate role in the initiation, orchestration and control of adaptive immune cell functions\textsuperscript{16,20}. At homeostasis, FRC produced CCL19 and IL-7 promote the survival of naïve T cells\textsuperscript{25}. With regards to influenza infection, Denton \textit{et al} demonstrated that lymph node FRCs are required to initiate adaptive immunity to influenza infection\textsuperscript{21}. FRCs produce homeostatic chemokines (CCL19 and CCL21) that recruit and position lymphocytes and dendritic cells in lymph nodes\textsuperscript{22,23}. FRCs also create a conduit network controlling the migration of T cell, dendritic cells and antigen in the lymph node paracortex and medulla\textsuperscript{24}. During the response to an infection, FRCs have been shown to elongate and expand, which is thought to increase space for the immune response to occur\textsuperscript{26–28}. Apart from enhancing immune cell interactions and survival, FRCs have been shown to control T cell mediated immunity, though a NOS2 dependent pathway\textsuperscript{29–31}.

Despite the in depth involvement of FRCs in development and control of T cell mediated immunity, it is unclear how aging impacts the numbers and function of these cells\textsuperscript{32–34}. We hypothesize that FRCs in aged lung draining, mediastinal lymph nodes (MLN), are an underlying factor in the attrition of T cell responses to influenza infection in aged mice. In this study, we rigorously characterize how aging impacts FRC number and morphology at homeostasis and during influenza infection. We also determine how aging impacts FRC functional ability to produce
homeostatic chemokines, important for T cell recruitment into the lymph node and tested the ability of aged FRCs to inhibit T cell proliferation.

We determined that at homeostasis young and aged MLNs have similar numbers of FRCs, but after infection aged FRCs take longer to peak in number in part due to decreased proliferation. Aged FRCs appear to be functionally deficient, with decreased CCL19 and CCL21 concentrations in aged MLNs at homeostasis that fail to dip after infection as is seen in young MLNs. Importantly, the decreased CCL19 and CCL21 concentrations correlate with reduced homing of young donor T cells into aged MLNs when compared to young MLNs. We also found that young influenza NP specific CD8+ T cells exhibit reduced activation and proliferation in aged compared to young MLNs. Yet from in vitro experiments, it appears as though aged FRCs maintain their ability to inhibit T cell proliferation and may not be the cause the in vivo T cell proliferation deficit. This research expands our understanding of the aged immune system and provides new insight into the biology of aging stromal cells.

MATERIALS AND METHODS

Mice/Infection

Young (2-4 month) male C57BL/6 mice were acquired from Jackson laboratories. Aged (19-21 month) male C57BL/6 mice were acquired from National Institute of Aging’s colony at Charles River Laboratories. F5 RAG KO TCR transgenic (TCR specific for NP of H17 influenza) mice were received as a generous gift from Dr. Linda Cauley at the University of Connecticut School of Medicine and were used in experiments at two months of age. Mice were housed under specific pathogen free conditions in sterilized, individually vented, HEPA filtered cages in the UConn Health animal facility. Mice were infected via intranasal administration with sublethal doses of either 400EID\(_{50}\) PR8 influenza or 600EID\(_{50}\) E61-13-H17 (H17) influenza. After infection, mice were monitored daily and sacrificed if they showed visible signs of distress or if their weight dropped
below 70% of their starting weight. Mice were sacrificed at time points listed after infection using CO$_2$ asphyxiation. The UConn Health Animal Care and Use Committee reviewed and approved all experimental procedures using animals.

**Lymph Node Stromal Cell Digestion/ Flow Cytometry**

Lymph nodes from individual mice were dissected and placed in RPMI-1640 on ice until digestion. Lymph nodes were placed into 5 ml Eppendorf tubes with 2 ml of freshly made enzyme mix comprised of RPMI-1640 containing 1.3 U/ml Liberase TL (Sigma) and 300U/ml of Benzonuclease (Millipore Sigma). Tubes were incubated at 37°C in a water bath and gently inverted at 5 min intervals. After 20 minutes, lymph nodes were very gently pipetted up and down once using a 1-ml pipette, which disrupting the capsule. The tubes were placed back into the water bath and fragments were allowed to settle for 30 seconds. Subsequently, the enzyme mix was removed and added to 10 ml of ice-cold FACS buffer (2% FCS, 5 mM EDTA in PBS) and centrifuged (300 g, 4 minutes, 4°C). Two milliliter of fresh enzyme mix was added to the digestion tube. After 10 minutes, the cells were mixed vigorously for 30 seconds using a 1-ml pipette. Fragments were again allowed to settle, the supernatant was removed and added to the previously spun cell pellet in FACS buffer. Two ml of fresh enzyme mix was added to the digestion tube. Until the digestion was completed, the samples were vigorously pipetted with a 1-ml pipette every 5 minutes until, when held up to light, no lymph node fragments were visible. In general, the aged lymph nodes took about five minutes longer to digest then young lymph nodes. Supernatants were centrifuged after each removal (300 g, 4 min, 4°C) until finally, each collection tube contained the entire cellular contents of an individual mouse’s harvested lymph nodes. Cells were filtered through 100 μm nylon mesh, counted and assessed for viability using AOPI on the Cellometer cell counter (Nexcelom). For staining of surface antigens only, 5 × 10$^6$ cells per sample were then incubated with 100 μl diluted antibodies for 20-30 min at 4°C in ice-cold FACS buffer before acquisition on the flow cytometer, without fixation. For measurement of stromal cell
proliferation, intracellular Ki67 was assessed using Foxp3 Fix/Perm kit (eBioscience) according to manufacture’s instructions. For measurement of death in stromal cell populations, cells were stained in Annexin V Binding buffer (BD Biosciences) according to manufacture’s protocol. Peripheral blood was acquired by cardiac puncture and red blood cells were lysed using ACK lysis buffer (Life Technologies). Surface antigens on peripheral blood cells were stained as described above. To quantify cell numbers in peripheral blood, CountBright Absolute Counting Beads (ThermoFisher) were used according to manufacture’s instructions. Antibodies used are listed in Table 3. Samples were acquired on a LSRII flow cytometer (BD Bioscience) and analysis was performed using Flowjo software v10 or v7.6.5 (Tree Star).

### Table 3. Antibodies used for flow cytometry of stromal cells

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Fluorophore</th>
<th>Clone</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45</td>
<td>PerCPCy5.5</td>
<td>30-F11</td>
<td>BD</td>
</tr>
<tr>
<td>PDPN</td>
<td>PE-Cy7</td>
<td>8.1.1</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD31</td>
<td>BV510 AF647</td>
<td>MEC13.3</td>
<td>BD Biolegend</td>
</tr>
<tr>
<td>TER-119</td>
<td>APC-Cy7</td>
<td>TER-119</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD21/CD35</td>
<td>BV421</td>
<td>7E9</td>
<td>Biolegend</td>
</tr>
<tr>
<td>Annexin V</td>
<td>BV711</td>
<td></td>
<td>BD</td>
</tr>
<tr>
<td>CD8α</td>
<td>BUV737</td>
<td>53-6.7</td>
<td>BD</td>
</tr>
<tr>
<td>CD45.2</td>
<td>PerCPCy5.5</td>
<td>104</td>
<td>BD</td>
</tr>
<tr>
<td>CD45.1</td>
<td>APC A20</td>
<td></td>
<td>BD</td>
</tr>
<tr>
<td>CD69</td>
<td>BV421 H1.23F</td>
<td></td>
<td>BD</td>
</tr>
<tr>
<td>CD44</td>
<td>BV650 IM7</td>
<td></td>
<td>Biolegend</td>
</tr>
<tr>
<td>NOS2</td>
<td>AlexaFluor®488</td>
<td>C-11</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Ki67</td>
<td>PE SolA15</td>
<td></td>
<td>eBioscience</td>
</tr>
<tr>
<td>Carboxylic Acid, Succinimidyl Ester (Live/Dead Indicator)</td>
<td>AlexaFluor®350</td>
<td></td>
<td>Life Technologies</td>
</tr>
<tr>
<td>CD16/CD32 (Fc Block)</td>
<td>Unconjugated 93</td>
<td></td>
<td>eBioscience</td>
</tr>
</tbody>
</table>

**Immunofluorescence**

Mediastinal lymph nodes were fixed overnight in medium containing 0.05 M phosphate buffer, 0.1 M L-lysine, pH 7.4, 2 mg/ml NaIO4, and 10 mg/ml paraformaldehyde then dehydrated with 30% sucrose in phosphate buffer. Lymph nodes were frozen in Tissue-Tek O.C.T. compound (Sakura...
Finetek) and 20μm sections were cut using a CM1850 cryostat (Leica). Sections were blocked for 30 minutes with 2% goat serum, stained overnight at 4°C in a humidified chamber with primary antibody cocktail, washed in PBS and then stained for 2 hours at room temperature with secondary antibodies. For detection of CCL21, sections were blocked as described and stained overnight with anti-CCL21 in 2% goat serum with 0.3% Triton X-100 (Sigma). After PBS washes, CCL21 signal was amplified using Alexa Fluor™ 594 Tyramide SuperBoost™ Kit, streptavidin (ThermoFisher Scientific) according to manufacture’s instructions. Antibodies used for immunofluorescence are listed in Table 4. Slides were mounted with Immu-Mount (Thermo Scientific), cover slipped and sealed. Images were acquired using the Zeiss 780 laser scanning microscope (Carl Zeiss; air objective 20× Plan-Apochromat with NA 0.5 or water objective 40× C-Apochromat NA 1.2 with 1.5 digital zoom) using multichannel frame scans. The ZEN Black software (Carl Zeiss) was used for image acquisition. For full lymph node images, the ZEN Black tile scan function was used to stitch individual 20x images together. For ER-TR7 analysis three z-stack (4-1μm steps) images per mouse were acquired of the T cell paracortex with the 40x objective. Volume and disconnection of ER-TR7 network was quantified using the isosurface tool in Imaris 8.1 software (BRITPLANE). Network length and branch point analysis was quantified using the filament tracer tool in Imaris 8.1 software.

Table 4. Antibodies used for immunofluorescence of stromal cells

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Fluorophore</th>
<th>Clone</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1° ER-TR7</td>
<td>Unconjugated Rat</td>
<td>ER-TR7</td>
<td>Acris</td>
</tr>
<tr>
<td>2° Goat-anti-Rat</td>
<td>AlexaFluor568</td>
<td></td>
<td>Invitrogen</td>
</tr>
<tr>
<td>B220</td>
<td>PE-Dazzle</td>
<td>RA3-6B2</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD31</td>
<td>AlexaFluor647</td>
<td>MEC13.3</td>
<td>Biolegend</td>
</tr>
<tr>
<td>Lyve-1</td>
<td>AlexaFluor488</td>
<td>ALY7</td>
<td>eBioscience</td>
</tr>
</tbody>
</table>

**T Cell Transfer**

CD45.1+ CD8+ T cells were isolated from pooled spleens and lymph nodes of F5 RAGKO mice using Mojosort Mouse CD8 T cell isolation kit (Biolegend) according to manufacturer’s instructions, with an ending purity of >90% (data not shown). To measure T cell proliferation *in vivo*, isolated
F5 T cells were labeled in PBS with 5μM CFSE for 10 minutes at 37°C then washed in PBS with 10% FCS. 8x10^5 CFSE+ CD45.1+ F5 CD8+ T cells were tail vein injected in 200μl of PBS into each mouse, and then rested overnight before infection with H17 influenza the next morning. To assess T cell entry into lymph nodes, 2.3x10^6 CD45.1+ CD8+ F5 T cells in 200μl of PBS were tail vein injected into recipient young or aged recipient mice. Recipient mice were then sacrificed exactly 30 minutes after transfer. Blood (cardiac puncture) and MLN were collected for flow cytometric analysis from those mice.

**CCL19/CCL21 ELISA**

MLNs were dissected, flash frozen and stored at -80°C. MLNs were homogenized in 400μl of TPER with EDTA (Invitrogen) and HALT protease and phosphatase inhibitor cocktail (Thermofisher), and then centrifuged at 1000g for 10 minutes. Supernatants were aliquoted and frozen at -80°C until analysis was performed. CCL21 was measured using mouse CCL21/6kine DuoSet ELISA (R&D Systems) according to manufactures instructions on MLN homogenates diluted 1:3 in reagent diluent. CCL19 was measured using mouse CCL19/MIP-3 beta DuoSet ELISA (R&D Systems) according to manufactures instruction on undiluted MLN homogenates. Chemokine concentrations were normalized to total protein concentration determined using Pierce BCA Protein Assay (Thermofisher).

**FRC Mediated T cell Proliferation Inhibition Assay**

Lymph nodes were harvested and pooled from either 10 young or 10 aged mice. Stromal cells were liberated by sterile digestion in 5 ml of enzyme mix per sample as described above. Total lymph node digested samples were cultured at 5x10^5 cells per cm^2 in αMEM with 10% FCS and 1% ABAM (Life Technologies). After 24 hours, non-adherent cells were removed and new media was added to the cultures. After five additional days of culture, cells were harvested with 0.2% trypsin, 5mM EDTA in PBS. LECs and BECs were removed from the harvested population by staining with anti-CD45 PE (Clone 30-F11) (BD) and anti-CD31 PE (Clone MEC13.3) (BD),
followed by anti-PE microbeads isolation (Miltenyi). FRC purity was confirmed at >90%. 5x10^4 FRC per well were plated in a flat bottom 96 well plates and rested overnight. The next day, CD8+ T cells were isolated from young C57BL/6 mice and labeled with CFSE as described above, with >90% purity. 5x10^5 CFSE labeled CD8+ T cells were added to wells with or without young or aged FRCs. To stimulate the CD8+ T cells in vitro, anti-CD3, anti-CD28 Dynabeads (ThermoFisher) were added at a 1:1 bead to T cell ratio to wells with or without FRCs. After 48 hours, T cells were removed from culture and proliferation was measured by flow cytometric examination of CFSE dilution. Division index, the average number of cell divisions that a cell in the original population has undergone, was calculated using Flowjo’s proliferation tracker tool. Culture supernatants were also collected and nitric oxide was measured using the Greiss Reagent system (Promega) according to manufacture’s instructions.

**Statistical Analysis**

Statistical significance was determined by Student’s t test, One way or Two way ANOVA, Repeated Measures ANOVA or Mantel Cox Log Rank Test as specified in the figure legends. Statistical analyses were performed with Prism 6 software (GraphPad Software). Differences were considered significant at p<0.05.
RESULTS

Altered kinetics of aged lymph node stromal cell expansion

To evaluate if stromal cells are an underlying contributor to impaired immune responses during influenza infection in aged mice, we first sought to determine how stromal cells respond in young and aged mice. Young and aged mice were infected with PR8 influenza (Fig. 17A). In agreement with previous reports, aged mice had decreased survival (Fig. 17B) and increased weight loss (Fig. 17C) after infection compared to young.

![Figure 17](image)

**Figure 17. Aged mice have decrease survival and increased weight loss after sub lethal infection with influenza.** A. Experimental design: Young and aged mice were infected with a sub-lethal dose of PR8 influenza infection and sacrificed at time points listed for analysis. B. survival C. weight loss was quantified. Data are from one representative experiment of at least four experiments performed independently. Statistical significance was determined by B. Mantel Cox Log Rank Test or C. Repeated measures Two Way ANOVA with Sidak multiple comparison. **p<0.01, ****p<0.0001. Error bars= ±SEM.

In order to determine how aging impacts the number of stromal cells in lymph nodes, we digested both the lung draining mediastinal lymph node (MLN) and the non-draining popliteal lymph node as a control to ensure that digestion was standard across time points. While some reports have suggested that aged lymph nodes fail to expand to the same extent as young lymph nodes after immune challenge, we found that young and aged MLNs expanded with similar kinetics and no significant difference was observed in total cell number at homeostasis or at any time point after influenza infection in young and aged MLNs or PLNs (Fig. 18A). In order to quantify lymph node stromal cell numbers, we used a slightly modified version of Fletcher et al's protocol for digestion of lymph nodes for stromal cells. With minor modifications, we were able to digest lymph nodes with high viability (Fig. 18B) and achieved similar frequencies of stromal cell populations (Fig.
18C) to what has been reported in the literature. Upon quantification of the total number of stromal cells (CD45-Ter119-) cells in MLNs and PLNs at homeostasis, we did not see any significant difference in young and aged samples. Interestingly, at day 10 post infection, a previously reported peak of stromal cell numbers after influenza infection, we found that aged MLNs had significantly fewer total stromal cells compared to young MLNs (Fig. 18D). By day 12 post infection, the aged MLN stromal cell numbers were equal to that of the young MLNs, suggesting a delayed expansion in aged lymph nodes (Fig. 18D). The total stromal cell population was further differentiated into FRCs (PDPN+CD31-CD21/CD35-), LECs (PDPN+CD31+), and BECs (PDPN-CD31+). At homeostasis in both MLNs and PLNs all of these populations were not significantly different in number in young and aged lymph nodes (Fig. 18E,F,G). After infection there were significantly fewer aged FRCs and LECs at day 10 post infection (Fig. 18E,F) but this difference was corrected at day 12 post infection when the aged numbers reached that of the young for both FRCs and LECs (Fig. 18E,F). BECs had different expansion kinetics with their numbers being similar in young and aged up to day 10 post infection, but higher in aged MLNs at day 12 post infection (Fig. 18G). Of note, PLN total stromal cell, FRC, LEC, and BEC numbers were not significantly different from the non-infected time point at any day post influenza infection (Fig 18. D,E,F,G), confirming that the digestions were consistent and the increase in MLN FRC, LEC and BEC numbers was not due to a technical artifact. We next sought to determine the mechanism behind the decreased numbers of FRCs and LECs at day 10 post infection.
Figure 18. Reproducible, high validity stromal cell digestion reveals altered stromal cell expansion kinetics in aged mediastinal lymph nodes after influenza infection. A. Total cell numbers of stromal cell digested MLNs (draining lymph node) and PLNs (non-draining lymph node) after infection with PR8 as in Fig 1. B. Viability of lymph nodes after stromal cell digestion. C. Flow cytometric gating strategy for lymph node stromal cell populations in young (top) and aged (bottom) lymph nodes. Number of D. total stromal cells E. FRCs, F. LECs, G. BECs in young and aged MLNs and PLNs after influenza infection. Data are pooled from two independent experiments n=10-18 mice per group, per time point. Statistical significance was determined by Two-Way ANOVA with Tukey post test. Error bars=± SEM.
Decreased proliferation contributes to reduced number of FRCs and LECs after influenza infection

It is possible that the decreased number of FRCs and LECs at day 10 post infection in aged MLNs may be due to either decreased proliferation or increased death or a combination of both. Upon analysis of proliferation, we found that at day 10 post infection, lower frequencies of FRCs (Fig. 19A), LECs (Fig. 19B) and BECs (Fig. 19C) were proliferating in aged MLNs compared to young MLNs, correlating with decreased cell numbers at this time point. Analysis of cell death in MLN FRCs (Fig. 19D), LECs (Fig. 19E), and BECs (Fig. 19F) showed no significant differences throughout the course of influenza infection. These results suggest that the mechanism behind the decreased number of FRCs and LECs at day 10 post infection is in part due to decreased proliferation and not increased rate of cell death. Thus, we next wanted to determine if the structure of the FRC network was altered with age.
Figure 19. Decreased proliferation, but not increased death, contributes to reduced stromal cell numbers in MLNs after influenza infection. Frequency of Ki67+ A. FRC, B. LECs, and C. BECs after influenza infection in draining (MLN) and non-draining lymph nodes (PLN). The frequency of apoptotic (Annexin V+, CASE L/D-), necrotic (Annexin V- CASE L/D+) and viable (Annexin V- CASE L/D-) D. FRCs, E. LECs and F. BECs in MLNs were quantified. Data are pooled from two independent experiments n=8-10 mice per group per timepoint. Statistical significance was determined by Two Way ANOVA with Tukey post test. ****p<0.0001. Error bars =±SEM.

Preserved T cell zone FRC network morphology in aged lymph nodes

It is well accepted that aging disrupts the architectural organization of secondary lymphoid organs. Several studies have described that aged lymph nodes have poorly defined B cell follicles that merge into the T cell paracortex\(^{12,34,35}\). To determine if similar architectural disorganization existed in aged mediastinal lymph nodes at homeostasis and after influenza infection, we performed confocal microscopy. At homeostasis, we found mild disruption of B cell follicles in aged (Fig. 20 bottom left) compared to young MLNs (Fig. 20 top left). After influenza infection, aged MLNs showed dramatic loss of B cell follicle organization (Fig. 20 bottom right), while B cells in young MLNs were confined to well organized follicles (Fig. 20 top right).
**Figure 20. Disrupted lymph node architecture in aged mice after influenza infection.** MLNs from non-infected young mice (top left) display organized B cell follicles, while B cell follicles in aged MLNs (bottom left) show mild disruption. Fourteen days after influenza infection B cell follicles in aged MLNs (bottom right) become highly disputed, while young MLN B cell follicles remain organized (upper right). Images shown are representative of 5 young and 5 aged mice at each time point from two experiments performed independently. Yellow-B220 (B Cells), Red-CD31 (Blood vessels), Blue-Lyve-1 (lymphatic vessels), Purple colocalization of CD31 and Lyve-1 (lymphatic vessels). Scale bar= 200µm.
FRCs are important for homeostasis and maintenance of B cell follicle organization\textsuperscript{36}. We sought to determine if the FRC network was preserved in aged lymph node at homeostatic and after influenza infection. Quantification of the T cell zone FRC network revealed no difference in density (Fig. 21D), network length (Fig. 21E), connectivity (Fig. 21F), or in the number of branch points (Fig. 21G) at homeostasis or after influenza infection in young compared to aged lymph nodes. After influenza infection both young and aged FRC network density (Fig. 21D) and length (Fig. 21F) decreased, supporting previous data that after LPS challenge FRCs become less tense and elongate\textsuperscript{37}. These data show that the T cell zone FRC network is preserved with age and responds similarly to influenza challenge. Next, we wanted to determine the effects of aging on FRC function.
Figure 21. Aging does not alter T cell zone FRC morphology. A. Representative confocal image of stromal cells in a young non-infected MLN denoted by ER-TR7 in yellow. The red insert is an magnification of the T cell zone FRCs, which are quantified in the remainder of the figure. B. Displays the image analysis pipeline for quantifying the FRC network. Z stack images of 4 one µm steps were taken using the 40x objective lens of the Ziess LSM780 (Left-staining). Imaris imaging software was used to create a digital rendering of the staining called an isosurface (middle) to quantify FRC volume and segmentation. The filament tracer tool in Imaris software was used to map the FRC network (right) to determine the network length and number of FRC branch points. C. Representative FRC images from young (top) and aged (bottom) non-infected (left), day 7 post infection (middle) and day 10 post infection (right) MLNs. No significant difference was discovered in the FRC volume D, segmentation E, network length F or branch points G in young and aged mice at steady state and after infection. Statistical significance was determined using two-Way ANOVA with Tukey post test. *p<0.001. n=10 young, n=10 aged mice per time point. Data shown are pooled from two experiments performed independently. Error bars= ±SEM. Scale bar=20µm.
Reduction in homeostatic chemokines and altered HEV architecture correlates with reduced T cell homing into aged MLNs

FRCs produce homeostatic chemokines CCL19 and CCL21 which facilitate the localization and homing of CCR7 expressing cells in the lymph node\textsuperscript{22,23}. We wanted to determine if young T cells had decreased homing into aged compared to young MLNs as previously reported for other lymph nodes\textsuperscript{12}. We transferred young CD8+ T cells into young and aged hosts and quantified their entry into MLNs (Fig. 22A). There was a higher frequency (Fig. 22C) and number (Fig. 22D) of young donor CD8+ T cells in the peripheral blood of aged compared to young hosts. This correlated with decreased frequency (Fig. 22F) and numbers (Fig. 22G) of donor cells in aged MLN when compared to young. These data show that there is a lymph node intrinsic defect that results in decreased recruitment of T cells.

Next, we went on to determine if the decreased T cell entry in aged MLNs correlated with reduced homeostatic chemokine concentrations. At homeostasis, we found that aged MLNs had decreased concentrations of both FRC produced homeostatic chemokines, CCL21 (Fig. 22H) and CCL19 (Fig. 22I). The location of CCL21 appeared to be similar in both young and aged MLNs and did not extend into the B cell follicles (Fig. 22J). Since FRCs have been shown to respond to immune challenge by temporarily decreasing their production of CCL21\textsuperscript{38,39}, we went on to determine if aged FRC responded to infection in a manner similar to young. Three days after infection, we found that young MLNs decreased their production of CCL21, but aged MLN CCL21 concentration did not change (Fig. 22H). These data show that aged stromal cells produce less homeostatic chemokines and are less responsive to challenge when compared to young stromal cells. These changes in FRC function may contribute to reduce homing of young T cells into aged lymph nodes. We next sought to analyze another component import for T cell entry into lymph nodes, high endothelial venules (HEV).
Since HEVs are the physical entry sites for lymphocytes into the MLN\(^7\), we assessed if HEV morphology was influenced by aging. We found that HEVs in aged naïve MLNs appear less cuboidal and were thinner when compared to those in young MLNs (Fig. 22K, L). Our attempts to quantify the number of HEVs in naïve MLNs using flow cytometry gave conflicting results, so the data is not reported here. ICAM-1 is an import adhesion molecule on HEVs and BECs which binds to LFA-1 on T cells to mediate arrest during transendothelial migration \(^{40}\). Interestingly we found no difference in ICAM-1 expression on young or aged BECs (Fig 22. M). These data show changes in stromal cell function and morphology, which may contribute to impaired homing of T cells into aged lymph nodes. Thus, we next performed experiments to determine if aged FRCs contributed to impaired T cell priming in aged lymph nodes.
Figure 22. Decreased homing of young T cells into aged lymph nodes correlates with decreased homeostatic chemokines and altered HEV structure. A. Experimental outline for T cell transfer. B. Representative flow plot, C. frequency, and D. number of young transferred CD8+ T cells in young or aged blood. E. Representative flow plot, F. frequency and G. number of young transferred CD8+ T cells in young and aged MLNs. H. CCL21 protein concentration in naïve and day three post H17 influenza infection MLNs. I. CCL19 protein concentration in naïve MLNs. J. Naïve MLNs stained for CCL21. K. Representative images of HEVs in naïve MLNs. L. Quantification of HEV thickness in naïve MLNs. M. ICAM-1 gMFI of BECs from naïve MLNs. A-G Data are from one representative of three experiments performed independently, n=4 mice per group. H, I, Data are pooled from two independent experiments, n=13-20 mice per group. J,K Representative images from one of n=6-10 mice per group, scale bar =200um. L. Each data point is the average thickness of three HEVs from one mouse. Each HEV was measured at 5 different points and the average thickness was taken, n=6-10 mice per group. M. Data are representative from one of two experiments performed independently n=5-8 mice per group. C,D,F, G, I, L, M statistical significance was determined by two tailed T-test. I. Statistical significance was determined by Two-way ANOVA with Tukey post test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Error bars =±SEM.
Reduced priming of young T cells in aged MLNs

Previous studies have reported defective priming of young T cells in aged inguinal lymph nodes\textsuperscript{10}, therefore we sought to determine if a similar phenomenon happened to T cells in the MLN in response to influenza infection. To test this, we used F5 TCR transgenic CD8+ T cells, which have a TCR specific for the influenza nucleoprotein peptide 366-374 (NP\textsubscript{366-374}/\textsuperscript{Db}) from the E61-13-H17 (H17) influenza virus\textsuperscript{41}. Young CFSE labeled CD45.1+ F5 CD8+ T cells were transferred I.V. into both young and aged CD45.2+ hosts, which were rested overnight and then infected with H17 influenza (Fig. 23A). Three days after infection, the response of the transferred cells was assessed using flow cytometry. The young F5 cells in aged MLNs had reduced proliferation compared to those in young MLNs (Fig. 23B). Furthermore, fewer F5 cells in the aged host expressed the activation markers CD69 (Fig. 23C) and CD44 (Fig. 23D) when compared to donor cells in young hosts. These data show that T cell priming is impaired in aged MLNs after influenza infection. To examine this further, we tested the hypothesis that FRCs may be the culprit behind this proliferation defect.
Figure 23. Reduced proliferation and activation of young influenza specific CD8+ T cells in aged lymph nodes after infection

A. Experimental outline

B. CFSE division of young F5 CD8+ T cells in young (grey) or aged (white) mediastinal lymph nodes, three days after influenza infection with representative flow plot (left) and quantification (right). C. CD69 and D. CD44 expression on young F5+ T cells in young (grey) or aged (white) MLNS. FMOs shown in black. Data are one representative experiment of two performed independently. n=4-5 mice per group. Statistical significance was determined by a two-tailed T-test. *p<0.05, **p<0.01. Error bars=±SEM.
FRCs maintain their inhibitory capacity with age

FRCs have been shown to reduce the proliferation of T cells in culture via production of nitric oxide (NO)\cite{29}. To test the inhibitory capacity of young versus aged FRCs, we used the *in vitro* system published by Lukacs-Kornek et al. in which CFSE labeled CD8+ T cells were stimulated in cultures with or without FRCs\cite{29}. Since aged T cells have intrinsic proliferation defects, we used only young T cells in our co-culture system to test the capacity of aged and young FRCs to inhibit T cell proliferation, with the hypothesis that aged FRCs would be more inhibitory\cite{42}. Young stimulated CD8+ T cells had high rates of proliferation as expected (Fig 24. A, B, C). Interestingly, both young and aged FRCs suppressed the division of these young CD8+ T cells equally (Fig. 24. A,B,C). We also examined whether these cultured young and aged FRCs produced similar quantities of NO. As reported by Lukacs-Kornek et al, FRCs only produced NO in the presence of activated T cells, not when they are cultured by themselves (Fig. 24D). Young and aged FRCs produced equal amounts of NO when cultured with young CD8+ T cells and stimulation (Fig. 24D), in agreement with their ability to equally suppress T cell proliferation (Fig. 24A,B,C). These experiments have the major caveat of being *in vitro*. Changes in aged FRCs may be lost during culture, so we wanted to determine what was happening *in vivo*. Therefore, we analyzed the expression of the protein responsible for NO production in FRCs, NOS2 in both MLNs and PLNs at homeostasis and after influenza infection\cite{29,43,44}. We found that both young and aged MLN FRCs upregulate NOS2 expression after influenza equally, with no age-related differences (Fig. 24E). These results are in agreement with our *in vitro* data in that it appears as though aged FRCs maintain their capacity to suppress T cell proliferation as equally as young FRCs and may contribute to the reduced T cell proliferation in aged MLNs after influenza via this mechanism.
Figure 24. Aged FRCs maintain their ability to suppress T cell proliferation Young CFSE+ CD8+ T Cells, with or without anti-CD3, anti-CD28 microbeads, were cultured for 48 hours with either young FRCs, aged FRCs or no FRCs at a 10:1 ratio (T cell/FRC). The ability of FRCs to suppress T cell proliferation was measured by CFSE dilution of young CD8+ T cells, representative flow plots shown in A. B. Division index of CD8+ T cell in the FRC co-culture. C. Percent of young CD8+ T cells that divided in co-culture. D. Nitric oxide in the co-culture supernatants was quantified using the Greiss reagent system as Nitrite. E. In vivo NOS2 MFI was measured on FRCs at homeostasis and after influenza infection in draining (MLN) and non-draining (PLN) lymph nodes. MFI was normalized to FMO at each time point listed. A, B, C, D Each point represents one experimental replicate, which is the average of three technical replicates, n=3 experiments. Statistical significance was determined by One-Way ANOVA with Tukey post test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 E. Combined data from two experiments performed independently, n=8-16 mice per group. Statistical significance was determined by Two-Way ANOVA with Tukey post test. No significant differences were found in young or aged PLNs at any time point tested. **p<0.01, ***p<0.001, ****p<0.001. Error bars =±SEM.
DISCUSSION

In this study, we addressed the hypothesis that age-related changes in lymph node stromal cells could be an underlying factor hindering T cell immunity to influenza infection in aged mice. These experiments provide the most comprehensive analysis of aged lymph node stromal cells to date. We determined that the number of stromal cells in young and aged MLNs at homeostasis is similar, but aged lymph nodes have decreased concentrations of CCL19 and CCL21, which correlate with impaired T cell homing into the lymph node. These results contradict those in the literature with regards to how aging impacts the number lymph node stromal cells. Several reports have stated that aged lymph nodes either have fewer FRCs or total stromal cells\textsuperscript{32-34}, but these decreases may be technical artifacts due to poor digestion techniques. Stromal cell digestion techniques are technically challenging and need to be optimized before reliable results can be obtained\textsuperscript{20}. Importantly we found that aged lymph nodes take five to ten minutes longer to digest then young lymph nodes. The published data so far was from lymph nodes digested for the same period of time and which may have under digested the aged compared to young lymph nodes. Although unlikely, it is also possible that MLNs and PLNs analyzed in this study, may age differently than cervical, inguinal, axillary and brachial lymph nodes analyzed by others\textsuperscript{32-34}.

After antigenic challenge or infection, lymph node stromal cells expand and proliferate\textsuperscript{13,21,26}. Here we determined that aged stromal cells have different kinetics of expansion compared to young after influenza infection. The peak of LEC and FRC expansion in aged MLNs is delayed compared to young MLNs, in part due to decreased proliferation. BECs in aged MLNs have similar expansion kinetics, but are actually increased in number in aged MLNs at day 12 post infection. The biological importance of stromal cell expansion is unclear, since there is currently no way to selectively inhibit their proliferation. Yet expansion of stromal cells is thought to be critical to hypertrophy and resolution of lymph nodes and provide additional space for the immune response.
to occur\textsuperscript{14}. What impact the altered expansion of stromal cell kinetics has on the immune response to influenza is unclear. FRCs were shown to be critical for the initiation of adaptive immunity to influenza infection, but when depleted after the immune response was initiated, adaptive immunity developed unhindered\textsuperscript{21}. It is possible that decreased numbers of FRCs and LECs at day 10 post infection, may alter the formation of memory responses in aged mice, by decreasing the entry new cells into the lymph node late during the immune response, which may differentiate into memory\textsuperscript{45}. In fact, it is well established that aged memory CD8+ T cells have reduced functionality, and stromal cells may contribute to this problem\textsuperscript{46}. It remains to be determined why aged FRCs and LECs have delayed proliferation after influenza infection. Further studies are required to elucidate the exact signals required for FRC and LEC proliferation.

Out of the lymph node stromal cell subsets, T cells have the longest potential for interaction with FRCs, since they are the conduit upon which T cells traffic\textsuperscript{24}. Morphologically we found that the T cell zone FRC network remains intact with age and influenza infection. Like previous reports we found that after infection, the density of the FRC network decreases, which may be in part due to relaxation of the FRC network tension\textsuperscript{37}. It remains to be determined if aged FRCs localization is altered and if they extend into the B cell follicles of aged lymph nodes to contribute to architectural disorganization found in aged lymph nodes. We found that the FRC produced chemokine CCL21 does not appear to extend into the B cell follicles of aged mice, so this may not be the case for aged FRCs.

FRCs also are known to control proliferation of activated T cells via production NO\textsuperscript{29}. We demonstrated that young influenza specific CD8+ T cells had reduced proliferation in aged MLNs and tested the hypothesis that aged FRCs may be the cells blocking this proliferation. Interestingly we showed that is most likely not the case, with young and aged FRCs have similar inhibitory
capacity and production of NO. *In vivo* studies linking FRCs to T cell proliferation suppression have used models of self-reactivity and whether or not this mechanism is important during viral infections is unclear. We determined that after influenza infection, FRCs in young and aged MLNs upregulate expression of NOS2, correlating with our *in vitro* results showing preserved suppressive functionality. This data also presents the possible role of FRC mediated T cell proliferation control during influenza infection. Why young T cells are not proliferating as well in aged lymph nodes remains to be determined as well. Most likely numerous factors contribute to this deficit including changes in the priming capacity of aged dendritic cells.

These data expand our picture of the aged immune system and elucidate possible roles of age related changes in stromal cells to alterations in adaptive immune function. This is just the beginning to our understanding of the impact of stromal cells on the aged immune response.

REFERENCES


CHAPTER 5
Discussion

The immune system is like a complex and elegant machine. When all of the components are fully operative, the system functions properly, protecting the host from disease. When one cog malfunctions, compensatory mechanisms can often overcome the deficit. Occasionally this is not the circumstance and a singular problem can cause cascading downstream effects. In the case of the aged immune system, with many broken or damaged gears, it becomes incredibly difficult to track which parts are the mediators of the problem and which are simply downstream consequences. Only by increasing our understanding of what changes are occurring in the aged immune system we can begin to dissect causality and consequences.

Although descriptive in nature, this thesis progresses our understanding of the aged immune system and provides important insight into how it responds to influenza infection. To model aging, we used a widely accepted biomedical research model of humans, the of C57BL/6 mouse. Not surprisingly, laboratory mice have a much shorter lifespan (about 24 months\(^1\)) than the average U.S. citizen (78.8 years\(^2\)). Mice reach sexual maturity and are analogous to human adults beginning at eight to 12 weeks of age\(^3\). Mice are considered aged or elderly starting at roughly 18 months\(^4\). One problem that is becoming more widely accepted and published in the immunology community with the laboratory mouse model is cleanliness\(^5\). Laboratory mice, including mice used for aging research, live in specific pathogen free (SPF) conditions, thus they are not exposed to a pathogen until an experiment is performed. This lack of pathogen exposure has been show to alter the functionality of the immune system, putting into question the ability of SPF mice to adequately represent the human immune system\(^6\). Exposure to chronic pathogens like cytomegalovirus (CMV) has been shown to dramatically alter immune system function and is neglected in SPF mice models\(^7\). Several groups are working to create mouse pathogen exposure
cocktails and models to more adequately represent the human immune system. Such models could potentially be applied to the aging mouse model to enhance its clinical relevance.

Although, immunologically the C57BL/6 mouse is a powerful tool, due to the tetramer reagents and TCR transgenic strains available on the background, it has the shortcoming of being inbred thus lacking genetic diversity. Several mouse models exist that recapitulate human genetic diversity such as diversity outbred mice, collaborative cross mice and four-way cross mice. Although not used in this thesis, several groups are currently using these models in aging systems. Despite the imperfections of the mouse model used in this thesis it is still a widely accepted method and good starting point for studying the aged immune system. Other more complex models may be used in the future to replicate these studies and enhance their validity.

In this thesis, we expand our understanding of how aging impacts multiple components of the immune response to influenza infection, an often lethal problem for the elderly population. In chapter two we start by characterizing how changes in CD4+ T cells may contribute to impaired humoral immunity to influenza infection. Our hypothesis predicted that alterations in CD4+ T cells may contribute to impaired B cell immunity during influenza infection. Before these studies were performed, it was unclear how aging impacted the maturation and function of T_{FH}, the CD4+ T cell subset most intimately involved in B cell immunity. Yet it was well established that CD4+ T cells had intrinsic defects that could potentially make them poor B cell helpers, including reduced upregulation of the important costimulatory molecule CD154 (CD40-L). We found that young and aged mice have equal numbers of influenza NP-specific CD4+ T cells after influenza infection in their spleens, but fewer of these mature into germinal center T_{FH} in aged mice. The NP-specific T_{FH} cells in aged mice also had altered costimulatory molecule expression and presented an inhibitory phenotype with increased expression of IL-10 but not PD-1. Upon further analysis, we found that more of the influenza NP-specific CD4+ T cells in aged mice were either regulatory T cells or T_{FR} at later time points after infection. From transfer experiments, we concluded that
increased active TGF-β in the aged splenic environment may be driving the accumulation of regulatory T cells, but not T_{FR}, in aged mice. Sage et al used an NP-OVA in CFA immunization model and had similar findings with increased T_{FR} in aged mice when compared to young. From *in vitro* experiments, this study also found that aged T_{FR} were potent suppressors of B cell function and that aged T_{FH} were poor promoters of B cell immunity. Previously we showed that aged CD4 TCR transgenic cells transferred into young CD4 KO host and immunized with their cognate antigen supported the development of a significantly weaker germinal center response compared to young CD4+ T cells. In chapter two, we carried out a similar experiment, but we transferred a high frequency of young polyclonal CD4+ T cells into young and aged hosts then infected the mice with influenza. We found that the young T cells in the aged host enhance the germinal center B cell responses, showing that some of the B cell defects can be overcome in the presence of sufficient CD4+ T cell help.

These findings present multiple targets that could be used to enhance immunity in the elderly. If T_{FH} development was enhanced it could potentially boost antibody mediated responses in the elderly. One way that this can be accomplished in response to vaccination, is increasing the dose of antigen used in the vaccination, since T_{FH} responses are proportional to the quantity of antigen available. This approach has shown promising results for influenza vaccination in the elderly. High dose Fluzone vaccination was shown to be 24.4% more effective at preventing influenza in the elderly population over the age of 65 when compared to a standard dose influenza vaccine. Another possible method of boosting immunity in the elderly would be by blocking inhibitory responses including the development of T regs.

One potential method to decrease regulatory T cell formation would be to deplete TGF-β, since it is an important factor in the development of peripheral T regs. This may enhance immunity, but it is possible that this may also result in unwanted autoimmune reactions if too much TGF-β is depleted. TGF-β KO mice die of autoimmunity by the age of three to four weeks, but targeted low
level depletion may have the potential to be therapeutically effective\(^{19}\). It also remains to be determined which cells are the source of the increased TGF-\(\beta\) in the aged splenic environment from our studies. Future studies will need to be performed to answer this question.

Another factor which we did not explore that may be contributing to impaired germinal center formation in response to influenza is changes in follicular dendritic cells with aging. It has been shown that aged FDCs have impaired antigen trapping, and disorganized morphology\(^{20-22}\) which may contribute to smaller disrupted germinal centers found in aged mice. Known changes in FDCs, a type of stromal cell, led us to hypothesize that other stromal cell populations in the spleen may be altered with age.

In chapter three, we characterize how aging impacts FRCs in young and aged spleens at homeostasis. FRCs are stromal cells in the T cell zone of the spleen, which are important conduits of immune cells and antigen into the white pulp\(^{23}\). FRC produced CCL19 and CCL21 are important chemotactic signals for the recruitment of responding immune cells into the T cell zone of the white pulp\(^{24,25}\). We have previously shown that aged spleens, after I.P. immunization with OVA and alum, had decreased concentrations of CCL19 and CCL21 compared to young spleens\(^{26}\). Homeostatic chemokine concentrations have been reported to dip after immune challenge\(^{27}\), so the decreased concentration in aged spleens after challenge may not have been indicative of what was happening at homeostasis. Decreased homeostatic chemokines in aged spleens correlated with reduced homing of young OTII TCR transgenic CD4+ T cells into aged spleens after challenge. Fewer of the young OTII cells in the aged spleens were recruited into the T cell zones of the spleens, which correlated with diffuse localization of CCL21\(^{26}\). Although this study indirectly suggested FRCs may be altered in the spleens of aged mice, it did not directly examine them, which we did in chapter 3. Another study by Aw et al used confocal microscopy to quantify splenic FRCs and showed increased FRC area in aged spleens at homeostasis, but their data had high variability and did not quantify number of FRCs using flow cytometry\(^{28}\). Thus, prior to
our current study, there was little information in the literature about how aging impacts splenic FRCs.

We found, by employing flow cytometry and confocal microscopy, that aged spleens had reduced FRCs compared to young spleens, which correlated with reduced homeostatic chemokine concentrations and homing of young T cells. Unlike our previous publication, we found that CCL21 only marginally extended into the B cell follicles of aged mice. Architectural disruption of splenic white pulp and CCL21 localization may become more diffuse after activation in aged spleens. We only noted mild merging of the T cell zone and B cell follicles in naïve spleens, whereas after influenza infection, as shown in chapter two, B cell follicles are considerably more disorganized. This disruption may be due to altered chemokine production by stromal cells or it could be due to defects in chemokine receptor expression or sensing in lymphocytes, but this remains to be determined.

These changes could have the potential to delay immune response formation in aged spleens. Disruption of splenic architecture could potentially increase the time it takes for immune cells to find each other and interact. Delayed entry into the spleen could also increase the time it takes for the rare naïve T cell to find the dendritic cell presenting its cognate antigen. Importantly, T cells transferred into mice deficient in CCL19 and CCL21 have also been shown to have reduced velocity, which could further impact potential interactions with dendritic cells. Thus, the decrease in these chemokines with aging could potentially reduce T-DC interactions and T cell speed, which may increase the time necessary for immune responses to initiate.

Many aspects of splenic FRC biology remain to be elucidated. Basic questions such as if FRCs proliferate in the spleen after immune challenge or infection are still unanswered. It is even unclear if lymph node and splenic FRCs are of the same origin. Future studies can be performed to answer these basic questions. The field of lymph node FRC biology is much more advanced than its
splenic counterpart and, thus, in chapter four we sought to determine if changes in lymph node stromal cells were an underlying factor in impaired T cell immunity to influenza infection in aged mice.

Our hypothesis predicted that changes in aged FRC functionality, including increased inhibitory capacity, could contribute the impaired formation of robust T cell responses in aged mice to influenza infection. We also sought to answer some basic questions to address if aging impacts the number of FRCs, LECs and BECs at homeostasis and after influenza infection. Within the past two years, three studies have been published that attempt to quantify the number of stromal cells in aged lymph nodes at homeostasis\textsuperscript{30–32}. All three of these studies employed lymph node digestion protocols that may have skewed their results. Davies \textit{et al.} claimed that there were fewer total stromal cells in aged cervical lymph nodes, mainly due to fewer DNCs in aged lymph nodes\textsuperscript{31}. Turner \textit{et al.} found that aged lymph nodes (pooled cervical, axillary, brachial and inguinal) had fewer DNCs, but equal numbers of FRCs, LECs and BECs\textsuperscript{30}. The last published study by Becklund \textit{et al} claimed that there are fewer FRCs in aged compared to young lymph nodes at homeostasis but failed to perform statistical analysis to support their result\textsuperscript{32}. After several months of optimization, we were able to digest young and aged lymph nodes with high viability and reproducibility.

To further validate the reproducibility of our lymph node digestions, we digested both draining and non-draining lymph nodes during influenza infection, with the idea that non-draining lymph node stromal cell numbers should not change during the course of infection and would be a way to monitor variability in digestions. We determined that there were no significant differences in the number of total stromal cells, FRCs, LECs or BECs in MLNs or PLNs at homeostasis. Interestingly after influenza infection we found that aged MLNs had fewer total stromal cells, FRCs and LECs at day ten, a previously reported peak\textsuperscript{33}. The number of FRCs and LECs in aged MLNs reached that of young mice by day 12 post infection, showing a delayed response. The number of BECs
was equal at day ten post infection but higher at day 12 post infection in aged lymph nodes. We also determined that reduced proliferation, but not increased death may contribute to decreased FRC and LECs numbers at day ten post infection. The exact signals responsible for stromal cell proliferation are currently unclear. We tried to repeat several methods reported in the literature to get aged FRCs to proliferate after influenza infection and failed to repeat what was published (data not shown)\textsuperscript{34–36}. Future studies need to be performed to uncover the exact signals required for proliferation. Another possible mechanism by which FRCs can increase in number during an immune response is by the recruitment of precursor cells from adipose tissue\textsuperscript{37,38}. It remains to be determined how aging impacts FRC precursors in adipose and if they have impaired migration into MLNs after infection. This could be a contributing factor in the reduced number of FRCs at day ten post influenza infection in aged mice. The physiological consequences for altered stromal cell proliferation kinetics are unclear, since there is currently no way of specifically blocking stromal cell proliferation during infection or antigenic challenge. Studies using mouse models to conditional deplete FRCs have shown that they are required at the start of the immune response to influenza infection to make a strong adaptive immune response. Once the response has been initiated, FRCs can be depleted without hindering adaptive immunity\textsuperscript{33}. We hypothesize that altered stromal cell proliferation kinetics may possibly not have impact on the primary response to influenza, but may actually be a factor contributing to impaired memory T responses in aged mice\textsuperscript{39}. Fewer FRCs could reduce the recruitment of naïve T cells into aged lymph nodes late during the immune response, which is important since it has been reported that “latecomer” T cells are more prone to becoming memory cells\textsuperscript{40}. Further studies will need to be completed to test this hypothesis.

Since T cells have the greatest potential to interact with FRCs due to their location in the lymph node, we sought to further characterize FRC morphology and function in aged mice. Our hypothesis predicted that the FRC network in aged lymph nodes may be more disconnected,
which could potentially impair the ability of T cells and dendritic cells to locate each other in the lymph node and delay T cell responses in aged mice. This was in fact not the case, we found that the T cell zone FRC network was similar in young and aged MLNs at steady state and during influenza infection. It should be noted that our analysis of FRCs was limited to the T cell zone and there may be age related changes in FRC morphology in the medulla or near B cell follicles, which could be determined with further imaging studies. Extension of FRCs into the B cell follicles could be a contributing factor to the altered B cell follicle morphology in aged lymph nodes. We did determine that the FRC produced chemokine CCL21 did not appear localized in the B cell follicles of aged MLN, so extension of FRCs into the B cell follicle is probably not occurring with aging. Aged MLNs did have decreased concentrations of CCL19 and CCL21, which correlated with decreased recruitment of young naïve transferred T cells. After infection, the concentration of CCL21 dipped in young lymph nodes, but not aged lymph nodes, suggesting that the ability of aged FRCs to respond to challenge is reduced. Muller et al. showed that CD4+ T cells and interferon gamma were required for FRCs to decrease their expression of CCL21 during immune challenge. It is possible that there may be reduced levels of interferon gamma or CD4+ T cells in aged lymph nodes, which may provide inadequate signals for aged FRCs to down regulate expression of CCL21. This mechanism needs additional studies to be determined.

Another factor that may contribute to the reduced recruitment of young T cells into aged lymph nodes are HEVs. HEVs are the entry sites for lymphocytes into lymph nodes and alterations in these vessels may impair T cell entering. We found that HEVs in aged lymph nodes were thinner in morphology but expressed similar levels of ICAM-1, which is needed for T cell entry. Whether the altered morphology of aged HEVs contributes to reduced T cell homing remains to be determined. The ability of young T cells to traffic though aged HEVs could be analyzed in vivo using intra-vital microscopy, which could determine the time it takes for T cells to pass through HEVs. Our imaging data is just the start to understanding how HEVs age.
We next hypothesized that FRCs could have increased inhibitory capacity and be a factor in the impaired T cell responses to influenza infection. FRCs have the capacity to reduce T cell proliferation via production NO$^43$. Similar to previous studies using other models$^26$, we found that young influenza specific CD8+ T cells transferred into aged mice had reduced proliferation in aged MLNs after influenza infection when compared to young MLNs. We first used an *in vitro* method developed by Lukacs-Kornek *et al.* to see if FRCs contributed to this reduced proliferation$^43$. Our *in vitro* studies determined that FRCs from both young and aged mice had a similar capacity to inhibit T cell proliferation, with similar production of NO. *In vivo* we found that FRCs in young and aged MLNs upregulate expression of NOS2 equally after influenza infection. This outcome correlated with our *in vitro* results which suggested that aged FRCs maintain their suppressive capacity. There are of course limitations to these studies. The *in vitro* experiments could potentially select for FRCs in aged lymph nodes with a more functional phenotype, especially since they are grown in culture for six days. We tried to directly isolate FRCs from mice and co-culture them with CD8+ T cells and stimulation, but found that the digestion was too rough on the FRCs and they did not inhibit T cell proliferation with many of them dying in culture (data not shown). Our *in vitro* data is only correlative as well. Currently there are no mouse models available to conditionally deplete NOS2 in FRCs, which could be used during infection to determine if T cell proliferation was altered in the absence of NOS2. There are inhibitors of NOS2 available, but if used *in vivo* they could block NOS2 expression in other immune cells like macrophages and neutrophils and influence the course of infection$^{44}$. Of note, this is the first time that in either young or aged mice that NOS2 expression has been examined during the course of influenza infection in FRCs. LECs also have the ability to suppress the proliferation of activated T cells and it remains to be determined if changes in aged LECs could contribute to reduced proliferation of T cells in aged lymph nodes.
Multiple other factors may contribute to the reduced proliferation of young T cells in aged lymph nodes after influenza. Regulatory T cells are increased in aged mice and could be impairing T cell proliferation\textsuperscript{45-48}. Changes in the ability of aged dendritic cells to prime young T cells could also contribute to impaired proliferation. Several studies have shown that in vitro, aged dendritic cells are capable of priming young T cells with the same efficiency as young dendritic cells\textsuperscript{49,50}. Additional studies are needed to determine how aging impacts dendritic cell responses to influenza infection and to determine the mechanism behind this impaired T cell proliferation.

Although it appears as though FRCs are not directly blocking T cell proliferation, they may be an underlying factor in other age related problems, such as autoimmunity\textsuperscript{51}. FRCs have been shown to have a role in mediating peripheral tolerance via expression of deformed epidermal autoregulatory factor 1 homologue, which allows FRCs to express self antigens\textsuperscript{52}. FRC expression of tissue restricted antigens allows for the depletion of autoreactive T cells which may be involved in the development of autoimmunity\textsuperscript{53,54}. Additional studies are required to determine if aging alters this functional ability of FRCs. Multiple other aspects of the immune response including maintenance of T cell homeostasis could also be influenced by age related changes FRCs\textsuperscript{55}.

Since the studies in this thesis are the first to examine in depth how aging impacts lymph node stromal cells, many questions remain to be answered. Since stromal cells are just beginning to be appreciated for their role in immune responses, it is not surprising that many aspects of their biology remain to be elucidated. This research has laid the ground work for others to further characterize and test stromal cells as a factor in aged related immune impairment.

Although the picture is still incomplete, the research in this thesis has significantly progressed our understanding of the age immune response to influenza infection. This thesis has progressed our understanding of how aging impacts T\textsubscript{FH}, T\textsubscript{FR}, T reg, germinal center, FRCs, LECs, BECs, and
HEV responses at steady state and influenza infection. These studies are the foundation for the development of future treatments that could help the lives of countless elderly people.

REFERENCES


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


