IMMUNOSENESCENCE IN B CELLS: A STUDY ON CHANGES IN IMMUNOREGULATOR EXPRESSION AND METABOLISM WITH AGE

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Dedicated to my parents and my brother.

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ABSTRACT

IMMUNOSENESCENCE IN B CELLS: A STUDY ON CHANGES IN IMMUNOREGULATOR EXPRESSION AND METABOLISM WITH AGE

Senthil Kannan

Dr. Hildegund C.J. Ertl, M.D.

There is a vital need for better vaccines for the aging population, and especially better vaccines to influenza viruses. To address this, I studied immunosenescence in B cells and antibody secreting cells (ASCs) in mice and humans. In humans, I measured humoral immune responses to the trivalent inactivated influenza vaccine (TIV) during the 2011-12 and 2012-13 influenza seasons. ASCs in the aged were observed to have decreased expression of the defining markers CD27 and CD38. Aged ASCs also expressed lower levels of B and T Lymphocyte attenuator (BTLA) on their surface. Expression of BTLA inversely correlated with age and appeared to be linked to shifting the nature of the response from IgM to IgG. High BTLA expression on mature B cells was linked to higher IgG responses to the H1N1 virus. Finally, high BTLA expression on isotype switched memory B cells was linked to better preservation of virus neutralizing antibody titers and improved recall responses to influenza vaccination given the following year. In mice, aged ASCs expressed high levels of PD-1 and this correlated with high levels of cellular reactive oxygen species (cROS), indicating a link between immunoregulator expression and metabolism. Micro-array studies of bone marrow-derived ASCs showed substantial differences in their gene expression profile in aged as compared to young mice, which may explain their deteriorating functions. In summary, expression levels of immunoregulators on ASCs or their precursor populations change upon aging. Aged ASCs or their precursors are metabolically different from their younger counterparts, and these two factors are linked.

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CHAPTER 1 - INTRODUCTION

Aging is an inevitable part of life. In 2014, there were 46.7 million individuals in the U.S, who were 65 years of age or older, and they account for nearly 15% of the current population of the country (1). As healthcare and medical technologies progress, this proportion will continue to increase. The strongest predictor of this is the significant increase in life expectancy that we've seen over the past century – from only 50 years in 1900 to 83 in the 21st century (2).

This increase in life expectancy can be attributed to many technological advances. But there are two major breakthroughs that have had a vital role in human longevity – the production and availability of clean drinking water and vaccines.

To merely say vaccines have played a central role in human health would be an understatement. They have been singlehandedly responsible for saving millions of lives. It is due to vaccines that diseases that were once fatal, like smallpox, have effectively been eradicated. Thanks to major scientific breakthroughs, we now have vaccines to a wide variety of pathogens – from pediatric killers like rotavirus and measles, to ones like influenza and yellow fever, which affect people of all ages.

Unfortunately, the efficacy of vaccines drops rather sharply with age, as exemplified by the trivalent inactivated influenza vaccine (TIV). In the 2012/13 Influenza season, final analyses of vaccine efficacy in the US and Canada reported an efficacy of ~ 32% for influenza A viruses in the general population. However, in people over 65 years of age, the efficacy against influenza A viruses only reached 9%. (3) . This decline in function of the immune system with age is called immunosenescence – the major topic of this thesis.

Immunosenescence affects multiple aspects of both innate (4) and adaptive (5) (6) immunity. A carefully orchestrated interaction between the innate and adaptive immune systems produces an effective immune response (7)

In this system, the “first line of defense” towards pathogen invasion is usually the innate immune system, which tends to respond consistently to repeated infections, while the adaptive immune system prefers a different approach. The adaptive immune system responds slower, but in a more specific manner to the initial infection, and upon repeated infection it displays immune memory and produces a rapid, specific response against the pathogen. This is the immunological basis for vaccines.

Immunosenescence has been observed to affect multiple facets of the innate immune system. Type 1 IFN production by plasmacytoid dendritic cells in response to herpes viral infections has been observed to be impaired in the aged. A recent study in mice showed that aging affected the upregulation of IRF-7 – a critical transcription factor in the type 1 IFN signaling pathway (8). Age-induced oxidative stress was also observed to play a role in this phenotype, and thus is of particular interest to this thesis. In humans, a recent study from the same investigators reported a reduced ability of plasmacytoid dendritic cells from older humans to upregulate IRF-7 in response to a viral infection compared to plasmacytoid dendritic cells from younger people (9).

Another aspect of the innate immune system – NK cells – has also been observed to exhibit defects upon aging. This has been well studied in the murine immune response to the ectromelia virus – the mouse equivalent of human smallpox. Aged mice exhibited increased lethality and defective viral clearance, and most interestingly, did so in a T cell-extrinsic manner. Specifically, the accumulation of NK cells at the site of infection (in

this case, the footpad) was severely impaired in the aged compared to the young mice and this was primarily due to the reduced migration of $CD27^{+}CD11b^{-}$ mature NK cells (10).

The second arm of the immune system is the adaptive immune system. It can be broadly divided into cellular immune responses (primarily by $CD8^{+}$ T cells) and the humoral immune responses (by B cells, with/without T cell help). Aging has been shown to affect both arms in multiple ways.

To start with T cells: Alterations within the bone marrow and thymus lead to a shift in the composition of the T cell repertoire from naïve to antigen-experienced T cells, thereby compromising the diversity of the T cell pool. Additional infections with latent pathogens such as cytomegalovirus aggravate this process. A decrease in the number of antigen-inexperienced naïve T lymphocytes combined with an increase in antigen-experienced memory and effector T cells are the most substantial age-related changes within the T cell compartment. The initial trigger responsible for the dysregulated balance within the composition of the T cell pool observed in elderly persons is the involution of the T cell maturation organ, the thymus gland. The functional mass of the thymus decreases with age and the consequent reduction in naïve T cell output necessitates the homeostatic forces to take more responsibility in ensuring survival of T cells and keeping their numbers constant. Another key player in the maintenance of the T cell compartment is apoptosis, Apoptosis controls the selection of the T cell repertoire in the thymus, the deletion of self-reactive lymphocytes, the regulation of immunological memory, and the deletion of effector T cells (11). Further effects of aging on the immune system are telomere shortening, changes in T cell signaling, impaired DNA repair, and antioxidant

mechanisms, which may all contribute in regulating T cell survival and shaping of the repertoire (5).

The output of peripheral naïve T cells is dramatically reduced in the aged (up to 80%), leading to a reduced ability to respond to new antigens. This can happen due to two reasons. Firstly, declining Hematopoietic Stem Cell (HSC) functions, due to deficiencies in DNA damage and repair, shortening of telomeres and an overall reduction in hematopoietic tissue. Secondly, the severe reduction in thymic mass contributes to reduced production of mature T cells. The thymus is fully developed at birth, and soon after birth it starts getting replaced slowly by fat; by 50 years of age this thymus is almost completely composed of fat tissue. In contrast to naïve T cells, memory T cells rely on IL-7 in concert with IL-15 to cycle and self-renew in vivo> Memory T cells divide three- to fourfold faster than naïve T cells, and are capable of vigorous proliferation under lymphopenic conditions. In addition to the decrease in naïve T cell numbers, antigenic stimulation by persistent viral infections can challenge the tightly regulated orchestra of clonal expansion, contraction, and homeostasis of memory T cell and may thus lead to the massive accumulation of clones of certain specificities, culminating in a dramatically reduced diversity of the memory T cell pool in elderly individuals (12).

The other arm of the adaptive immune system – B cells – also exhibits a significant decline in function upon aging. Since B cells produce antibodies, the prime correlates of vaccine-induced protection against viral infections, they are of particular interest to vaccinologists, and thus this thesis. Multiple descriptive studies have shown the effects of immunosenescence on various steps in B cell development (13). Commitment to the B cell lineage from HSC progenitors involves key transcription networks (14). Once committed, the cells enter the pro-B cell stage where recombination activating gene

(RAG)-mediated IgH (heavy chain) gene rearrangement occurs. Upon successful IgH rearrangement, the cell surface expressed Ig heavy chain is associated with a surrogate light chain and the Ig-α and Ig-β signaling complex. B cells in this stage are called pre-B cells. Pre-B cells proliferate briefly, and rearrange their light chains to express a complete B cell receptor on their surface. They then transition into their next stage, immature B cells (13).

Immature B cells expressing a complete B cell receptor (BCR) on their surface now undergo a stringent specificity based selection, and mature upon exit from the bone marrow. This positive selection is vital in B cell development and involves a necessity for persistent low level BCR signaling, which results in survival-promoting signals. Thus, amongst peripheral B cells, there is a fitness hierarchy based on BCR specificity, with primary B cells competing against each other. (13).

This forms the next pool of B cells – the antigen-experienced B cell subsets –, which include memory cells, antibody secreting plasma cells (ASCs) and recently activated B cells. B cell responses to a pathogen can be either T cell dependent or independent, based on their requirement for cognate T cell help. T cell-independent responses generate short-lived IgM-secreting plasma cells, yield little humoral memory and lack substantial affinity maturation. On the other hand, T cell-dependent responses produce germinal centers, where activated B cells undergo class switch (CSR) and somatic hypermutation (SHM), culminating in cells producing the high-affinity class-switched antibodies crucial to effective long-term humoral immunity.

Immunosenescence affects B cells at multiple points along their developmental pathway. Upon aging, the frequency of HSC precursors that are capable of generating B cells is

reduced, similar to defects seen in T cell development. This results in smaller pools of pre-B and immature B cells (15) and thus bone marrow output. B cell lymphopoiesis is reduced with aging, leading to a decline of naïve B cells (16). Primary B cell responses in the elderly are commonly low and short-lived, resulting in antibodies with low affinity (17). Formation of germinal centers is decreased (18), antigen transport is impaired and follicular dendritic cells have reduced capacity to form antigen depots (19). Autoantibodies are more common (20) and the B cell repertoire becomes more restricted (21). Expression of the E2A-encoded transcription factor E47 is decreased in old splenic B cells, which causes a reduction in the activation-induced cytidine deaminase, needed for class switch recombination and Ig somatic hypermutation (22). Some of the defects of B cell responses are secondary to an age-related decline of helper functions from $CD4^+$ T cells, which show reduced expression of critical co-stimulatory receptors (23) (24). $CD4^+$ T cells in turn are essential for activation of B cells, germinal center formation, rearrangement and hypermutation of immunoglobulin (Ig) genes.

Since correlates of protection for most vaccines are neutralizing antibodies, the question how antibody secreting cell (ASCs), which are responsible for the production of neutralizing antibodies, change with age needs to be addressed. This led to the main hypotheses of this thesis:

a. *Expression levels of immunoregulators on ASCs or their precursors change upon aging.*
b. *Aged ASCs or their precursors are metabolically different from their younger counterparts*
c. *These two factors are linked.*

To address these hypotheses, I carried out a number of studies with human samples and in mice. In humans, I adopted an antigen-specific approach, using TIV, and studied immune responses to this vaccine. In mouse studies, due to the increased experimental freedom and availability of more complex sources of cells and tissues, such as spleen and bone marrow isolates, I was able to ask questions that could not be addressed with the more limited samples from human studies. This included a detailed look into age-related differences in gene expression profiles, metabolic changes upon aging, and their relation to immunoregulator expression.

Why did I choose to study influenza? Influenza is one of the top 10 causes of death in older adults. TIV consisting of two strains of influenza A and one or two strains of influenza B virus is approved for use in the elderly, but affords incomplete protection (25) (26). This incomplete protection to TIV has been linked in part to poor stimulation of B cells producing virus-neutralizing antibodies.

I started this study in 2011, to assess B cell responses of the aged to TIV in the post 2009 pandemic phase. I tested these responses in aged individuals of or above 65 years of age to the influenza A virus components of the 2011/12 TIV in comparison to a cohort of middle-aged individuals of 30-40 years of age.

The primary objective of the study was to compare antibody and B cell responses of the two cohorts with regard to magnitude and kinetics of responses using three complementary assay systems.

As expected, most individuals of the middle-aged cohort responded to both influenza A virus strains. Aged individuals more commonly responded to the H1N1 virus than to the

H3N2 virus. Interestingly within responders, vaccine-induced neutralizing antibody titers to H3N2 were comparable in magnitude between aged and younger individuals while the aged cohort mounted significantly lower neutralizing antibody titers to the H1N1 virus. At baseline, the aged had significantly higher levels of circulating IgG to both viruses compared to younger individuals. Analyses of peripheral blood mononuclear cells (PBMCs) by ELISpot assays showed no difference in responses between younger and aged individuals suggesting that low antibody responses in the aged related to cell intrinsic defects rather than lack of responding cells. Analysis of $CD19^+$ PBMCs by staining for CD27 and CD38 to detect ASCs by flow cytometry, a method, which upon influenza virus vaccination primarily detects vaccine-induced cells, showed only marginal increases in circulating ASCs in the aged while younger individuals showed far more pronounced increases. There was a pronounced discrepancy in the aged between results obtained by flow cytometry and ELISpot assays in that the former appeared to underestimate the vaccine-induced increases in ASCs observed with the latter. Further analysis suggested that in the aged, ASCs express reduced levels of CD27 and CD38. This is an important finding as loss or reduced expression of these two molecules, which are both involved in crucial signaling pathways, may negatively affect ASC functions. It also made the flow cytometric analysis of ASC frequencies in the aged unreliable as it is based on the detection of these two markers. A secondary objective of this study was to optimize time points post vaccination for optimal ASC retrieval, the completion of which directly led me to pursue my next human study.

In the second study conducted during the 2012-13 influenza season, I focused on expression of immunoregulators on different B cell populations, i.e., transitional B cells, mature naïve B cells, unswitched and switched memory B cells, double-negative B cells,

and antibody secreting cells (ASCs) defined by stains for CD19, IgD, CD20, CD27, and CD38 from aged and younger individuals before and on days 7 and 14 after TIV. In addition, I analyzed the VNA response to the two influenza A virus strains of TIV, i.e., H1N1 A/California/7/2009 pdm09-like virus and H3N2 A/Victoria/361/2011 virus to assess if the expression of immune regulators was linked to responsiveness to vaccination.

My results show that, as observed in the study of the previous year, the younger individuals mounted higher VNA responses to both viruses and had higher numbers of naive B cells compared to the aged. The B and T lymphocyte attenuator (BTLA), which upon interactions with the herpes virus entry mediator (HVEM) provides inhibitory signals (27) was significantly higher expressed on B cells from younger compared to aged individuals at baseline. BTLA, as well as percentages of $BTLA^{hi}$ B cells, selectively increased in the aged after vaccination so that differences between the two cohorts cease to be significant. Individuals with high BTLA on their mature B cells had better IgG responses to the H1N1 virus, compared to those with low BTLA. Further analysis showed that individuals with high BTLA expression levels showed better preservation of H1N1- and H3N2-specific VNA titers and superior booster response to the next annual dose of TIV compared to those with $BTLA^{lo}$ switched memory B cells. Overall these results show that a decline of BTLA during immunosenescence might contribute to lack of sustained antibody responses in the aged and to a reduction in their ability to mount recall responses.

Another hypothesis that I addressed was that immunosenescence causes metabolic changes in aging B cells and ASCs, which may in part be linked to differential

expression of immunoregulators. Immunosenescence involves a gradual deterioration of many biological processes. For example, the genome becomes unstable and accumulates mutations due to exogenous insults as well as endogenous sources of DNA damage such as increased production of reactive oxygen species (ROS), replication errors and declines in DNA repair mechanisms (28) (29). Mitochondrial DNA also becomes increasingly damaged in a similar fashion and energy production through the respiratory chain deteriorates (30).

Hence, in the third chapter, I further elucidated age-related defects in B cell metabolism using mice. In addition I tested if changes in metabolism are linked to changes in co-inhibitor expression. Whole genome expression arrays comparing bone marrow-derived ASCs from young and aged mice showed disparity of expression in over 1500 genes and distinct clustering. Genes that were differentially expressed participate in numerous pathways and functions including immunological and metabolic functions. Aged B cells have increased levels of ROS and show differential expression of immunoregulators such as PD-1. Expression of PD-1, which is known to affect key metabolic pathways, is strongly correlated with levels of cellular (c) ROS. Defects in mitochondrial functions were further confirmed upon *in vitro* stimulation of B cells.

In summary, this thesis addresses immunosenescence in B cells, specifically ASCs and their precursors from two perspectives – changes in expression of immunoregulators, their metabolism, and the relationship between these two factors.

CHAPTER 2 – B CELL RESPONSES TO THE 2011-12 INFLUENZA VACCINE IN THE AGED

Abstract:

Antibody and B cell responses to influenza A viruses were measured over a period of 2 months in 30 aged and 15 middle-aged individuals following vaccination with the 2011/12 trivalent inactivated influenza vaccine by micro-neutralization assays, ELISAs, ELISpot assays and cell surface staining with lineage-defining antibodies followed by multicolor flow cytometry. Both cohorts developed comparable antibody responses to the H3N2 virus of the vaccine while responses to the H1N1 virus were compromised in the aged. ELISpot assays of peripheral blood mononuclear cells (PBMCs) gave comparable results for the two cohorts. Analysis by flow cytometry upon staining of $CD19^{+}IgD^{-}CD20^{-}$ PBMCs with antibodies to CD27 and CD38 showed markedly reduced increases of such cells following vaccination in the aged. Additional analysis of cells from a subset of 10 younger and 10 aged individuals indicated that in the aged a portion of IgG producing cells fail to express CD27 and have reduced expression of CD38.

Introduction/purpose of study:

The aim of this study was twofold. Firstly, I wanted to study the kinetics of the humoral immune response to the TIV. Specifically, how different are the kinetics between the two aged groups. Would the aged respond poorer and slower? Secondly, I needed to optimize time points post vaccination for optimal ASC retrieval in subsequent studies.

Study design:

To assess responses of the aged to TIV in the post 2009 pandemic phase, we tested B cell responses of 30 aged individuals of or above 65 years of age to the influenza A virus

components of the 2011/12 TIV in comparison to a cohort of 15 middle-aged individuals of 30-40 years of age. As stated earlier, the objective of the study was to compare antibody and B cell responses of the two cohorts with regard to magnitude and kinetics of responses using three complementary assay systems. Details of our enrollment criteria are specified in the materials and methods section of this chapter. Basically, we only included immunocompetent individuals who did not have any chronic or other illnesses that would prevent completion of the study such as respiratory infections, and those with a history of substance abuse, and allergies to the components used in the TIV.

The subjects were vaccinated with the TIV by our collaborators at Duke University, and blood was collected from them before vaccination and at five time-points post vaccination – days 7, 10, 14, 28 and 60.

Results:

Human subjects:

Twenty subjects reported influenza vaccination during the previous 5 years, 3 were unsure and 2 reported influenza-like illnesses. Thirteen of those that reported previous vaccinations had been immunized in 2010 when the pandemic H1N1 strain was incorporated into the vaccine. Sixteen individuals between 30-40 years of age with an average age of 36 were enrolled and 15 completed the study. 9 of the 15 younger individuals were female and 6 were male, 9 were Caucasian, 5 were African American and 1 was of mixed ethnicity. The average age of the aged cohort was 74 ranging from 65-87. 20 individuals were female, 10 were male. The majority [20] was Caucasian, two

were African Americans, one was of mixed ethnicity and one was either American Indian or Alaskan Native.

There were no serious adverse events (SAEs) following vaccination in either age group.

Neutralizing antibody responses to the vaccine are comparable:

Serum was isolated from the blood collected at the previously mentioned time points, and tested for vaccine-specific antibodies by two methods. Microneutralization assays were performed to measure levels of neutralizing antibodies and Enzyme Linked Immuno-Sorbent Assays (ELISAs) were performed to measure levels of virus-binding antibodies. Both assays were conducted in a virus-specific manner against the two A strains of the influenza virus in the 2011-12 TIV (H1N1 A/California/04/2009 and H3N2 A/Perth/16/2009).

At baseline, both cohorts had comparable antibody titers to the two viruses. Percentages of individuals with antibody titers of or above 1:40 to either virus were slightly higher in the aged than in the younger cohort with 33% of younger vs. 40% of aged being positive for H1N1 virus ($p=0.725$) and 40% of younger vs. 60% of older individuals being positive for H3N2 virus ($p=0.466$). Aged subjects, who were enrolled towards the end of the study, more commonly had neutralizing antibody titers of or above 1:40 than those that were enrolled earlier. More specifically of the 11 elderly individuals that were first tested after Mid-March of 2012, 91% were sero-positive for the H1N1 virus while 72% were positive for the H3N2 virus. In contrast of the 19 aged individuals recruited early only two (i.e., 11%) was positive for antibodies to H1N1 at the time of vaccination ($p<0.001$) while 10 (52%) were sero-positive for the H3N2 virus ($p=0.490$). This bias at baseline was not seen for younger individuals all of whom had been enrolled by the end of February.

Upon vaccination 8 aged individuals failed to respond to the H1N1 virus and 10 were unresponsive to the H3N2 virus.

Importantly, kinetics of antibody responses differed between the two cohorts, albeit slightly. (**Figure 1**) Average titers peaked in the younger cohort by day 10 after vaccination and only marginally declined by the end of the study. In the aged peak titers to H1N1 were also reached by day 10 after vaccination while average peak responses to the H3N2 virus were delayed to day 14 reflecting a significant difference in time to peak response ($p = 0.0316$). The decline of antibody titers was more pronounced in the aged although this did not reach significance ($p = 0.057$).

To summarize, neutralizing antibody responses were comparable between the two age groups, but a potential shift in kinetics was observed.

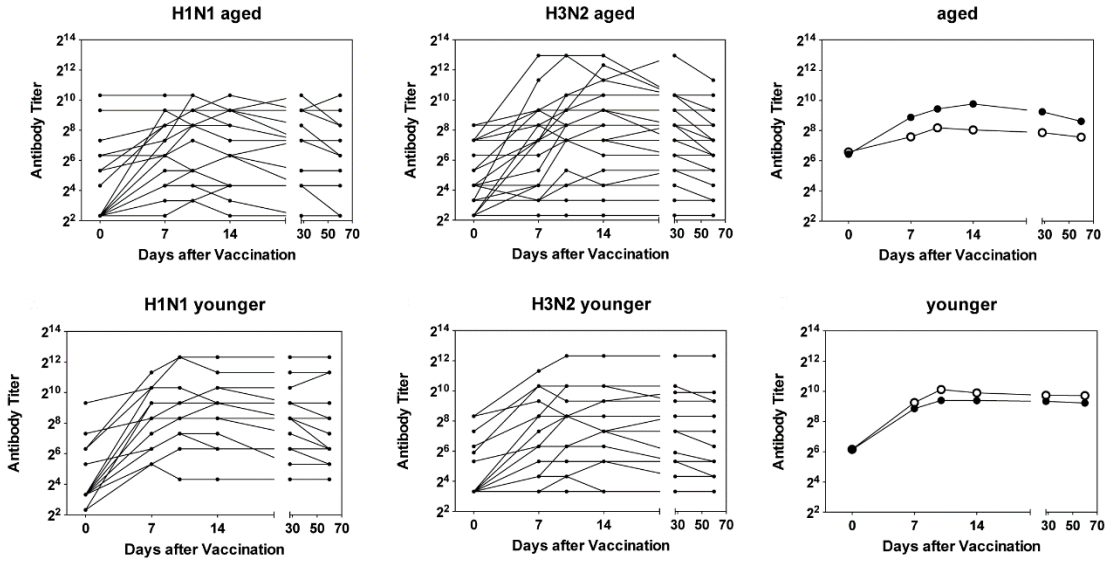

Figure 1: Antibody Titers. Titers of H1N1-specific antibodies in individual sera are shown for aged and younger subjects in the first column. Titers of H3N2-specific antibodies in individual sera are shown in the second column. Mean titers of H1N1 (closed circles) and H3N2 (open circles) are shown in the column on the right. * indicates significant differences from baseline/ day 0.

Binding antibody responses to the TIV are comparable, but the kinetics of the response are different:

To test for antibody isotypes, sera collected at baseline and on days 10 and 28 following vaccination were tested by ELISAs on plates coated with H3N2 or H1N1 virus (**Figure 2**).

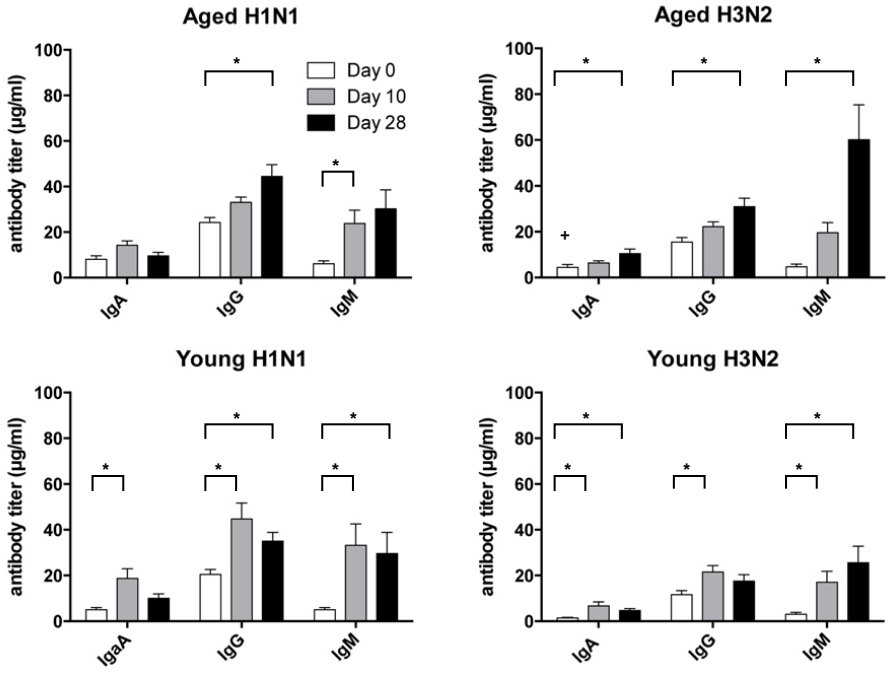

Figure 2. Antibody Isotypes. Serum samples collected on days 0, 10 and 28 following vaccination were tested for IgA, IgG and IgM to H1N1 and H3N2 viruses by ELISAs. Graphs show average titers ± SEM. * indicates statistical significance ($p<0.05$) as calculated using the Mann Whitney test. + indicates statistical significance ($p<0.05$) as difference between age groups as calculated using the Mann Whitney test.

At baseline, antibody responses were slightly higher in the aged although this only reached significance for H3N2-specific IgA. The younger cohort responded quicker,

showing significant increases by day 10, in all three isotypes (IgA, IgG and IgM) to both viruses. The aged, on the other hand, mainly showed significant increases in binding antibody titers by day 28. The only exception was the IgM response to H1N1, which increased by day 10.

Responsiveness by ELISA differed between the two cohorts. Responsiveness is defined as a 4 or more fold increase in circulating antibodies of either isotype at either of the two time points. One of the younger individuals failed to show responsiveness to H3N2 virus, while three failed to respond to H1N1; the non-responder to H3N2 virus also failed to respond to H1N1 virus. Non-responsiveness by ELISA was more common in the aged; 3 and 11 individuals failed to show significant increases of antibodies of either isotype to H3N2 and H1N1 virus, respectively on either day 7 or 28. Of the non-responders, the 3 individuals that failed to respond to H3N2 virus also did not develop increased antibodies to H1N1 virus. Aged individuals, who did not mount a recall response to H1N1 virus, had significantly higher levels of specific IgG at baseline ($p<0.05$) compared to responders. The same trend was seen for H3N2 virus but failed to reach significance.

In summary, binding antibody responses to the TIV were comparable between the two age groups, but the kinetics were considerably different, with the younger cohort responding quicker to both the H1N1 and H3N2 viruses.

Circulating B cell populations are lower in the aged:

The two B cell subsets that serve as precursors to my population of interest (ASCs) are naïve mature B cells and memory B cells. So, I measured these two subsets. Aged individuals had significantly lower number of naïve B cells at baseline ($p = 0.004$) and numbers remained stable after vaccination (**Figure 3**).

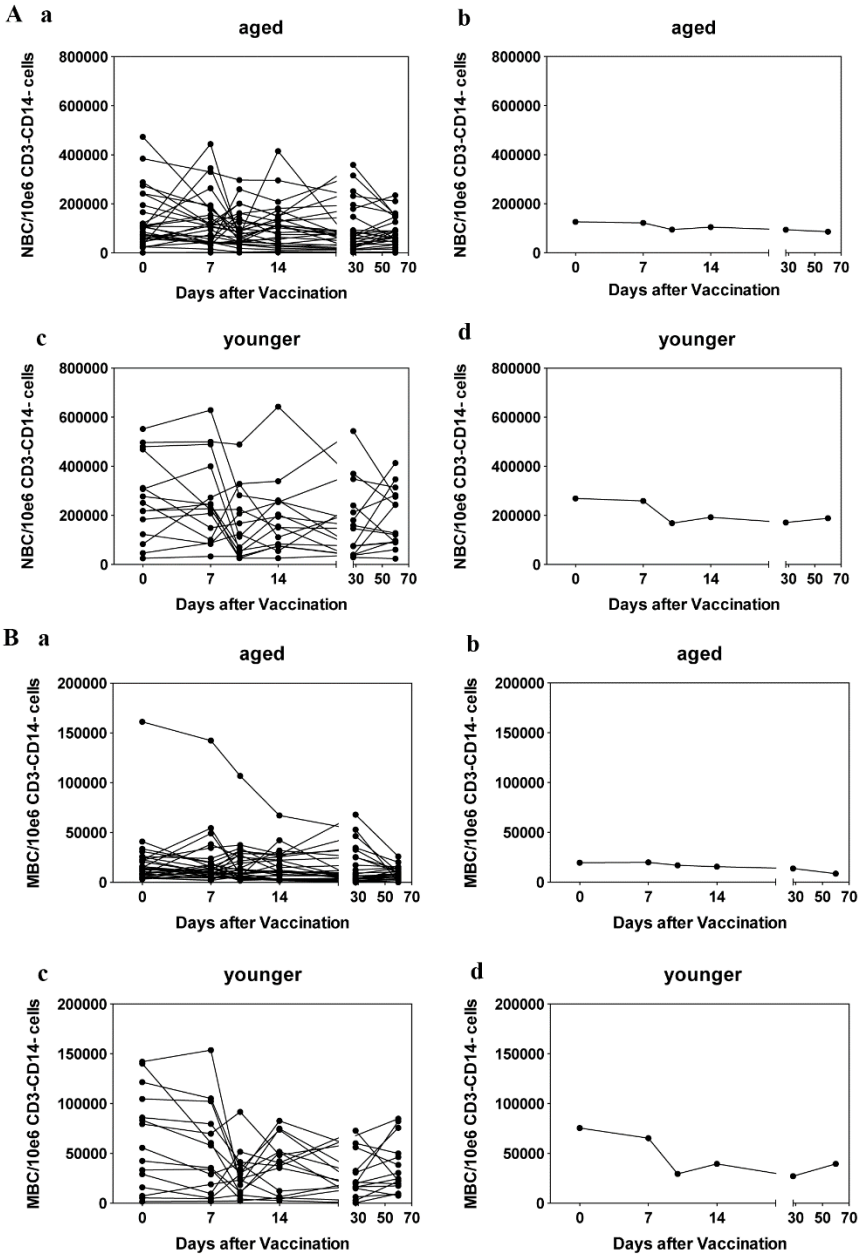

Figure 3. Circulating B cells. 3A shows numbers of naïve B cells per 10^6 $CD3^-CD14^-$ live PBMCs. Naïve B cells (NBCs) were identified by gating on $CD19^+CD20^+CD27^+CD38^-IgD^+$ cells. (**a**) shows data for PBMCs from individual aged subjects, (**b**) shows means of the same set of data, (**c**) shows data for PBMCs from individual younger subjects, (**d**) shows means for the same data. **3B** shows numbers of memory B cells per 10^6 $CD3^-CD14^-$ live PBMCs. Memory B cells were identified by gating on $CD19^+CD20^+CD27^+CD38^-IgD^-$ cells.

In younger individuals, numbers significantly (p=0.045) declined by day 10 following vaccination and for the duration of the study remained below levels seen at baseline. Memory B cells were also higher in younger than aged individuals (p = 0.002) at baseline and in the both cohorts significantly declined by day 10 following vaccinations (p = 0.016 for the younger and 0.036 for the aged).

Vaccine-induced circulating influenza virus-specific ASCs are comparable:

PBMCs from 10 younger and 28 aged individuals collected at the 6 time points mentioned were analyzed for vaccine-induced responses to the two influenza A viruses of the vaccine by ELISpot assays (**Figure 4**). Responders based on this assay had to show at least a 2-fold increase in ASCs and a minimum of 10 influenza virus-specific spots per 10^6 PBMCs following vaccination.

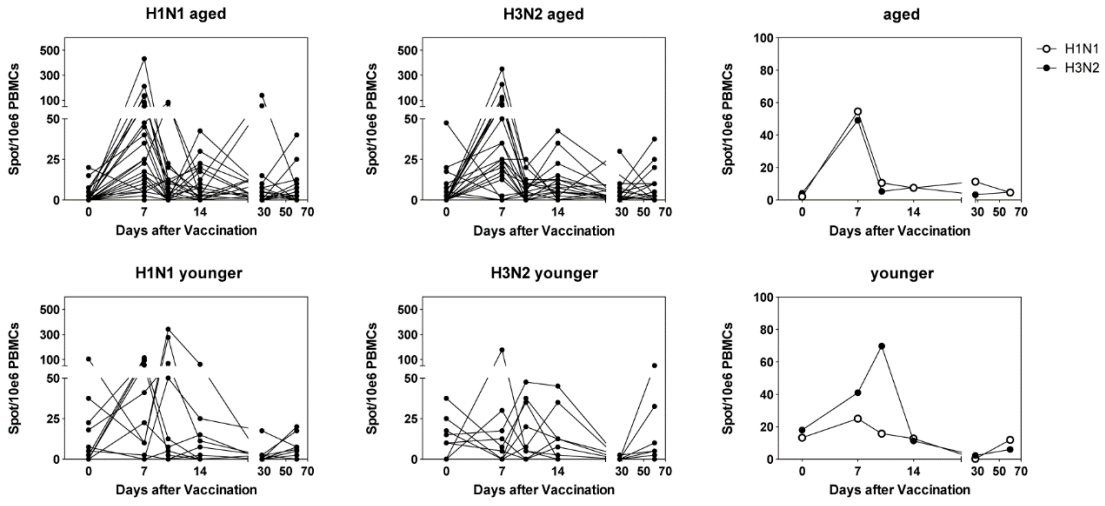

Figure 4. ELISpot Results. Spots per 10^6 live PBMCs are shown for individual samples in the first two columns. Mean results for aged and younger subjects are shown in the column on the right. The graphs are arranged as in Figure 1.

At baseline only two versus four aged individuals had ≥10 spots to H1N1 or H3N2 virus, respectively. Three aged individuals failed to respond to the H1N1 vaccine; seven aged subjects did not show an increase in H3N2-specific spots. Of the 10 younger individuals, 3 had positive spots for H1N1 virus while 6 were positive for H3N2 virus at baseline. Two younger subjects failed to respond to H1N1 virus; five younger individuals failed to respond to H3N2 virus. The kinetics of responses in the aged showed a sharp peak on day 7 to both viruses, which was followed in some individuals by additional smaller peaks on days 14 or 28. Younger individuals showed peaks either on days 7 or 10. There was no significant difference in magnitude or time to peak responses between the two cohorts. In summary, virus-specific ASCs were comparable between the two cohorts when measured by ELISpot assays.

Circulating ASCs measured by flow cytometry are significantly lower in the aged:

ASCs can be identified by cell surface markers that distinguish different B cell populations. Specifically, in humans ASCs are negative for IgD and CD20 but express high to intermediate levels of CD19 and high levels of CD38 and CD27. Upon influenza vaccination a transient rise in circulating ASCs is mainly reflective of vaccine-induced cells and up to 80% of such cells secrete antibodies specific to the antigens in the vaccine. PBMCs were therefore stained with lineage-defining antibodies before and at the indicated time points after vaccination. Samples were analyzed by flow cytometry and the post-acquisition-gating scheme is shown in **Figure 5**, data are shown in **Figure 6**.

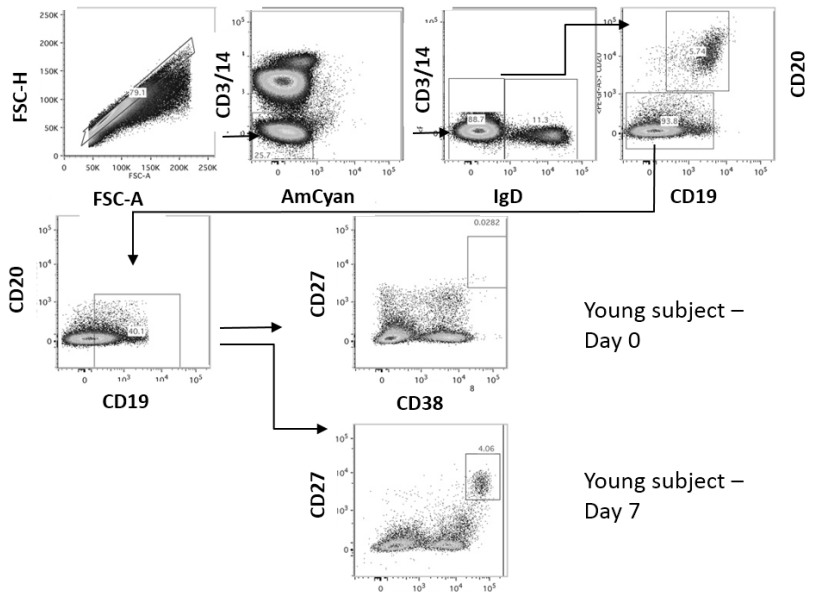

Figure 5. Gating scheme for ASCs. Graphs on the bottom right shows results of a young individual at baseline and 7 days post-vaccination

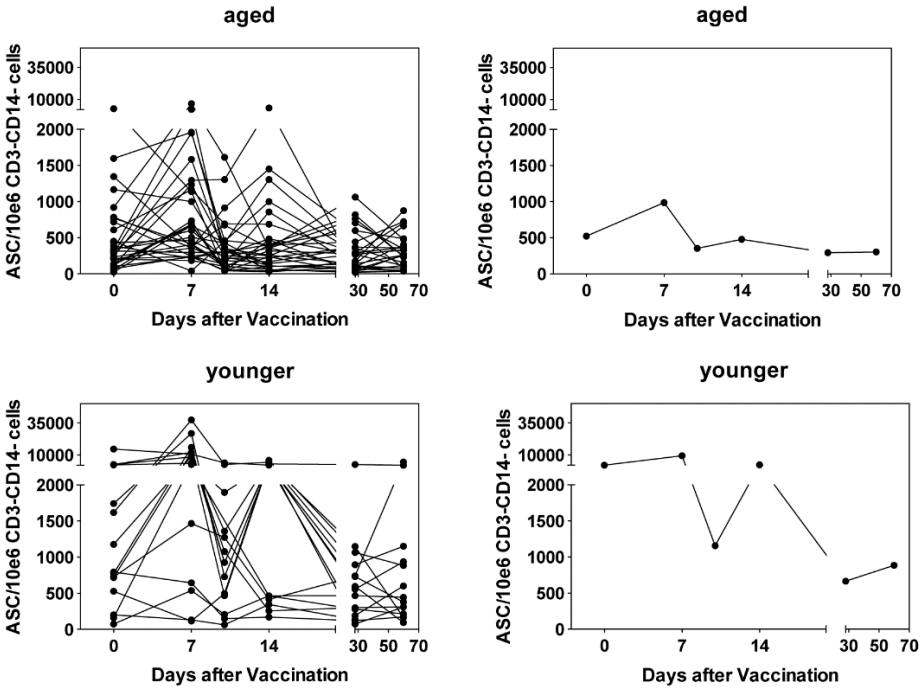

Figure 6. Circulating ASCs. Graphs show numbers of circulating ASCs per 106 CD3-CD14- live PBMCs.

Individuals with more than 1000 circulating ASCs over 10^6 $CD3^-CD14^-$ live lymphocytes were defined as high baseline responders; subjects that showed an at least 2-fold increase in ASCs over baseline within 14 days following vaccination were deemed responders.

The younger cohort developed significantly higher numbers of ASCs upon vaccination than the aged ($p = 0.013$) despite having a significantly higher portion of subjects with high ASC counts at baseline (7/15 vs 4/30 in the aged; $p = 0.04$ by Fischer exact t-test) Four younger subjects failed to show increases in ASCs within 2 weeks after vaccination, two of those had high ASC counts at baseline. Sixteen of the aged individuals failed to show a two-fold increase in ASCs and this included all of the subjects with high ASC numbers at baseline. Again correlation with antibody responsiveness to vaccination was poor, only one of the subjects that failed to show increases in ASC numbers failed to show an antibody response to the H3N2 virus of the vaccine.

Several human subjects reported symptoms indicative of an infection during the 60-day follow-up period of the study. Specifically 8 aged individuals reported coughs, runny noses or sinusitis at the 5^{th} or 6^{th} study visit by when vaccine-induced changes in ASC counts should have subsided. Of those 5 showed delayed increases in ASC counts, which coincided with their cold symptoms. Of the 6 younger individuals, who reported cold-symptoms at either of the last two visits, none developed accompanying increases in ASC counts.

Expression of CD27 and CD38 is decreased on aged ASCs:

Poor responses in the aged as measured by increases in circulating ASCs detected by high expression of CD38 and CD27 on $CD20^{-}IgD^{-}CD19^{+}$ cells following vaccinations did not correspond to the results obtained by ELISpot assays, which revealed comparable influenza virus-specific ASC frequencies in blood of younger and aged individuals by day 7 following vaccination. This may have reflected that key markers, which were used for identification of ASCs by flow cytometry, were differentially expressed depending on age. We therefore repeated the cell stains with day 7 cryopreserved PBMC samples from 10 younger and 10 aged individuals including an intracellular stain for IgG, which is only present in isotype-switched ASCs. After flow cytometry, blots were gated on ASCs based on high expression of CD38 and CD27 as shown in **Figure 4**. Alternatively they were gated onto $CD3^{-}CD14^{-}CD20^{-}IgD^{-}CD19^{+}IgG^{+}CD38^{+}$ cells relaxing the CD38 gate and including cells that showed intermediate expression (**Figure 7**). In either cohort, cells that showed high expression of CD38 also carried high levels of IgG. A second population that was mainly detected in the aged expressed intermediate levels of CD38 and carried intermediate levels of IgG. Cells of this subset were largely $CD27^{-}$. Of note $CD20^{+}$ cells were IgG^{-}, indicating that IgG^{+} cells that were detected by the analysis did not belong to the memory B cell pool, which express CD20 and surface IgG.

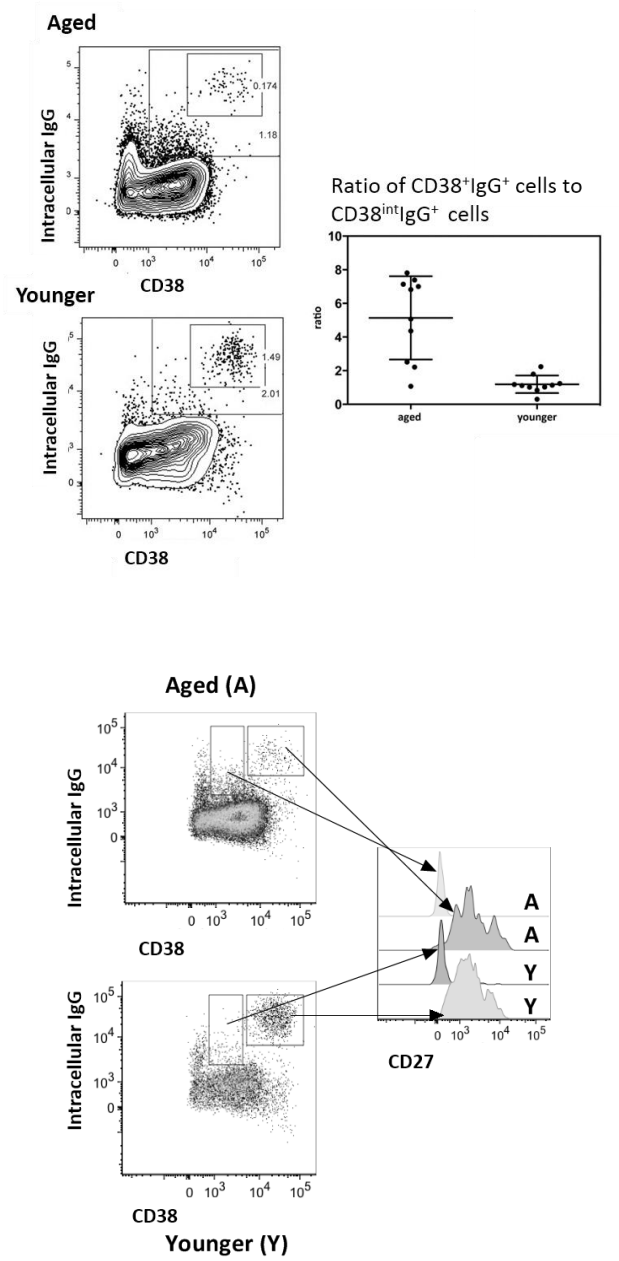

Figure 7: Expression of intracellular IgG: Graphs on top left show day 7 ASCs the graphs on the left show PBMCs of representative aged/younger individuals harvested 7 days after TIV vaccination. Cells were gated onto live CD3-CD14-IgD-CD20-CD19+ cells. They were then gated onto CD38 over IgG. The smaller gate shows $CD38^{hi}IgG^{hi}$ cells, the larger gate shows cells that are $CD38^{int\text{-}hi}$ and $IgG^{int\text{-}hi}$. Graph on top right shows the ratios of normalized cells obtained with the $CD38^{int\text{-}hi}$ $IgG^{int\text{-}hi}$ gate or the traditional ASC gate as shown in Figure 5. Means and SDs are shown. Graphs on bottom show another representative younger/aged individual as described in the top half, with histograms showing CD27 expression on the right.

Calculating numbers of ASCs based on either gating scheme, and then comparing the results showed that in younger individuals, both gating schemes resulted in approximately equal numbers of ASCs indicating that cells positive for intracellular IgG were also high in CD38 and CD27. In the aged, the latter gating scheme in most individuals resulted in markedly higher numbers of cells ($p = 0.001$) (**Figure 7**) indicating that portion of the IgG-producing cells in the aged had reduced expression of CD38 and lacked expression of CD27 (shown in the second part of the figure with CD27 histograms).

Discussion:

Here, I tested acute B cell responses following TIV vaccination given in winter and spring of 2011/12 to cohorts of aged and middle-aged human subjects residing in the Triangle area of North Carolina. The study was undertaken to compare antibody and B cell responses of the two cohorts with regard to magnitude and kinetics of responses using three complementary assay systems.

A higher percentage of the aged cohort (63%) compared to the younger cohort (27%) reported influenza vaccinations during the 5 years prior to the study. However, average neutralizing antibody titers and percentages of individuals with neutralizing antibody titer ≥1:40 at baseline to either of the two strains of influenza A viruses present in the vaccine were comparable between the two groups. In contrast, binding antibodies of the IgG isotype to H1N1 and H3N2 were significantly higher prior to vaccination in the aged. This discrepancy may reflect that IgG antibodies in the aged at baseline were primarily directed to conserved non neutralizing epitopes that were shared between the vaccine strains and other previously circulating strains. Aged subjects that reported vaccinations

or infections during the 5 years prior to this study had on average higher neutralizing and binding antibody titers at baseline as compared to individuals that had not been exposed to antigens of influenza virus. This difference reached significance for H3N2-specific neutralizing antibodies ($p = 0.035$) and for binding IgG to both viruses. Aged subjects that were first vaccinated after mid-March of 2012 showed increased rates of neutralizing antibodies to the two influenza A virus strains prior to vaccination and differences in titers were significant for both viruses. In most years influenza activity is highest in January or early February and then declines. In the 2011/12-influenza season, positive tests reported to the CDC did not peak till mid-March. It is thus tempting to speculate that an increased portion of aged subjects that were not vaccinated till mid-March had experienced subclinical infections, which had elicited B cell recall responses.

Upon vaccination most subjects of the younger age group showed increases in neutralizing antibody responses that were comparable between the two influenza A virus strains at all time points tested. Lack of neutralizing antibody responses was more common to the H3N2 virus. 2 out of the 3 younger non-responders to H3N2 were noteworthy since they lacked specific antibodies at baseline in contrast to the one younger non-responder to H1N1 who had robust baseline titers. The aged cohort also had a higher proportion of individuals who failed to show increases in neutralizing antibodies to both viruses - H1N1 (27% of aged vs. 7% of younger) or the H3N2 (33% vs. 20%). After vaccination, neutralizing antibody responses to H3N2 virus of aged individuals were comparable in magnitude to those of younger individuals. Peak antibody responses to H3N2 virus were seen at day 14 in the aged and were slightly delayed compared to the younger cohort, which reached maximal responses by day 10. Antibody responses to H1N1 virus were compromised in the aged. Average titers upon

vaccination, comparing titers either in all individuals or only in responding individuals were both significantly below those in younger individuals ($p = 0.037$ for all individuals, $p = 0.011$ excluding non responders) at all time points tested. These results are in contrast to a previous study, which reported increased antibody responses in the post pandemic phase to H1N1 vaccination (31) but in agreement with a study conducted in Singapore which also reported decreased responses to the H1N1 vaccine in aged individuals (32) and another study conducted prior to the 2009 pandemic, which showed good responses to H3N2 and poor responses to H1N1 in the aged (33).

In this study, aged subjects that reported neither influenza vaccinations nor infections during the 5 years prior to this study developed significantly lower titers to H1N1 upon vaccination ($p = 0.011$) as compared to those who recalled either. Yet again, this is in contrast to a previous study, which showed that in adults between 22-49 years of age recent vaccination decreased antibody responses to vaccination with TIV (34) and may indicate that the aged are able to mount recall responses but have defective primary responses to TIV. Previous studies have reported that vaccine-induced antibody responses decline more rapidly in the aged (35). In our study, although average titers in the aged declined more markedly by two months after vaccination than in younger individuals this difference was subtle and did not reach significance ($p = 0.057$).

Initially at day 7 after vaccination both cohorts showed significant increases in IgM to H3N2 and H1N1 suggestive of primary responses. Both cohorts also mounted significant IgG responses to both viruses, which are likely to reflect recall responses. Interestingly, the pattern of the IgG and to some degree the IgM responses differed between the younger and aged cohorts; IgG and IgM responses to H1N1 and IgG responses to H3N2

were higher on day 7 than day 28 in younger individuals while in the aged IgG and IgM titers to both viruses increased between days 7 and 28. The pandemic H1N1 virus was routinely incorporated into the annual vaccine as of 2010. Thirteen of the aged individual received the influenza vaccine in 2010. These individuals had significantly higher levels of H1N1-specific IgG at baseline ($p = 0.001$). Upon vaccination their IgG responses to H1N1 were initially higher on day 7 than those of aged individuals that had not been vaccinated in 2010 but by day 28 after vaccination responses including isotypes of H1N1-specific antibodies were indistinguishable between the two aged subgroups. Neutralizing antibody titers became significantly higher in 2010 vaccine recipients on days 10 and 14 following vaccination in 2011. Again, this difference was not sustained.

Patterns of responsiveness assessed by the ELISpot assay, which detects all antibodies to influenza virus regardless of their function, differed from those of circulating antibodies. Younger individuals more commonly had positive responses at baseline and were less likely to respond with 20% and 50% showing no responses to H1N1 and H3N2 virus respectively. Non-responsiveness in the younger cohort was with one exception only observed in subjects that had either ≥ 10 spots per 10^6 PBMCs at baseline or had neutralizing antibody titers ≥1:40 at baseline; the one exception reported annual influenza vaccinations. Within the aged cohort only 11% failed to show increases of circulating B cells to H1N1 virus while 24% lacked responses to H3N2 virus. Lack of responsiveness by ELISpot in the aged was only seen in individuals with high numbers of specific spots at baseline or recent vaccinations.

In the younger cohort, peak ELISpot responses to both viruses were observed between days 7-14 with subjects that had high spot numbers at baseline typically responding

later. The aged showed a dominant peak on day 7 followed in some individuals by a less pronounced increase on day 14. Two aged individuals showed delayed peaks on day 28 following vaccination, and both reported previous vaccinations and had high antibody titers at baseline. Magnitude of responses to either virus was not significantly different between the two cohorts.

The most pronounced age-related defects were seen upon staining of $CD19^{+}$ PBMCs with antibodies to CD27 and CD38, which are typically used to identify ASCs. Upon exposure to antigen, naïve and memory B cells are stimulated and after undergoing antigen-driven proliferation and/or hypermutations can transiently be detected in blood. It has been reported that, in young individuals by day 7 following influenza vaccination, up to 80% of circulating ASCs are specific to antigens of the vaccine (36). Younger individuals had significantly higher baseline numbers of circulating ASCs as compared to the aged, as has been reported previously (37). Upon vaccination 73% of the younger individuals showed marked increases in $CD38^{hi}CD27^{hi}$ ASCs by day 7, which then decreased by day 10 and again in 73% of individuals increased by day 14. Lack of increases on day 14 were seen in younger individuals that reported recent vaccination suggesting that the ASCs circulating on day 7 were primarily derived from memory B cells while those on day 14 reflected de novo stimulated B cells. Of the aged, only 37% vs. 30% showed increases in $CD38^{hi}CD27^{hi}$ ASCs by day 7 and 14 respectively.

Contradicting results obtained by two of the antigen-specific methods of antibody and B cell detection, i.e., the microneutralization and the ELISpot assays, with the former unlike the latter showing reduced responsiveness mainly to the H1N1 virus in the aged can easily be explained; the microneutralization assay only detects antibodies to neutralizing

epitopes of the viral surface antigens while the ELISpot measures circulating ASCs to influenza virus regardless of their fine-specificity or function and thus allows for detection of B cells producing antibodies to non-neutralizing and more conserved epitopes. Furthermore, influenza virus-specific ASCs of the aged may have produced on average lower numbers of antibody molecules, which would have resulted in reduced neutralizing antibody titers without affecting numbers of specific ASCs. We view this as unlikely as increases of H1N1-specific antibodies following TIV measured by ELISA were similar in aged and younger individuals, suggesting that aged B cells more commonly produced non neutralizing antibodies directed presumably to epitopes that are conserved between the 2011 H1N1 vaccine strain and previously circulating strains, a principle long known as "antigenic sin" (38). Such cross-reactive antibodies may be less suited to provide protection against infection. Contradicting results obtained by surface staining and ELISpot assays, with the former indicating defective responses in the aged while the latter showed comparable responses in both cohorts, are harder to reconcile. We, like others, identified ASCs by expression of CD19, a pan B cell lineage marker, and high expression of CD27 and CD38 on $CD3^{-}CD14^{-}IgD^{-}CD20^{-}$ cells. It is feasible that upon aging expression of these markers declines on ASCs. It is well known that in the elderly expression of the co-stimulator CD28 declines on T cells resulting in reduced T cell responsiveness (39). CD27 is a member of the tumor necrosis family and signals through the tumor necrosis factor receptor-associated factors (TRAF) 2 and 5 to activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinase (MAPK) 8 and JUN (40). On B cells it is a marker of previous encounter with antigen and thus used to define memory B cells. $CD27^{-}$ memory B cells have been described and it is thought that such B cells are either induced outside

germinal centers (41) or prematurely leave germinal centers before they acquire CD27 (42). $CD27^-$ memory B cells show lower frequencies of somatic hyper-mutations compared to their $CD27^+$ counterparts (43) suggesting either the lack of or incomplete maturation within germinal centers. In the aged, numbers of circulating IgD^-CD27^- memory B cells increase (44), which presumably reflects defects in germinal center formation and our results suggest lack of CD27 on recently activated circulating ASCs. CD38, which distinguishes ASCs from memory B cells, is also expressed on activated T cells and previous studies have shown a decline of $CD8^+CD38^+$ T cells during aging (45). Cell surface expressed CD38 upon its ligation activates the phosphoinositide 3-kinase (PI3K) pathway, which through Protein Kinase B (Akt) and the mammalian target of rapamycin (mTOR) increases glucose uptake and glycolysis thus meeting the increased energetic needs of activated lymphocytes. Intracellular CD38, which was not assessed by our staining method, also cannibalizes nicotinamide adenine dinucleotide (NAD) (46) and thereby reduces the activity of sirtuin-1, a nuclear energy sensor which increases stress-resistance and influences metabolism through promotion of gluconeogenesis, fatty acid oxidation, and mitochondrial biogenesis (47) (48). Reduced surface expression of CD38 by aged ASCs would thus be expected to negatively affect cell metabolism, which is compatible with our finding of markedly lower levels of IgG in $CD38^{int}$ as compared to $CD38^{hi}$ cells.

In summary, in the 2011/12 influenza season aged individuals responded well to the H3N2 virus of TIV but mounted lower responses to the H1N1 virus. ASCs of the aged showed reduced expression of two signaling molecules which corresponded with reduced levels of IgG production, revealing a novel and hitherto undescribed defect of adaptive immunity that arises during immunosenescence.

The goal of this study was to compare the responses to the TIV in the aged cohort to the young, and also establish post-vaccination time points for future studies. *I successfully achieved both goals, and identified days 7 and 14 post vaccination for optimal ASC retrieval. This helped me focus on the next study – measuring immunoregulator expression levels in aged B cells – the next chapter in this thesis.*

Materials and methods:

Viruses: Stocks of H1N1 A/California/04/2009 and H3N2 A/Perth/16/2009, the two influenza A virus strains of the 2011 influenza vaccines, were obtained from the Center for Disease Control, Atlanta, Georgia. Viruses were expanded in 10 day-old specific pathogen-free (SPF) embryonated eggs for 48hrs at 35°C. After 48hrs allantoic fluid from the infected eggs was isolated and concentrated by centrifuging at 20,000rpm for 1 hour at 4°C. The pellet was re-suspended in PBS and further purified by a 55-10% sucrose density gradient. The virus was titrated by hemagglutination assay with chicken red blood cells. The mean tissue culture infective dose (TCID50) was determined by serially diluting virus on Madin-Darby Canine Kidney (MDCK) cells and screening cells 3 days later for viral plaques. Infectious virus was used for neutralizing antibody assays or inactivated by a 45-minute treatment with betapropionolactone for ELISpot assays.

Human Subjects: Blood was collected after informed consent from community dwelling persons in the Durham-Raleigh-Chapel Hill area of North Carolina. Younger individuals were 30-40 years of age; older individuals were ≥ 65 years of age. The following subjects were excluded from the study: [1] humans with immunosuppression resulting from diseases (e.g., clinically active malignancy, HIV/AIDS, immune disorders) or drugs (e.g., cancer chemotherapy, corticosteroid use); [2] individuals with significant underlying diseases that would be expected to prevent completion of the study; [3] subjects, which were bed-ridden or homebound or had intercurrent illnesses that might interfere with interpretation of study (e.g., urinary tract infection, respiratory tract infection); [4] individuals that were unlikely to adhere to protocol follow-up; [5] subjects that were involved in a conflicting study; [6] subjects that had a history of alcohol or substance abuse; [7] subjects with contraindication for influenza vaccination such as anaphylactic

hypersensitivity to eggs or to other components of the influenza vaccine, and moderate or severe acute illness with or without fever, and Guillain-Barre Syndrome within 6 weeks following a previous dose of influenza vaccine. Persons with moderate to severe acute febrile illness were not vaccinated until their symptoms have abated.

From enrolled subjects demographic data and medical history including medical diagnoses, medications, vaccination to influenza and other infectious diseases, and history of influenza or influenza-like diseases during the last 5 years were recorded. Subjects were bled and then vaccinated with TIV via the intramuscular route in the deltoid muscle. Subjects were bled again on days 7, 10, 14, 28 and 60 following TIV vaccination. Change in medical history, change in medications, influenza-like illness, adverse events (AE) and SAEs were assessed by solicited reports at each study visit and unsolicited reports from subjects at any time after study enrollment. Solicited AEs included symptoms of influenza-like illness and injection site complaints, including pain, tenderness, redness, and swelling.

Collection of Blood and Isolation of PBMCs and Plasma: Blood was collected into heparinized tubes and shipped overnight to Philadelphia. A 2 ml aliquot of each sample was set aside for serum collection. PBMCs were isolated from the remaining samples using established protocols. Specifically, blood was overlaid onto Ficoll-Paque Plus (GE Healthcare Biosciences, Piscataway Township, NJ) and spun for 30 minutes at 2000 rpm, with brake off, and at 50% acceleration. The PBMC layer at the Ficoll interface was then collected and washed twice with Hank's Balanced Salt Solution (Gibco, Grand Island, NY), by centrifuging at 2000 rpm. The washed, pelleted cells were then treated with 10 ml of red blood cell lysis buffer (eBioscience, San Diego, CA). Lysis was stopped by adding 5 ml of Roswell Park Memorial Institute (RPMI) medium supplemented with

10% fetal bovine serum [FBS] and washed using Hank's Salt (HBSS). Cells were then resuspended in 5ml of Dulbecco's modified Eagles medium (DMEM), live cells were counted using Trypan Blue as a diluent.

Micro-Neutralization Assay: Influenza specific micro-neutralization assay was performed in 96 well plates. Briefly, heat-inactivated human sera were serially diluted (1:10 to 1:5120) in serum-free Minimal Essential medium (MEM) in a 96 well plate. Equal volume of the two Influenza strains, Influenza A/H1N1/2009/California and Influenza A/H3N2/2009/ Perth at 50 $TCID_{50}$ was added to serum samples and incubated for 1 hour at 37°C. After 1 hour, serum-virus mixtures were added to Madin Darby canine kidney (MDCK) cells and further incubated for 2 hours at 37°C with 5% CO2. The plates were washed and replaced with MEM containing Tosyl phenylalanyl chloromethyl ketone-modified trypsin after incubation and scored for cytopathic effect after 3 days. The highest serum dilution in which 50% of the MDCK cells were intact was scored as the neutralization titer.

ELISA: To measure H1N1/California and H3N2/Perth-specific antibody isotypes, wells of Nunc Maxisorp™ plate were coated with 10µg/ml of each virus in bicarbonate buffer overnight at 4°C. Isotype standards for IgA1, IgG and IgM (Athens Research & Technology, Inc., Georgia, USA) were also included in each plate. After coating, plates were washed and blocked with 3%BSA in PBS containing 0.05% Tween. Heat-inactivated sera of young and old subjects from day 0, 10 and 28 were diluted to 1/250 and added to the plate for 2h at room temperature, followed by washing 4X with PBST. Alkaline phosphatase conjugated mouse anti-human IgA1 at 1:1000, IgG at 1:3000 and IgM at1:1000 (SouthernBiotech, Alabama, USA) dilutions were added to the plates and incubated for 1h at room temperature. Plates were further washed 4X with PBST and

developed using alkaline phosphatase substrate containing pNPP tablets (Sigma Aldrich, Missouri, USA) in DEA buffer and absorbance was recorded at 405nm. The absorbance values were plotted against standard curves from each plate for every isotype and the concentration was determined and is expressed in µg/ml.

ELISpot Assay: 96 well immobilin-P membrane plates (Millipore, Billerica, MA) were coated with 10µg/ml of H1N1 A/California/04/2009 or H3N2 A/Perth/16/2009 virus overnight. Negative control (PBS alone) and positive (total human Igs) wells were also included. The plates were washed 4X with PBS and blocked with 10% RPMI medium for 2hrs at 37°C. Freshly isolated PBMCs from human subjects were added onto the plate at 2x105 cells/well in duplicates and incubated overnight in a humidified 5% CO2 incubator at 37°C. The plates were further washed 6X with PBS containing 0.05% Tween and incubated with alkaline phosphatase conjugated anti-human IgG (Sigma Aldrich, Missouri, USA) at 1:1000 dilution for 1 hrs at 37°C. After the incubation the plates were washed 6X with PBST and developed using alkaline phosphatase substrate kit (Vector Labs, Burlingame, CA) the spots were analyzed using CTL Immunospot (Cellular Technology, Ltd., Cleveland, OH). The spots/well was calculated by subtracting spots in negative control wells and normalizing spots to 10^6 PBMCs cells.

B cell Detection by Flow Cytometry: 3 x 10^6 cells of each sample were used for flow cytometric analyses. Cells were initially treated with Human TruStain FcX Fc Receptor Blocking solution (BioLegend, San Diego, CA) for 30 minutes, washed with PBS at 1500 rpm for 5 minutes and then stained with fluorochrome-conjugated antibodies. The following antibodies were used: CD19-APC-Cy7, CD20-PE (BioLegend), IgD-APC CD38-PerCPCy5.5, CD3-Pacific Blue, CD14-Pacific Blue, CD27-FITC, and AmCyan Aquablue as a live cell stain. The optimal concentrations of these antibodies were

determined experimentally prior to the study. Samples were stained for 30 minutes at room temperature, washed with PBS and then resuspended in 150µl of fixative (BD Pharmingen). All antibodies were obtained from BD Biosciences (San Jose, CA) unless specified differently. The stained samples were analyzed in a LSRII flow cytometer (BD Biosciences, San Jose, CA).

Cells were gated on lymphoid single cells and then on live cells that were negative for CD3 and CD14. For ASC identification IgD^{+} and $CD20^{+}$ cells were excluded and $CD19^{hi}$ cells were gated on $CD38^{hi}$ and $CD27^{hi}$. In some samples a stain for intracellular IgG was included. In these samples $CD19^{hi}CD38^{+}$ samples upon exclusions of cells expressing CD3, CD14, IgD or CD20 were gated onto IgG^{+} cells. Memory B cells were identified by gating on $CD19^{+}CD20^{+}CD27^{+}CD38^{-}IgD^{-}$ cells. Naïve B cells were identified by gating onto $CD19^{+}CD20^{+}CD27^{+}CD38^{-}IgD^{+}$ cells. To detect intracellular IgG, samples were stained for extracellular markers, as described above. The cells were then permeabilized using Cytofix/Cytoperm (BD Bio-sciences) for 30 minutes at 4°C. Cells were washed with Permwash (BD Biosciences) and stained with anti-human IgG-Alexa 700 (BD Biosciences) with Permwash as a diluent for 30 minutes. Samples were fixed, as described above.

Portions of this chapter were adapted from:

B cell responses to the 2011/12-influenza vaccine in the aged. Kannan S, Kurupati RK, Xiang ZQ, Doyle S, Ratcliffe S, Schmader KE, Ertl HC. Aging (Albany NY). 2013 Mar; 5(3):209-26.

CHAPTER 3 – BTLA EXPRESSION DECLINES ON B CELLS OF THE AGED AND IS ASSOCIATED WITH LOW RESPONSIVENESS TO THE TRIVALENT INFLUENZA VACCINE

Abstract:

Virus-neutralizing antibody and B cell responses to influenza A viruses were measured in 35 aged and 28 middle-aged individuals following vaccination with the 2012 and 2013 trivalent inactivated influenza vaccines. Antibody responses to the vaccine strains were lower in the aged. An analysis of B cell subsets by flow cytometry with stains for immunoregulators showed that B cells of multiple subsets from the aged as compared to younger human subjects showed differences in the expression of the co-inhibitor B and T lymphocyte attenuator (BTLA). Expression of BTLA inversely correlated with age and appears to be linked to shifting the nature of the response from IgM to IgG. High BTLA expression on mature B cells was linked to higher IgG responses to the H1N1 virus. Finally, high BTLA expression on isotype switched memory B cells was linked to better preservation of virus neutralizing antibody titers and improved recall responses to vaccination given the following year.

Introduction/Purpose of study:

The aims of this study were to measure the expression levels of immunoregulators on ASCs, how their expression changed with age, and if expression levels influenced antibody responses to TIV.

Study design:

35 aged individuals of or above 65 years of age and 28 younger individuals between 30-40 years of age were enrolled in the Durham-Raleigh-Chapel Hill of North Carolina. Briefly, the aged were on average 76 years of age ranging from 66 to 88 years of age,

with 64% females and 95% Caucasians. Most individuals but for 4 had been vaccinated in 2011, the majority (58%) reported annual vaccinations with TIV. Younger individuals were on average 35 years of age with 64% females, 75% Caucasians, 21% African Americans and 4% Asians. All but two individuals received the 2011/12 Flu vaccine and 43% reported annual vaccination to influenza. Some of these individuals were re-vaccinated the following year with the 2012/13 TIV. VNA responses were tested against the two influenza A virus strains of the vaccine, i.e., H1N1 A/California/7/2009 pdm09-like virus and H3N2 A/Victoria/361/2011 virus. Immune responses were tested from blood at baseline and on days 7 and 14 after TIV.

Results:

Younger subjects responded better to the H1N1 vaccine:

Both cohorts responded to the to H1N1 California//7/2009 (from here on referred to as H1N1), but the younger subjects responded better. The younger subjects had higher baseline VNA titers to H1N1 ($p = 0.0025$ by Wilcoxon Rank analysis); titers to H3N2 Victoria/361/2011 (from here on referred to H3N2) were comparable (**Figure 8**). After vaccination both cohorts developed increased VNA titers to H1N1 and H3N2. VNA responses to H1N1 tested at baseline or on days 7 and 14 after vaccination were significantly higher in younger than aged individuals (**Figure 8A**). Responses to H3N2 virus were also higher in the younger cohort, although this only reached significance for day 7 (**Figure 8B**).

Younger individuals have more mature B cells:

The previous chapter showed that mature B cells were significantly lower in the aged subjects. In order to verify that this was a consistent observation and to investigate if

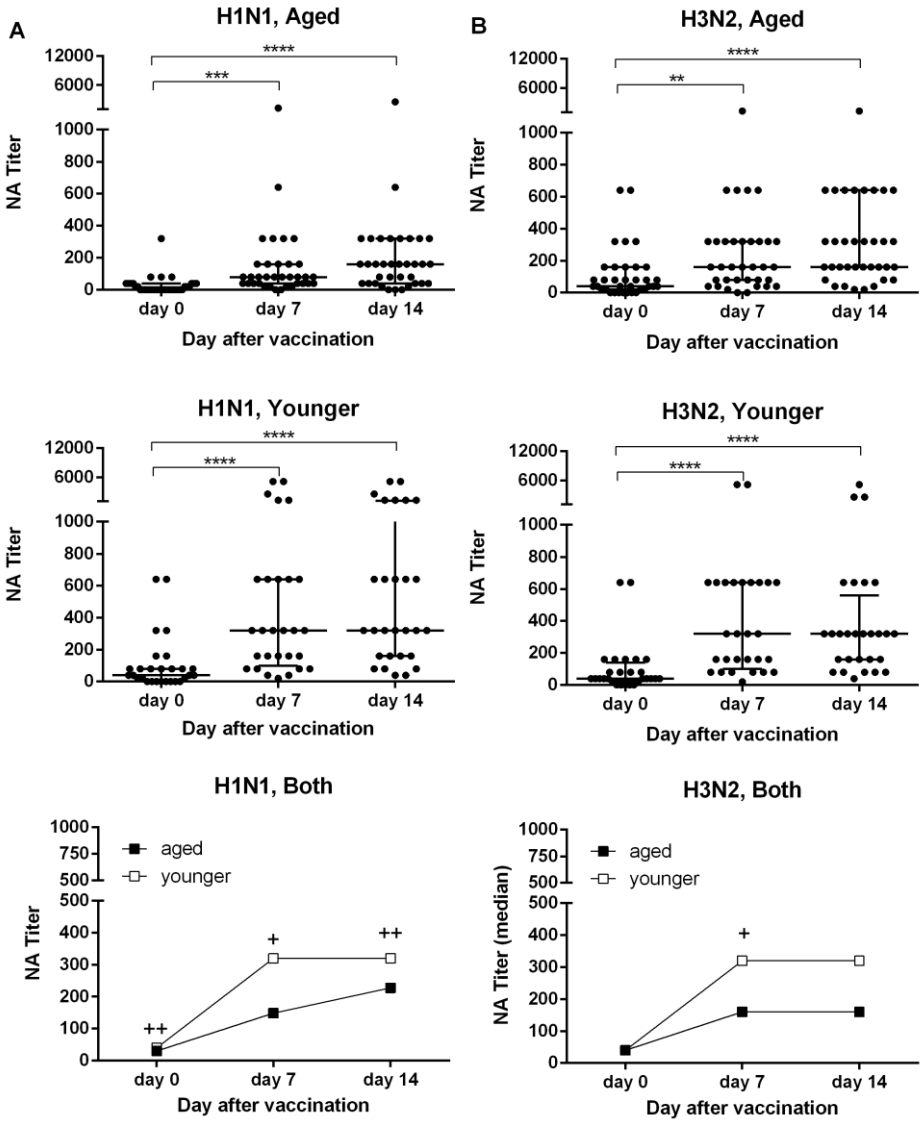

Figure 8: VNA Responses to Influenza A Viruses. Sera were tested by a microneutralization assay on H1N1 California/7/2009 [**A**], and H3N2 Victoria/361/20011 [**B**] virus. Graphs on the top and in the middle show VNA responses of aged and younger individuals at baseline and on days 7 and 14 after TIV. Data are shown for individual sera, lines show medians ± Interquartile range (IQR). Graphs on the bottom show median titers of the two cohorts. (*) Indicates significant differences between titers on day 0 compared to days 7 and 14 in graphs that show individual sera calculated by Friedman test with Dunn correction with the following p - values: age, H3N2: d7 $p = 0.0013$, all other comparisons $p < 0.0001$. In the graphs showing median titers for both cohorts, significant differences between the young and aged indicated by (+) were calculated by Wilcoxon matched-pairs signed rank test with the following p-values. H1N1: d0 $p = 0.0025$, d7 $p = 0.0038$, d14 $p = 0.037$.

other B cell subsets exhibited a similar pattern, we tested for a numbers of different B cell subsets by flow cytometry upon staining of PBMCs with antibodies to lineage defining markers (**Figure 9**).

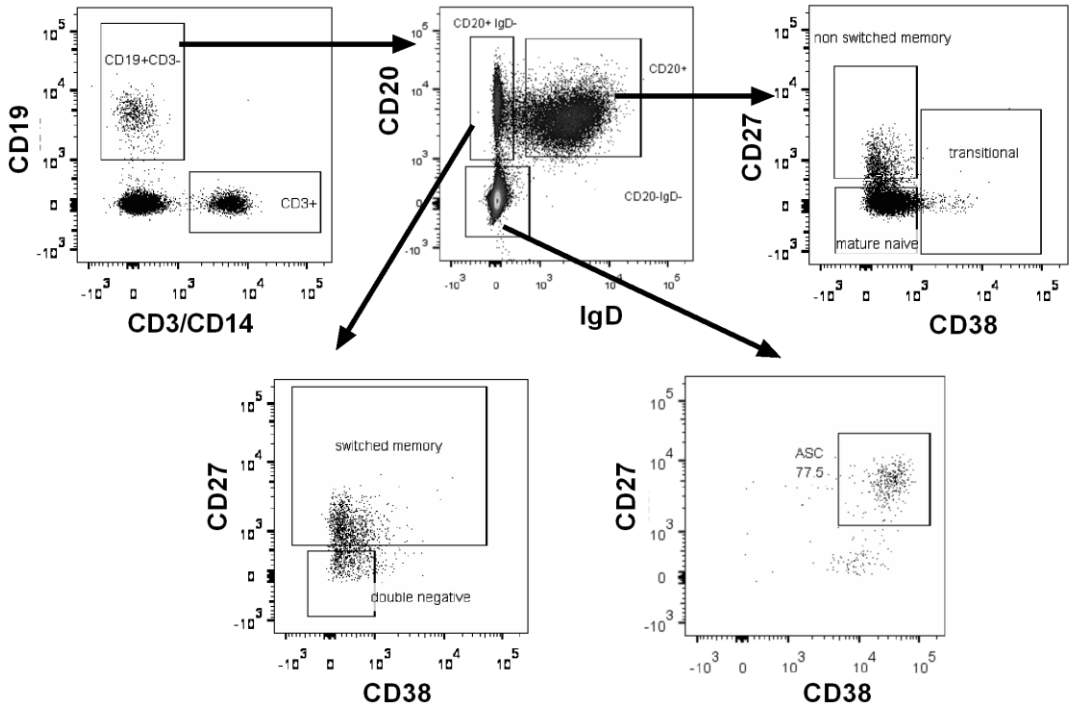

Figure 9: Flow cytometry gating scheme: This figure shows the gating scheme for the different B cell subsets, i.e., transitional B cells ($CD19^+CD20^+IgD^+CD27^{+/-}CD38^{+/-}$), mature naïve B cells ($CD19^+CD20^+IgD^+CD27^-CD38^-$), non-switched memory B cells ($CD19^+CD20^+IgD^+CD27^+CD38^-$), switched memory B cells ($CD19^+CD20^{+/-}IgD^-CD27^+CD38$, double-negative B cells ($CD19^+CD20^+IgD^-CD27^-CD38^-$) and antibody secreting cells.

Numbers of naïve B cells ($CD19^+IgD^+$) in blood were at all three time points higher in the younger individuals ($p < 0.0001$) (**Figure 10**). There was a trend towards increased numbers of transitional B cells, which form a link between immature B cells in bone marrow and mature naïve B cells in the periphery, in younger individuals; this failed to reach significance. The same was seen for double-negative IgD^-CD27^- B cells, which

may reflect exhausted B cells that have previously been described to be more common in the aged (49), as well as for switched memory B cells. Numbers of cells within the individual subsets were stable over time but for ASCs which in younger and aged individuals showed a non-significant trend towards increases on day 7 after vaccination as compared to baseline.

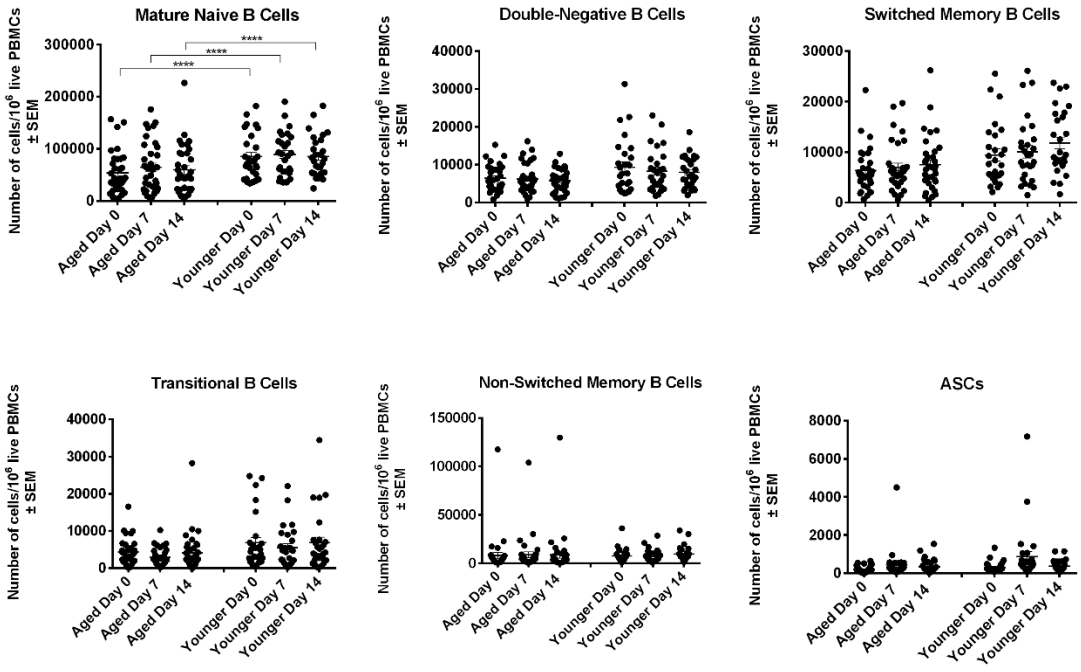

Figure 10: Circulating B cell subsets: Graphs show the cell counts of different B cell subsets, i.e., transitional B cells ($CD19^+CD20^+IgD^+CD27^{+/-}CD38^{+/-}$), mature naïve B cells ($CD19^+CD20^+IgD^+CD27^-CD38^-$), non-switched memory B cells ($CD19^+CD20^+IgD^+CD27^+CD38^-$), switched memory B cells ($CD19^+CD20^{+/-}IgD^-CD27^+CD38$, double-negative B cells ($CD19^+CD20^+IgD^-CD27^-CD38^-$) and antibody secreting cells. Cell counts are normalized to 10^6 live PBMCs, with error bars indicating Standard Error of the Mean (SEM). **** indicates p-values <0.0001 as calculated by two-way ANOVA, corrected for multiple comparisons with Tukey.

BTLA is significantly lower on aged B cell subsets:

It has been shown previously for T cells that the expression of co-inhibitors changes upon aging (50). We tested B cells for a number of different markers at baseline and upon vaccination, BTLA was found to show significant age-related differences (**Figure 11**).

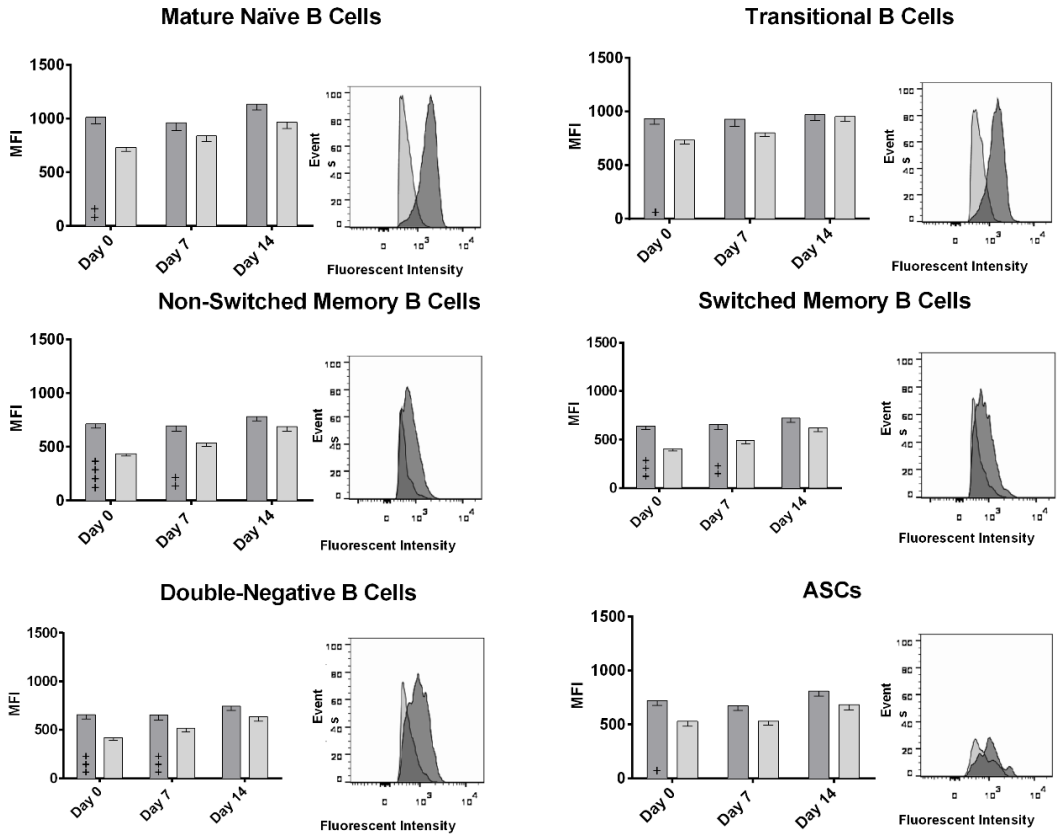

Figure 11: BTLA expression on B cell subsets: Mean Fluorescent Intensity (MFI) of BTLA on various B cell subsets at all three time points tested, are shown. Darker bars/histograms represent the younger cohort, and the lighter bars/histograms represent the aged cohort. Histograms of a representative pair show the MFI intensity on the x-axis and events normalized to Mode on the y axis. Within each cell subset, the graph shows the MFI with error bars denoting Standard Error of Mean (SEM). (+) within the bars indicate statistically significant differences between the two age groups at that time point. P-values were calculated using two way ANOVA, corrected for multiple comparisons using the Holm-Sidak correction.

At baseline, BTLA expression and percentages of BTLA^{hi} cells were significantly higher for B cells of all subsets from younger than aged individuals (**Figure 12**). After vaccination, expression levels slightly increased on aged B cells while percentages of BTLA^{hi} cells increased significantly in the aged for several of the B cell subsets. By day 7 after vaccination, differences between aged and younger B cells were only significant for non-switched memory B cells, switched memory B cells and double-negative B cells. By day 14 after vaccination, both levels of BTLA expression and percentages of BTLA^{hi} cells became comparable for all B cell subsets between the two age groups.

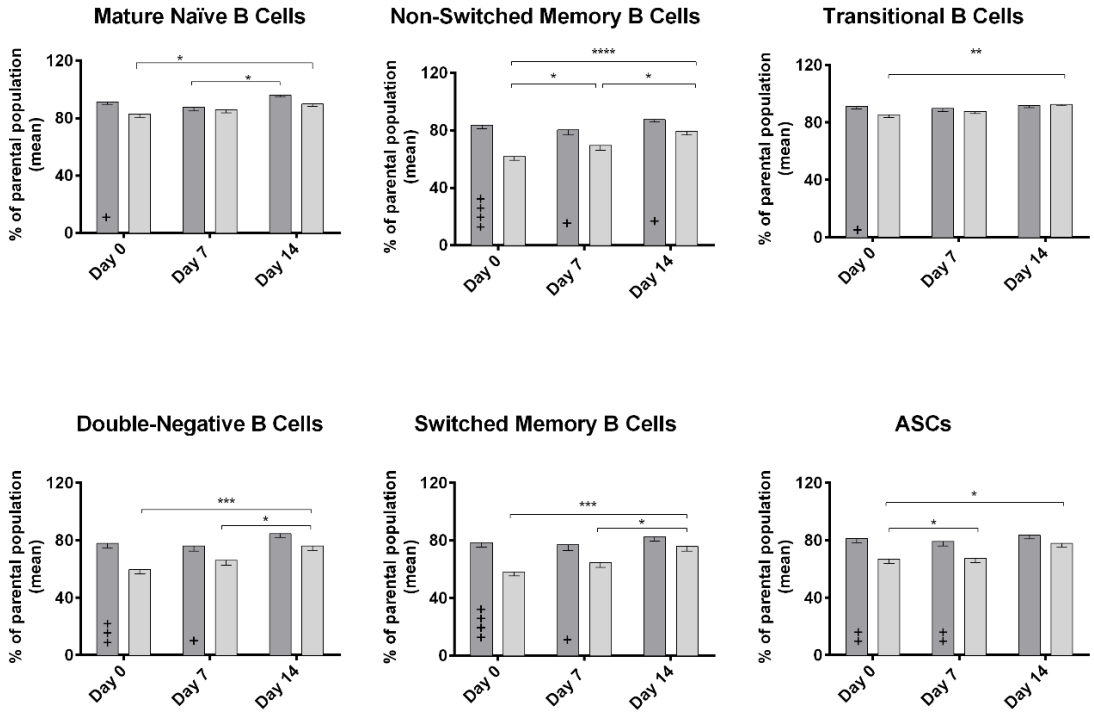

Figure 12: Percentage of BTLA^{high} cells is lower in the aged: Figure shows the % of cells that were BTLA^{hi} over the parental population. (+) within the bars indicate statistically significant differences between the two age groups at that time point. (*) above the bars indicate statistically significant differences within the same age group, between different time points as indicated by the lines. p-values were calculated using two way ANOVA, corrected for multiple comparisons using the Holm-Sidak correction.

BTLA expression at baseline showed a strong negative correlation with the biological age of the individuals on all B cell subsets again supporting that declines in expression of the co-inhibitor are age-related (**Figure 13A**).

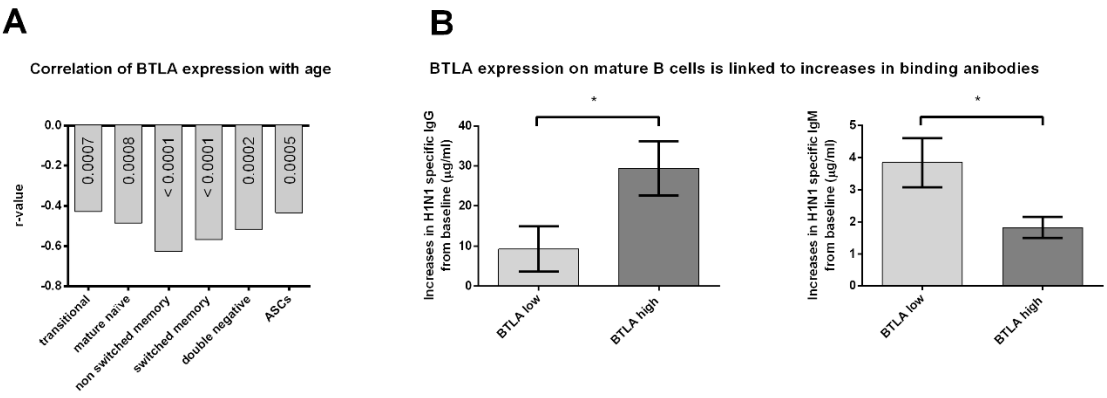

Figure 13: Correlation of BTLA expression with age and BTLA related increases in antibody titers: **A** shows the r- values of correlation of BTLA Mean Fluorescent Intensity (MFI) with age, as calculated by nonparametric Spearman correlation. All p-values were highly significant (<0.00001). Mature naïve: p=0.00008; Non Switched Memory: $p=7.89e^{-7}$; Transitional: p=0.00066; Double negative: p=0.00002; Switched memory: $p=2.21e^{-6}$; ASC: p=0.00053.

B shows the mean (±SEM) increases in antibody titers to the H1N1 influenza virus, in individuals with either high BTLA on their mature B cells (BTLA high) shown in dark grey or low BTLA on their mature B cells (BTLA low) shown in light grey. * indicates statistical significance ($p<0.05$) as calculated using the Mann Whitney test. p=0.028 for IgG. p=0.035 for IgM. Antibody titers were measured by ELISA.

BTLA expression on mature B cells is linked to higher increases in antibody titers:

To see if BTLA expression was related to antibody production, we looked at antibody titers of individuals with either high or low levels of BTLA on their mature B cells. We tested this in an age independent manner, to remove the age bias as the focus of the question was on the link between BTLA and antibody production, irrespective of age. We selected ten individuals who had either the highest (BTLA high) or lowest (BTLA low)

expression on their mature B cells, at baseline/, and looked at their virus specific antibody titers by ELISA, at baseline and two weeks after vaccination. Individuals with high BTLA on their mature B cells, showed a higher increase in IgG antibodies to the H1N1 virus, compared to those with low BTLA on their mature B cells (**Figure 13B**). This trend was reversed for IgM titers, indicating a possible role for BTLA in class switching of antibody responses.

Follicular T helper cells express the binding partner of BTLA:

To more formally test if BTLA signaling might affect induction or functions of ASCs we tested if HVEM, the binding partner of BTLA, is expressed on follicular T helper (T_{FH}) cells. T_{FH} cells interact with B cells during their differentiations into ASCs in lymph node follicles through bindings between PD-1 and PD-L2 (51) or ICOS and ICOS-ligand (52) expressed on T_{FH} and B cells, respectively. T_{FH} cells, which generally reside in lymph nodes, can be detected at low frequencies in blood (53). They were identified by positive staining for CD4, CXCR5 and PD-1. B cells were identified by staining for CD19. A stain for BTLA was included in the experiment, which used PBMC samples from 13 aged and 6 younger individuals that were not part of the original cohort shown in Figures 8-13. As shown in **Figure 14**, BTLA was expressed at high levels on B cells while T_{FH} cells were $BTLA^{lo}$. Results from this new cohort again confirmed higher BTLA expression on B cells from younger individuals. This was also seen for T_{FH} cells. HVEM was expressed at approximately equal levels on T_{FH} cells between the younger and the aged individuals, while B cells from younger individual expressed slightly higher levels. Overall these results indicated that BTLA on B cells could interact with its binding partner HVEM on T_{FH} cells. This in turn together with or in place of other receptor-ligand interaction may transfer of signals from T_{FH} to B cells.

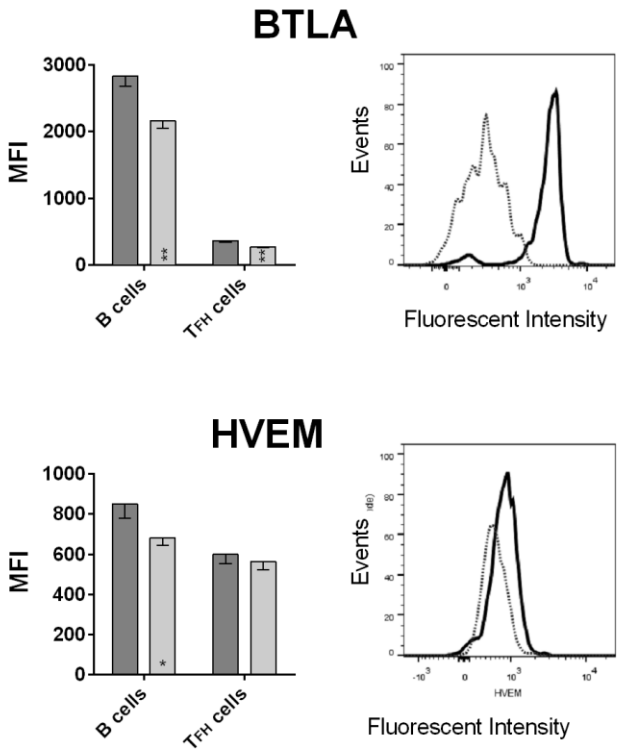

Figure 14: BTLA and HVEM expression on B cells and T_{FH} cells. Graphs show the MFI of BTLA and HVEM on $CD19^+$ B cells and $CD3^+CD4^+CXCR5^+PD\text{-}1^+$ T_{FH} cells, with error bars showing SEM. Dark grey bars are show data from younger subjects, and light grey bars aged subjects. (*) within bars, indicate statistical significance ($p<0.05$) between the young and aged cohorts, within that cell subset. P-values were calculated using multiple t-tests, corrected for multiple comparisons with Holm-Sidak correction. On B cells: p=0.002; BTLA on Tfh cells: p=0.001; HVEM on B cells: p=0.023

Histograms show BTLA or HVEM expression on B and Tfh cells of a representative young subject. The solid black line histogram shows the respective MFI on B cells and the dotted grey line histogram shows T_{FH} cells. HVEM MFI. Both histograms show intensity on x-axis and Events normalized to Mode on the y-axis.

High BTLA expression on memory B cells influences VNA responses:

Many of the individuals that we vaccinated in the 2012/13 season were vaccinated again by our team in the 2013/14 season. This gave us the opportunity to follow the evolution of antibody titers after an additional boost. To assess if levels of BTLA expression in

2012/2013 influenced VNA responses to the two influenza A virus strains of the vaccine in 2013/2014 we selected 10 individuals (regardless of age) that at baseline in 2012 had the highest (4 aged, 6 younger) or lowest (9 aged, 1 younger) levels of BTLA expression on their switched memory B cells. We analyzed their VNA titers on days 0, 7 and 14 after vaccination with 2012/13 TIV and then again 1 year later on day 0, 7 and 28 in relation to the 2013/14 dose of TIV. Increases in VNA titers to the influenza A virus strains of TIV in 2012 were similar in individuals with $BTLA^{hi}$ or $BTLA^{lo}$ switched memory B cells although there was an insignificant trend towards higher responses of the former to H3N2 virus. More impressive was that individuals with $BTLA^{hi}$ switched memory B cells maintained higher titers of H1N1 and H3N2-specific VNAs for the one year period between their seasonal Flu shots (compare d0 levels in 2013) and in addition mounted more robust recall responses in the following season compared to those that had $BTLA^{lo}$ switched memory B cells at baseline in 2012 (**Figure 15**).

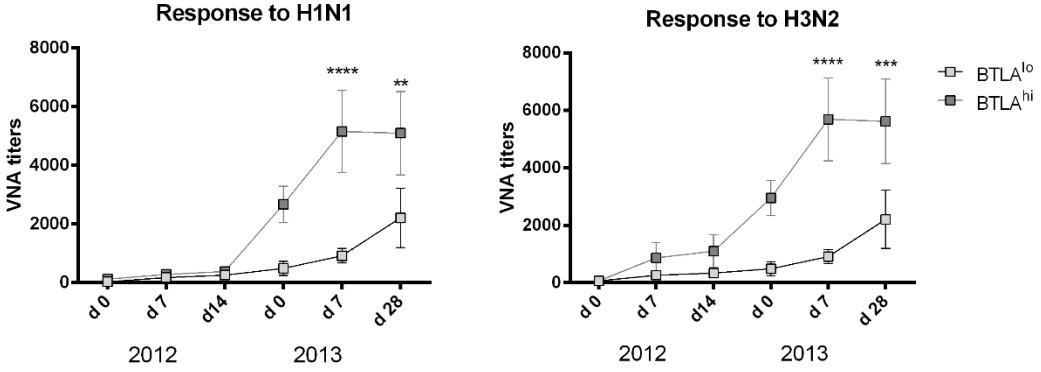

Figure 15: Correlation of BTLA expression and VNA responses. Graphs shows VNA titers over time of selected patients with either $BTLA^{hi}$ or $BTLA^{lo}$ switched memory cells (as determined at baseline 2012). The x-axis shows the dates of vaccination and follow-up over the two consecutive years of vaccination. Statistically significant differences are indicated by (*) with the following p-values: Response to H1N1 2013 d7: $p=2.55e^{-5}$; d28: p=0.003; Response to H3N2 2013 d7: $p=6.025e^{-6}$; d28: p=0.0009.

The goal of this study was to measure the expression of immunoregulators on ASCs and how they changed with age. *I successfully achieved that with this* study, showing a decline in BTLA expression on B cells upon immunosenescence that may be linked to decreased induction of long-lived plasma cells, memory B cells, and antibody titers in the aged.

Discussion:

The aims of this study were to measure the expression levels of immunoregulators on ASCs, study how their expression changed with age, and assess if and how they influenced antibody responses to the 2012-13 TIV. A cohort of middle-aged individuals was chosen for comparison to the elderly under the expectation that these subjects also had previous although less numerous exposures to influenza viruses or vaccines. Antibody responses were analyzed to identify non-responders by neutralization assays. As expected antibody responses of the aged tended to be lower than those of younger individuals.

My main emphasis was to identify differences in B cell subsets at baseline and upon immunization between aged and younger individuals and to assess if and how the differences may relate to low responsiveness of the aged. Confirming previous results, numbers of circulating naïve B cells were lower in the aged (54) while numbers of some of the other subsets only showed a trend towards an age-related decline. The other major finding from this study was the decreased expression of BTLA on aged B cells, and its possible role in the lowered virus neutralizing antibody responses to the TIV.

The key finding in this study was the decrease in expression of BTLA levels on various B cell subsets upon aging. BTLA binds to HVEM, but not exclusively. HVEM can also bind

CD160, LIGHT and lymphotoxin A (55). BTLA and CD160 bind to overlapping sites while LIGHT and lymphotoxin B bind to a different domain. CD160 and BTLA provide inhibitory signals while LIGHT and lymphotoxin A act as co-stimulators, with negative signaling prevailing in the case of simultaneous binding. The BTLA signaling pathway has mainly been studied for T cell responses where its blockade was shown to increase T cell responses to vaccines (56) and resistance to infections (57). Negative signaling through BTLA seems to be conserved in $CD8^+$ T cells of aged mice as gD-mediated blockade markedly improves their response to vaccination (58).

Downstream events of BTLA, a member of the CD28 family, remain controversial. The cytoplasmic tail of BTLA similar to that of the well characterized PD-1 immunoregulator carries a membrane proximal immunoreceptor tyrosine-based inhibition motif (ITIM) and a membrane distal immunoreceptor tyrosine-based switch motif (ITSM). These motifs through recruitment of the Src homology (SH) proteins (SHP)-1, and SHP -2 could inhibit the activating effects of CD3/CD28 ligation leading to reduced production of cytokines and cell proliferation (59). BTLA also contains a sequence in its cytoplasmic domain that may interact with the Grb-2 adaptor protein and either directly or indirectly with the p85 subunit of PI3 kinase, which in turn could provide survival signals (60). Indeed although BTLA is generally viewed as a co-inhibitor one report showed that this may depend on the strength of antigenic stimulation, - specifically in the setting of cardiac allograft rejection - where BTLA was found to have an inhibitory role in weak alloantigen stimulation, while upon potent alloantigen stimulation BTLA appeared to promote the T cell response (60). Other data showed that lack of BTLA reduced rather than augmented pathology in a colitis model (61) and promoted survival of antigen-specific $CD8^+$ T cells

induced by bacterial infection (62) again suggesting that BTLA may have disparate roles in maintaining homeostasis of the immune system.

An interesting observation was the relationship between BTLA expression on mature B cells prior to vaccination, and increases in H1N1-specific IgG antibody titers with concomitant decreases in IgM titers. This seems to indicate that high BTLA expression on mature B cells might play a role in augmenting IgG production, specifically in shifting the response from IgM to IgG. The fact that we only saw this for the H1N1 virus, and not the H3N2 virus needs to be noted. It may reflect that H1N1 underwent a dramatic change in 2009 and is thus a fairly recent virus while variants of H3N2 have been circulating since 1968.

Another intriguing observation from this study was the high expression of BTLA on switched memory B cells, the main subset that responds to vaccination against influenza virus to which adult humans in general have immunological memory, was linked to better preservation of VNA responses to the two influenza A virus strains of TIV and to higher recall responses upon the next dose of TIV given one year later. This in turn suggests that BTLA may promote rather than suppress the induction of long-lived plasma cells and memory B cells, a hypothesis that remains to be investigated further.

The goal of this study was to measure the expression of immunoregulators on ASCs and how they changed with age. *I successfully achieved that with this study, showing a decline in BTLA expression on B cells upon immunosenescence that may be linked to decreased induction of long-lived plasma cells, memory B cells, and antibody titers in the aged.*

Materials and methods:

Virus strains: The two influenza A vaccine strains of the 2012/13 seasonal influenza vaccine, A/California/7/2009 (H1N1) pdm09-like virus and A/Victoria/361/2011 (H3N2)-like virus were obtained from the Center for Disease Control, Atlanta, Georgia. Viruses were expanded by injecting 100 µl of 4 hemagglutinating units (HAU) of virus/ml into 10 day-old specific pathogen-free embryonated eggs, which were then incubated for 48 hrs at 35°C. After 48 hrs, the eggs were chilled at 4°C overnight. The allantoic fluids from the infected eggs were harvested and concentrated by centrifuging at 20,000rpm for 1 hour at 4°C. The concentrated viral pellet was resuspended in PBS and further purified by fractionation over 10-55% sucrose density gradients at 25,000rpm for 2 hrs. Purified viruses were serially diluted on Madin-Darby Canine Kidney (MDCK) cells to determine mean tissue culture infective dose (TCID50) after 3 days of infection by screening for cytopathic effects (CPE).

Human subjects: Blood was collected after informed consent from community dwelling persons in the Durham-Raleigh-Chapel Hill area of North Carolina. Younger individuals were 30-40 years of age; older individuals were > 65 years of age. The following subjects were excluded from the study: (1) humans with immunosuppression resulting from diseases (e.g., clinically active malignancy, HIV/AIDS, immune disorders) or drugs (e.g., cancer chemotherapy, corticosteroid use); (2) individuals with significant underlying diseases that would be expected to prevent completion of the study; (3) subjects, which were bed-ridden or homebound or had intercurrent illnesses that might interfere with interpretation of study (e.g., urinary tract infection, respiratory tract infection); (4) individuals that were unlikely to adhere to protocol follow-up; (5) subjects that were involved in a conflicting study; (6) subjects that had a history of alcohol or

substance abuse; (7) subjects with contraindication for influenza vaccination such as anaphylactic hypersensitivity to eggs or to other components of the influenza vaccine, and moderate or severe acute illness with or without fever, and Guillain-Barre Syndrome within 6 weeks following a previous dose of influenza vaccine. Persons with moderate to severe acute febrile illness were not vaccinated until their symptoms had abated.

From enrolled subjects demographic data and medical history including medical diagnoses, medications, vaccination to influenza and other infectious diseases, and history of influenza or influenza-like diseases during the last 5 years were recorded. Subjects were bled and then vaccinated with TIV via the intramuscular route in the deltoid muscle. Subjects were bled again on days 7 and 14 following injection of TIV. Some of the subjects were revaccinated with TIV the following years using the same procedures.

Collection of blood and isolation of PBMCs and plasma: Blood was collected into heparinized tubes and shipped overnight to Philadelphia, PA. A 1.5 ml aliquot of each sample was set aside for serum collection. PBMCs were isolated from the remaining samples using established protocols. Specifically, blood was overlaid onto Ficoll-Paque Plus (GE Healthcare Biosciences, Piscataway Township, NJ) and spun for 30 minutes at 2000 rpm, with brake off, and at 50% acceleration. The PBMC layer at the Ficoll interface was then collected and washed twice with Hank's Balanced Salt Solution (Gibco, Grand Island, NY), by centrifuging at 2000 rpm. The washed, pelleted cells were treated with 10 ml of red blood cell lysis buffer (eBioscience, San Diego, CA). Lysis was stopped by adding 5 ml of Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum [FBS]. Cells were washed with Hank's Salt

(HBSS). Cells were resuspended in 5ml of Dulbecco's modified Eagles medium (DMEM), live cells were counted using Trypan Blue as a diluent.

Micro-neutralization assay: Two-fold serially diluted (1:20 to 1:10240) heat-inactivated human sera were tested for neutralizing antibodies to influenza A virus strains by micro-neutralization assays. Equal volume of 100TCID50 per well of the titrated virus was added to the diluted serum in 96 well plates and incubated at 37°C. After 1hr, serum-virus mixtures were added to MDCK cells that had been washed twice with serum-free Dulbecco's Modified Eagles Medium (DMEM). The cells were incubated for 2 hrs at 37°C with 5% CO2. The cells were washed and re-incubated with DMEM supplemented with L -1-Tosylamide-2-phenylethyl chloromethyl ketone (TPCK) trypsin for 3 days. CPEs were scored under a microscope. Neutralization titers were defined as the dilution of the serum that resulted in 50% inhibition of CPE formation.

B cell detection by flow cytometry: Each subject sample was initially treated with Human TruStain FcX Fc Receptor Blocking solution (BioLegend, San Diego, CA) for 30 minutes, washed with PBS at 1500 rpm for 5 minutes and then stained with fluorochrome-conjugated antibodies. Samples were stained first for their extracellular markers using the following antibodies: CD19-Brilliant Violet^{TM} 650, CD20- Brilliant Violet^{TM}570, IgD-PeCy7, CD38-PerCPCy5.5, CD3-Pacific Blue, CD14-Pacific Blue, CD27-FITC, BTLA-APC, and AmCyan Aquablue as a live cell stain. The optimal concentrations of these antibodies were determined experimentally. Samples were stained for 30 minutes at room temperature and washed with PBS. The cells were then permeabilized using Cytofix/Cytoperm (BD Biosciences) for 30 minutes at 4°C, washed with Permwash (BD Biosciences) and stained with their specific panel of intracellular antibodies. The samples were stained with the intracellular stains for 30 minutes at 4°C,

washed with PBS and then resuspended in 150µl of fixative (BD Pharmingen). All antibodies were obtained from BD Biosciences (San Jose, CA) unless specified differently. The stained samples were analyzed in a LSRII flow cytometer (BD Biosciences, San Jose, CA).

ELISA: H1N1- and H3N2-specific binding antibody isotypes were measured by ELISA. Briefly, wells of Nunc Maxisorp™ plate were coated with 10µg/ml of influenza H1N1 or H3N2 virus along with isotype standards for IgA1, IgG and IgM (Athens Research & Technology, Inc., Georgia, USA) in bicarbonate buffer overnight at 4°C. The plates were blocked with 3% BSA in PBS and incubated for 2 hrs at room temperature with heat-inactivated sera of young and aged subjects at a dilution of 1/250. The plates were washed 4X with PBS containing 0.05% tween (PBST) and incubated for 1 hour at room temperature with alkaline phosphatase conjugated mouse anti-human IgA1 at 1:1000, IgG at 1:3000 and IgM at 1:1000 dilutions (SouthernBiotech, Alabama, USA). Following the incubation, plates were washed 4X with PBST and developed using alkaline phosphatase substrate containing pNPP tablets (Sigma Aldrich, Missouri, USA) dissolved in DEA buffer. Adsorbance was recorded at 405nm. The adsorbance values were plotted against standard curves from each plate for every isotype. Antibody concentrations were determined and are expressed in µg/ml.

Portions of this chapter were adapted from:

BTLA expression declines on B cells of the aged and is associated with low responsiveness to the trivalent influenza vaccine. Kannan S, Kurupati RK, Doyle SA, Freeman GJ, Schmader KE, Ertl HC. Oncotarget. 2015 Aug 14; 6(23):19445-55.

CHAPTER 4: B CELL IMMUNOSENESCENCE IN MICE: CHANGES IN GENE EXPRESSION PROFILES, EXPRESSION OF IMMUNOREGULATORS AND METABOLISM

Abstract:

I analyzed age-related defects in B cell populations from young and aged mice. Microarray analysis of bone marrow resident antibody secreting cells (ASCs) showed significant changes upon aging, affecting multiple genes, pathways and functions including those that play a role in immune regulation, humoral immune responses, chromatin structure and assembly, cell metabolism and the endoplasmic reticulum (ER) stress response. Aged ASCs expressed higher levels of immunoregulators including PD-1. Increased PD-1 expression was also seen on mature aged B cells indicating early defects in the lineage. High PD-1 expression on the aged ASCs correlated with high cellular reactive oxygen species levels. Mitochondrial defects in aged B cells were demonstrated by the absence of an increases in basal respiration observed upon in vitro stimulation of young B cells.

Introduction/Purpose of study:

While the previous chapters addressed the effect of aging in the human immune system, the aim of this study was to study how immunosenescence in mice – with the overall hypothesis that *expression levels of immunoregulators on ASCs change upon aging, and that aged ASCs are metabolically different compared to the younger and that changes in immunoregulators are linked to metabolic defects or vice versa.*

Here I further elucidated in mice the age-related defects in B cell populations. Whole genome expression arrays comparing bone marrow-derived ASCs from young and aged mice showed disparity of expression in over 2000 probes and distinct clustering. Genes

that were differentially expressed participate in numerous pathways and functions including immunological and metabolic functions. Aged B cells have increased levels of ROS and show differential expression of immunoregulators such as PD-1. Expression of PD-1 in turn is strongly correlated with levels of cellular (c) ROS. Defects in mitochondrial functions were demonstrated upon *in vitro* stimulation of B cells.

Results:

ASCs and their precursor populations are decreased in aged mice

Previous studies have shown that bone marrow output of naïve B cells is decreased upon aging (63). So, the first goal was to measure the number of ASCs in the spleens and bone marrow of young and aged mice using multicolor flow cytometry (**Figure 16**). ASCs were significantly reduced in the aged mice in both the bone marrow and the spleen – the two primary reservoirs for ASCs (**Figure 17**). The reduction was especially pronounced in the bone marrow, suggesting either reduced generation of long-lived ASCs or a decline in their life span.

In order to see if this discrepancy in ASC numbers was a remnant of a difference in their precursor population, we enumerated mature B cells and $CD23^+$ B2 cells. We noticed significantly lower percentages of mature B cells and $CD23^+$ B2 cells in the aged mice (**Figure 18**). This corroborates previous observations showing that bone marrow output of naïve B cells is decreased upon aging.

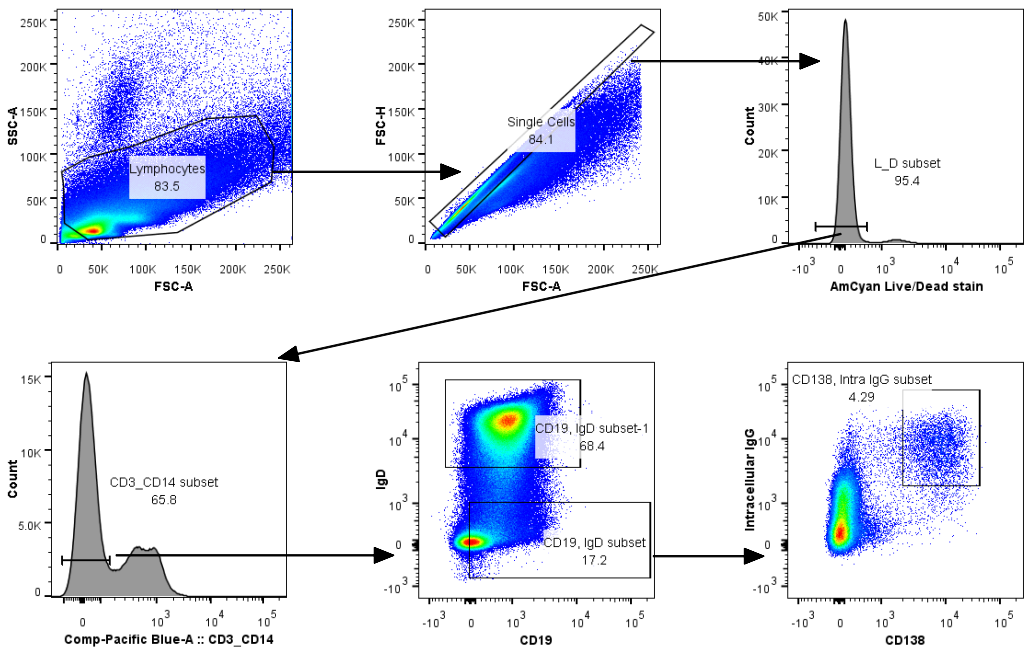

Figure 16: Gating strategy for ASCs: Figure shows the gating strategy used to identify ASCs (Antibody Secreting Cells) – $CD3^-$ $CD14^-$ Live IgD^- $CD19^+$ $CD138^+$ Intracellular IgG^+. A representative young mouse sample is shown.

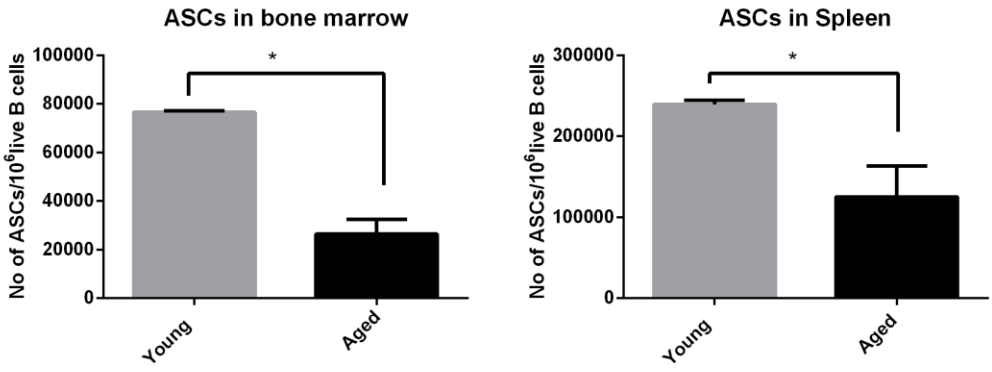

Figure 16: Aged mice have lesser ASCs. Figure shows numbers of ASCs in the spleen and bone marrow of young and aged mice. Aged mice are shown in black columns and young mice in grey columns. * denotes significance by t tests with Mann Whitney correction (ASCs in bone marrow: p=0.0012; ASCs in spleen: p=0.03)

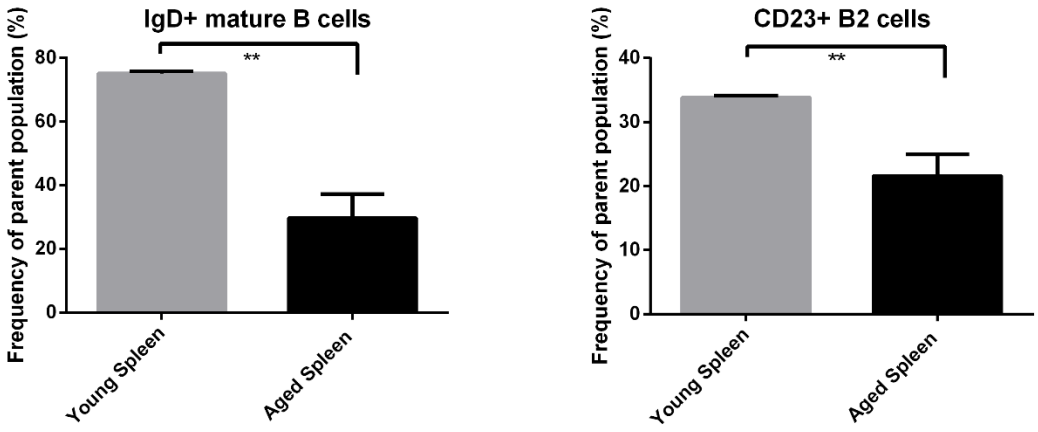

Figure 18: Aged mice have lower frequencies of mature B cells. Figure shows frequencies of mature B cells and $CD23^+$ B2 B cells. Aged mice are shown in black columns and young mice in grey columns. * denotes significance by t tests (Frequencies of mature B cells: p=0.0079; $CD23^+$ B2 B cells: p=0.0079).

Microarray analysis of ASCs shows multiple differences in transcriptome:

To elucidate age-related changes in ASCs, I isolated bone marrow resident ASCs from 12 young and 10 aged mice by cell sorting and profiled whole genome cDNA expression using Illumina Bead Arrays. I tested cells from individual mice or if cell numbers were low from pooled samples, resulting in 9 unique young samples and 6 unique aged samples. The aim of these experiments was to compare cells at the gene expression level and identify differently expressed genes, pathways and functions. The data was submitted to GEO database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE72224.

A total of 2175 probes were differentially expressed in ASCs from young mice compared to aged mice, at a p-value of 0.05. Of these probes, 1411 (65%) were more highly expressed in the aged mice while 764 (35%) had higher expression in the young mice.

Principle component analysis using 715 ($p < 0.01$) gene probes showed that young and aged ASCs clustered as 2 well separated groups (**Figure 19**).

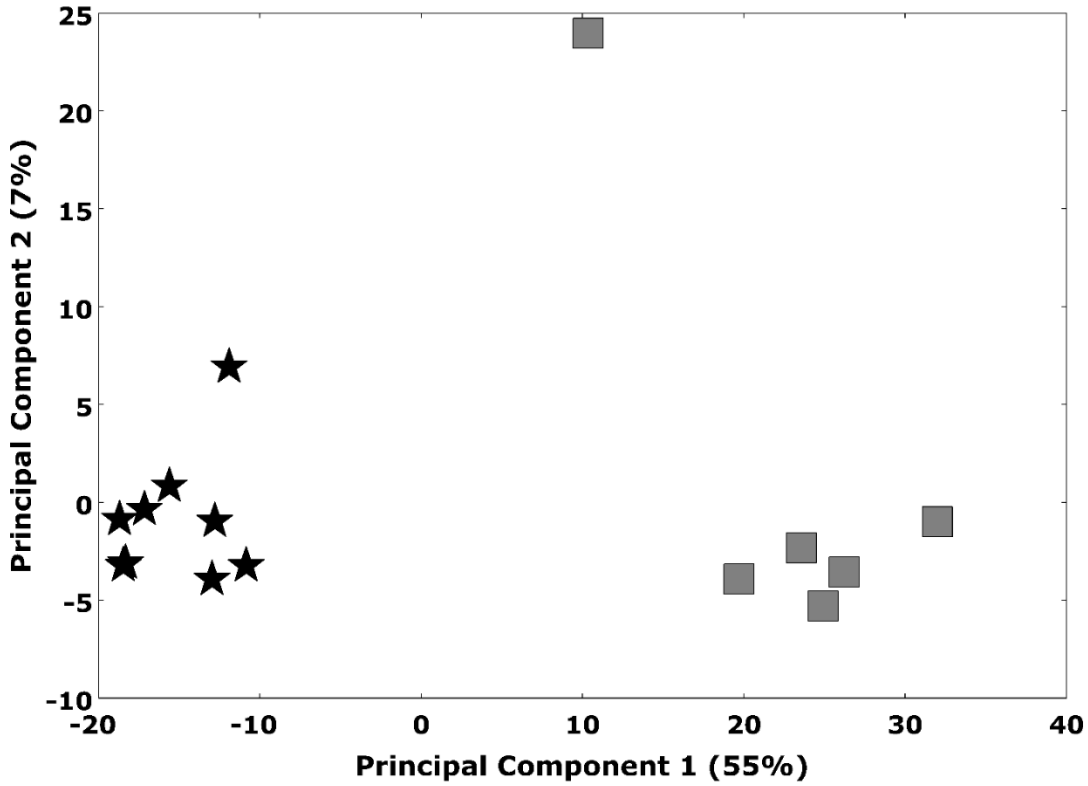

Figure 19: Principal component analysis: Figure shows clustering of genes significant at a p-value of 0.01 along the first two principal components.

A heatmap of the expression of the 100 genes that were the most significantly differentially expressed (by p-value) between the two groups is shown in **Figure 20**. Using Ingenuity Pathway Analysis, a number of functions distinguished the aged mice from the young mice. The most significant differences were observed for cell death and survival, cellular growth and proliferation, hematological system development and function, tissue morphology, cellular development, humoral immune response and protein synthesis.

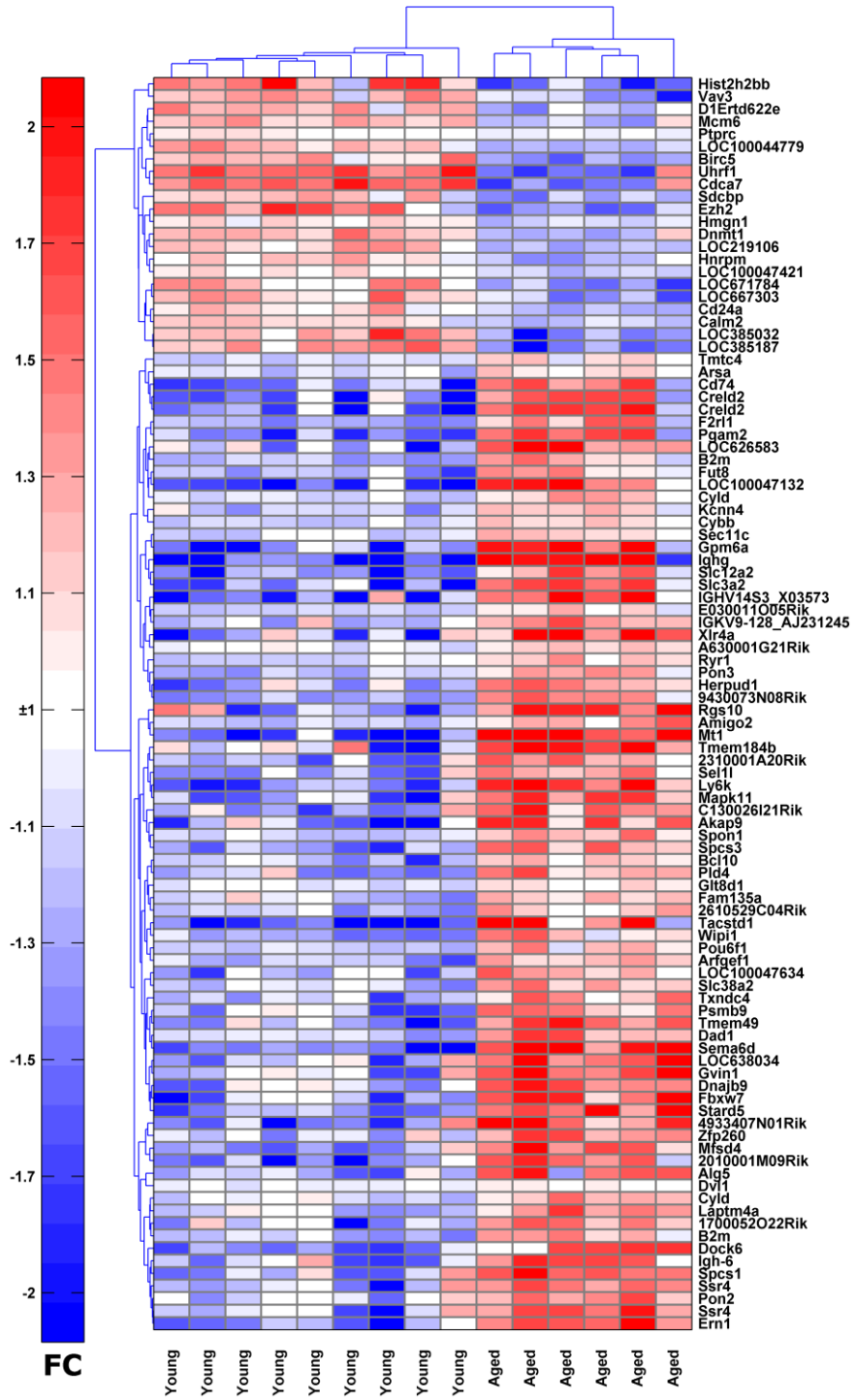

Figure 20: Heatmap of the top 100 genes that are differentially expressed between young and aged ASCs. Transcript names are shown along the right axis. Red: increased expression, blue: decreased expression.

Immunoregulators: Transcripts for a number of immunoregulators including CD markers, interleukin receptors, natural killer cell receptors and members of the TNF family were differentially expressed in aged and young ASCs (**Figure 21**).

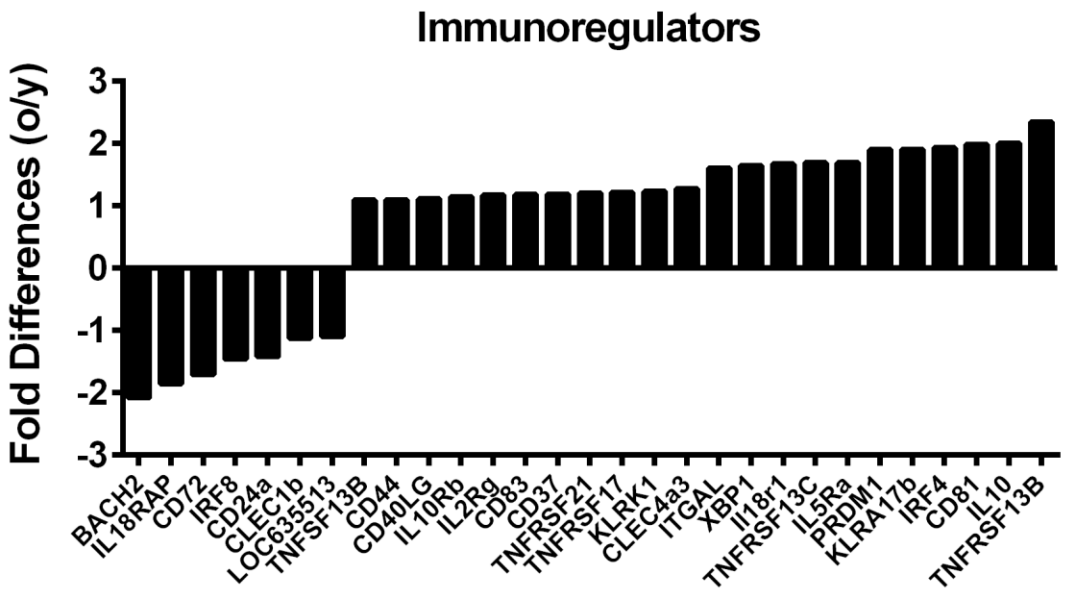

Figure 21: Differences in the expression of transcripts that encode immunoregulators between young and aged ASCs. Positive numbers show higher expression in the aged ASCs, negative numbers show high expression in young ASCs.

Most were higher in the aged (19/24). Several are known to affect B cell fate decisions. BACH2, which is essential for class switching, was expressed higher in young ASCs. XBP1, which becomes crucial at late stages of plasma cell development, was higher in aged ASCs. IRF4 and IRF8 were also differentially expressed; the former was overexpressed and the latter under expressed in aged B ASCs. Both play critical non-redundant roles in plasma cell development and germinal center formation. IRF8 induces expression of Bcl6. IRF4 down-regulates Bcl6 and instead induces Blimp-1

encoded by the PRDM1 gene. Transcripts for PRDM1 were increased in aged ASCs. While Bcl6 promotes germinal center formation and proliferation of B cells Blimp-1 drives terminal differentiation of plasma cells. These data support reduced class-switching in aged B cells and more terminal differentiation of aged plasma cells.

IPA also showed significant ($p \leq 0.01$) differences in ATM and p53 signaling and antigen presentation (**Figure 22**). Most of the differentially expressed transcript encoding proteins involved in ATM signaling, which is activated by double-stranded DNA breaks, were higher in young mice (5/7) while transcripts for proteins of the p53 pathway were more commonly high in aged cells (5/8). Transcripts for antigen presentation pathways, such as those encoding histocompatibility antigens, were also higher in aged ASCs.

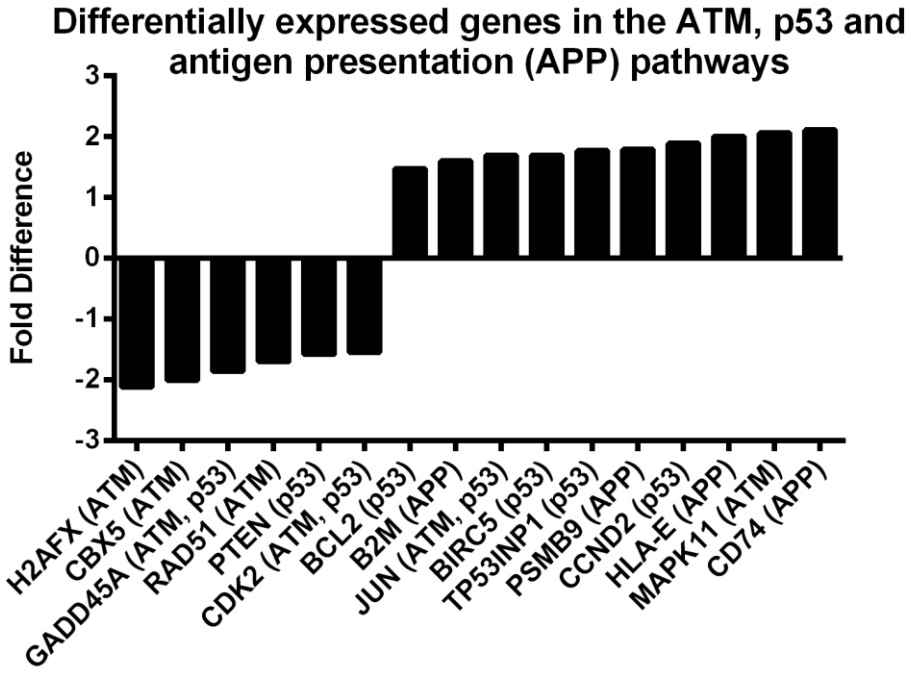

Figure 22: Differences in the expression of genes in the most significantly altered canonical pathways identified using Ingenuity. Positive numbers show higher expression in the aged ASCs, negative numbers show high expression in young ASCs.

Enrichment of genes involved in the humoral immune responses (**Figure 23**) were identified by Ingenuity Pathway Analysis (IPA), and again most of the involved genes had higher expressed in aged ASCs.

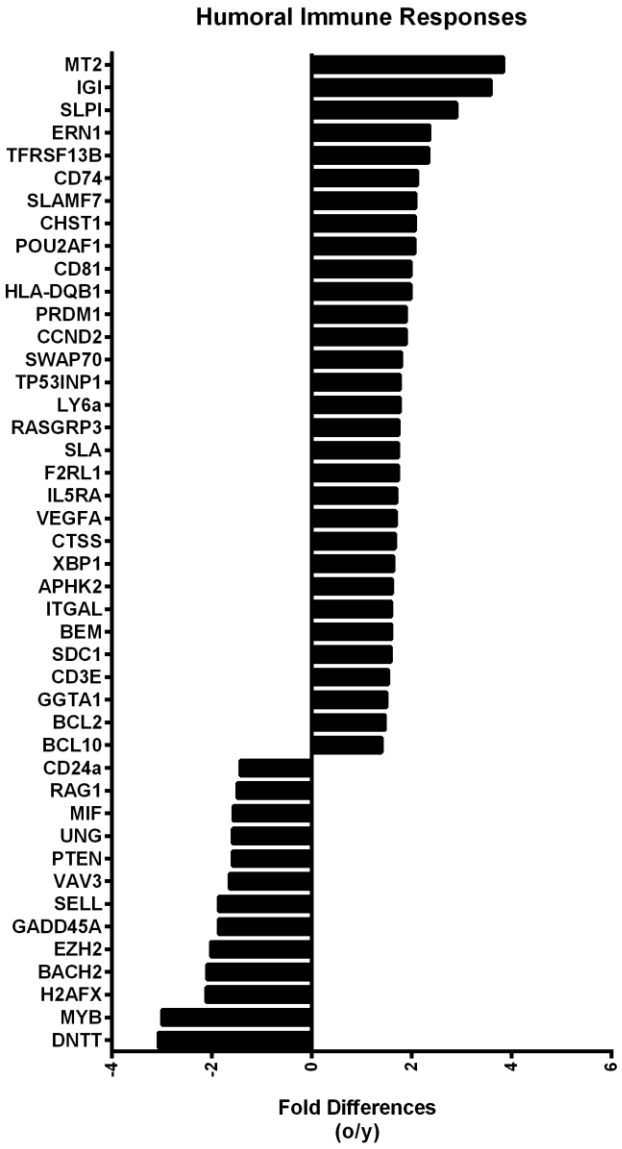

Figure 23: Differences in the expression of genes involved in the humoral immune responses biological function identified using Ingenuity. Positive numbers show higher expression in the aged ASCs, negative numbers show higher expression in young ASCs.

Metabolism and metabolic disorders: Differences in metabolic diseases, carbohydrate and lipid metabolism were also observed (**Figure 24**). Most transcripts for metabolic disorders (42/55), carbohydrate (19/22) and lipid (4/4) metabolisms were expressed at higher levels in aged as compared to young ASCs.

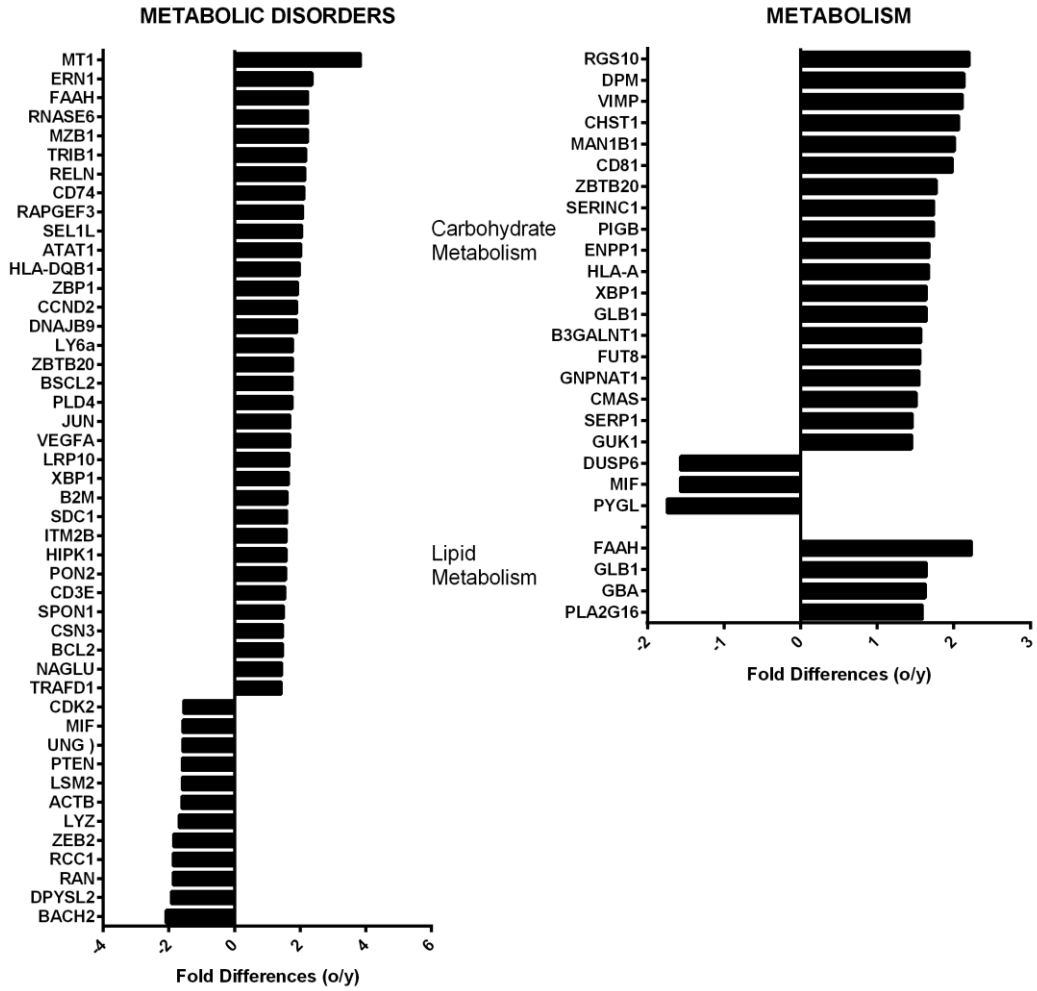

Figure 24: Differences in the expression of genes involved in metabolic disorders (on the left) or lipid and carbohydrate metabolism (right) identified using Ingenuity. Positive numbers show higher expression in the aged ASCs, negative numbers show high expression in young ASCs.

Several other genes involved in lipid and carbohydrate metabolism were also differentially expressed between young and aged ASCs at a more lenient p-value threshold of 0.05 and without fold change restrictions.

Another interesting observation was the differences in expression seen in mitochondrial proteins encoding components of the electron transport chain (**Figure 25**), and mitochondrial fission (FIS1), which affects mitochondrial morphology (64). These transcripts were more highly expressed in aged ASCs. One mitochondrial ribosomal protein [MRLP33 (-1.59)] and one key ATP synthase [ATP5j (-1.48)] were under expressed in aged ASCs.

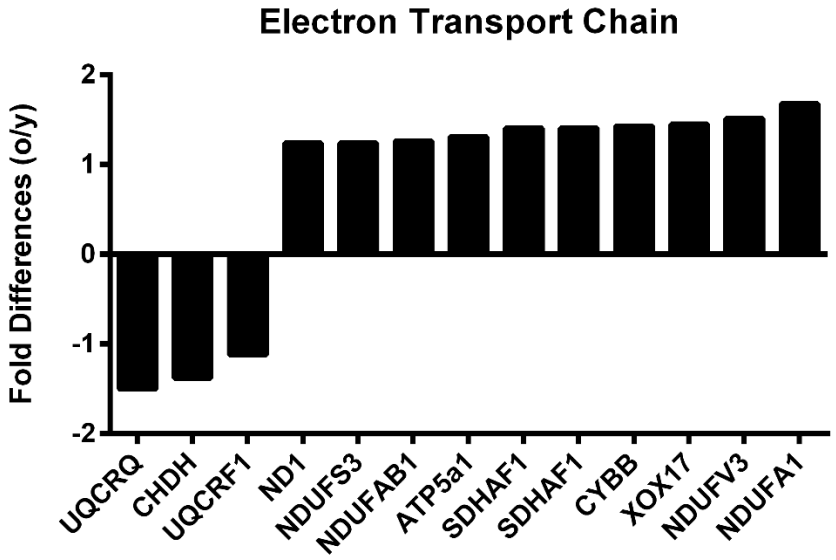

Figure 25: Differences in the expression of genes involved in the electron transport chain. Positive numbers show higher expression in the aged ASCs, negative numbers show higher expression in young ASCs

Functional analysis by DAVID: Database for Annotation, Visualization, and Integrated Discovery - (65) showed significant differences in expression levels for the endoplasmic reticulum with most genes having higher expression in the aged (51/52). At a lower significance threshold (genes with $p < 0.05$), transcripts indicative of ER stress were increased in the aged (5/5) while those involved in protein folding were in part higher in the younger (4/10). (**Figure 26**).

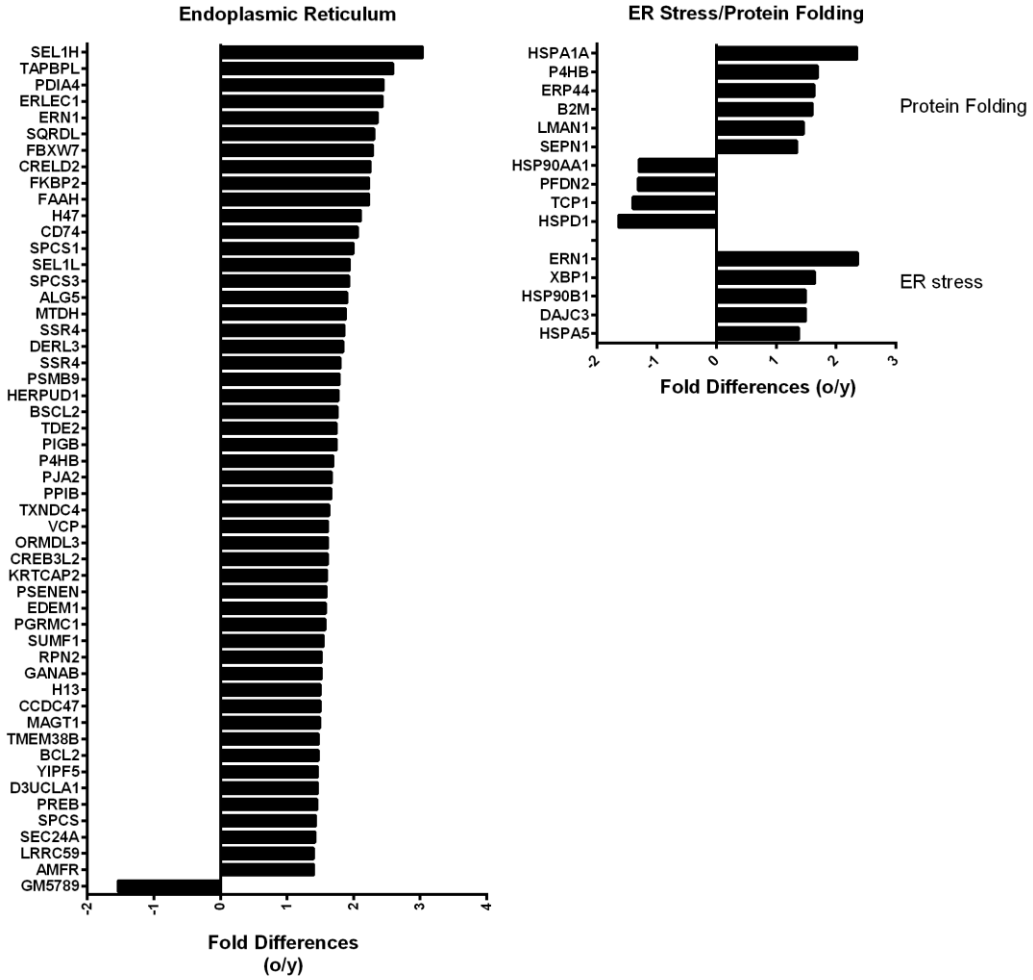

Figure 26: Differences in the expression of genes involved in the Endoplasmic Reticulum (ER), ER stress and Protein folding: Positive numbers show higher expression in the aged ASCs, negative numbers show higher expression in young ASCs

Differences were also seen in genes controlling functions of peroxisomes, which are involved in fatty acid metabolism and reduction of ROS. These included VCP (1.61), involved in assembly of peroxisome (66), PPARD (1.24), a regulator of energy metabolism (67) and ACBD5 (1.27), a peroxisome receptor for acyl-CoA-esters (68).

We selected a number of immunoregulators that were differentially expressed in the microarray, and measured their protein levels by flow cytometry. Flow cytometry analysis confirmed differences that were observed at the transcriptome level. Mainly, CD52 and CD98 were significantly higher on the aged ASCs (**Figure 27**).

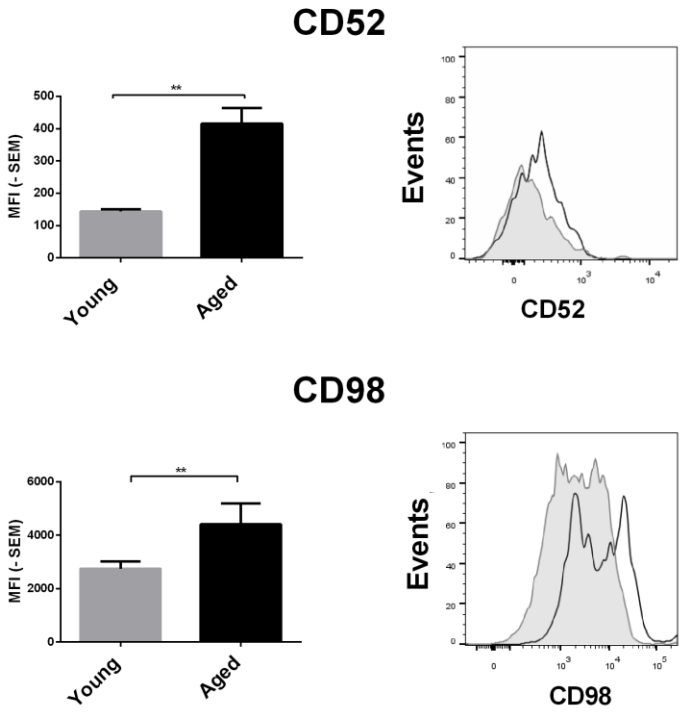

Figure 27: Expression levels of CD52 and CD98 on bone marrow derived ASCs from young and aged mice. Graphs on the left show Mean Fluorescent Intensity (MFI) of the specified molecule. Samples from aged mice are in black, and from young mice in grey. Error bars represent standard error of the mean (SEM). Representative histograms are shown on the right. Samples from aged mice are shown with a solid black line. Samples from young mice are shown in grey. * denotes significant differences as determined by Mann-Whitney test. (CD52: p=0.0079; CD98: p=0.005)

PD-1 expression is higher on aged ASCs:

The microarray analysis had indicated that immunological and metabolic pathways were both changed upon aging in ASCs. PD-1 is an immunoregulator that has been shown to have significant metabolic functions by inhibiting glycolysis in activating T cells (26). To assess if PD-1 plays a potential role in regulating metabolism in ASCs, we measured its expression levels and observed that PD-1 levels were significantly higher on aged ASCs, a difference that was also observed on mature naïve B cells (**Figure 28**).

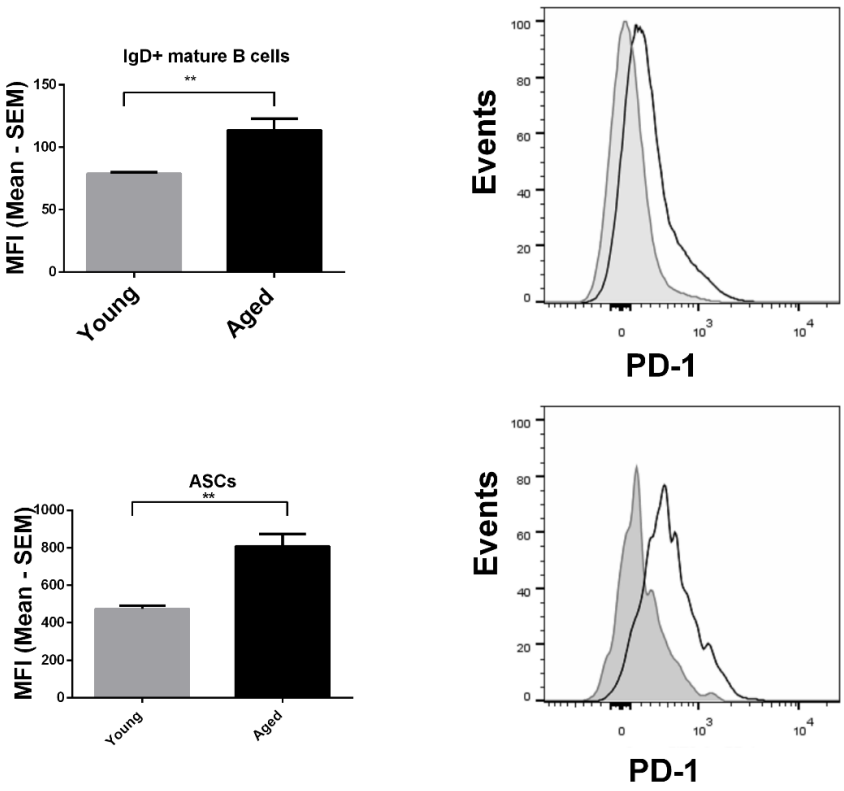

Figure 28: PD-1 expression on mature B cells and ASCs. Graphs on the left show the MFI of PD-1 expression, with error bars representing SEM. Aged samples are shown in black and the young in grey. Representative histograms are shown alongside with the aged samples in a solid black line and the young samples in grey. * denotes significant differences as determined by Mann-Whitney test. (Mature B cells: p=0.0079; ASCs: p=0.0159)

Aged ASCs have high cellular ROS levels, which correlate with PD-1 expression:

Since PD-1 plays a role in cellular metabolism of T cells and is expressed higher on aged B cells, we tested splenic ASCs for metabolic parameters and found that they carry higher levels of cROS, indicative of increased oxidative stress compared to cells from young mice (**Figure 29A**). ROS is produced within cells by numerous pathways with major sources being the mitochondrial electron transport chain, peroxisomes and the endoplasmic reticulum (ER) (69; 70; 71). Peroxisomes, which play a central role in lipid metabolism, contain a number of ROS producing and detoxifying enzymes. As mentioned previously, genes that control peroxisome assembly and size were differentially expressed between ASCs of the two age groups. During oxidative protein folding, ROS such as H_2O_2 is produced in the ER. Accumulation of misfolded proteins triggers an ER stress response (72) to reduce protein production, enhance production of chaperones that assist in protein folding, increase production of enzymes that ubiquinate and degrade misfolded proteins and initiate apoptosis.

Two enzymes that participate in cytoplasmic, peroxisomal and mitochondrial ROS metabolism were higher in the aged. These were PRDX4 (1.85) (73), which reduces hydrogen peroxide to water and CCS (1.25) (74), a copper chaperone for superoxide dismutase (SOD), a key group of enzymes that destroy ROS in the cytoplasm, mitochondria and peroxisomes. During oxidative protein folding, ROS such as H_2O_2 is produced in the ER. Accumulation of misfolded proteins triggers an ER stress response (72) to reduce protein production, enhance production of chaperones that assist in protein folding, increase production of enzymes that ubiquinate and degrade misfolded proteins and initiate apoptosis.

Levels of cellular ROS in ASCs correlated with PD-1 expression indicating a link between the immunoregulator and metabolism (**Fig. 29B**).

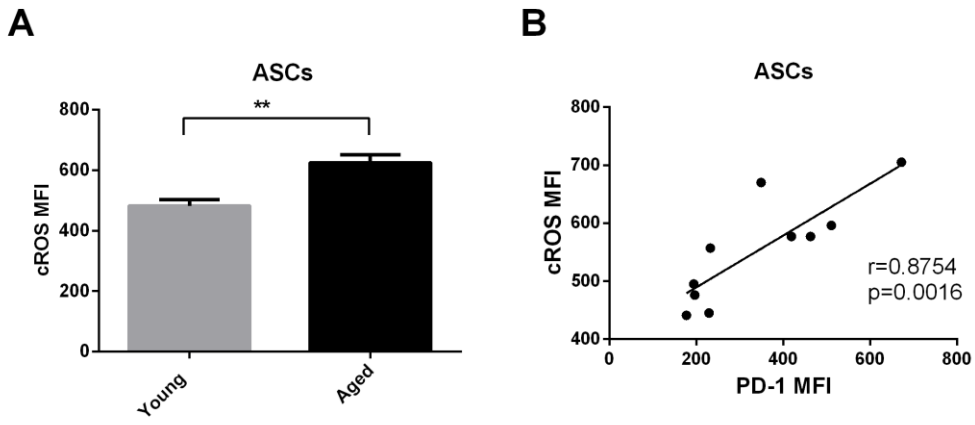

Figure 29: cROS expression is higher on aged ASCs and correlates with PD-1 expression. A shows cROS levels in young and aged ASCs. Samples from aged mice are shown in black and from younger mice in grey. Graph on left shows MFI, with error bars indicating standard error of the mean. * denotes significant differences as determined by Mann-Whitney test. (p=0.0079). **B** shows the positive correlation between cROS and cell surface expressed PD-1 using ASC samples from young and aged mice. Correlation was determined by Spearman. R and p-values are shown within the graphs.

Aged B cells upon activation exhibit defects in mitochondrial respiration:

The arrays indicated marked differences in carbohydrate and lipid metabolism as well as in the electron transfer chain between ASCs from young and aged mice. To further assess metabolic differences I tested bead purified resting and mitogen-stimulated B cells from young and aged mice for basal respiration and lactate production by Seahorse XF flux analyzer (75) (**Figure 30**). Aged resting B cells showed higher oxygen consumption rates (OCR), which mainly measures oxidative phosphorylation (OXPHOS) through the electron transfer chain. In addition, there were higher extracellular

acidification rates (ECAR), as a measure of lactate production by glycolysis, in B cells of aged mice compared to young B cells, suggestive of higher metabolic activity of aged compared to younger B cells. The OCR to ECAR ratio was slightly higher in younger B cells indicating a higher contribution of OXPHOS to their overall energy production.

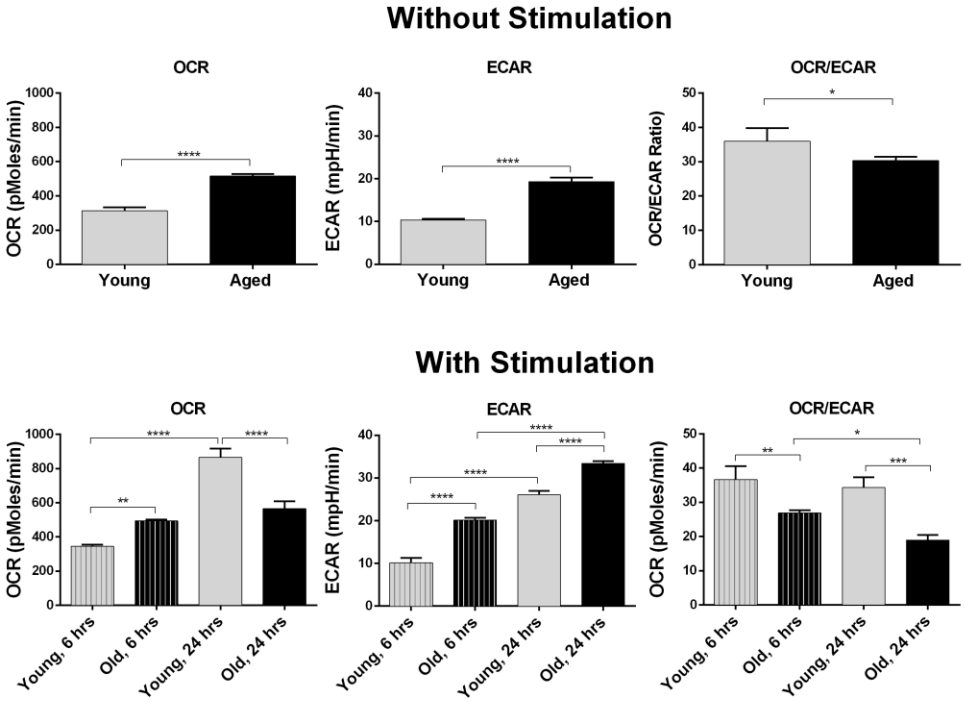

Figure 30: Defects in mitochondrial respiration: OCR and ECAR as well as ratios of OCR/ECAR are shown upon measuring splenic B cells without stimulation (top panels) or after stimulation for 6 or 24 hours (lower panels).Columns with vertical lines indicate 6 hour time points. Lines indicate significant differences between groups calculated by t-tests or 1-way ANOVA. * p values between 0.01 – 0.05, ** p-values between 0.001 – 0.01, *** p-values between 0.0001 – 0.001, **** p – values < 0.0001.

B cells were then stimulated with a cocktail of mitogens and tested 6 or 24 hours later. After 6 hours OCR and ECAR remained comparable to those of unstimulated young and old B cells. After 24 hours OCR markedly increased in younger but not aged B cells,

while ECAR increased in both. These data indicate that upon aging, OXPHOS cannot satisfy increases in energy demands under challenging conditions.

In summary, the goal of this study was to investigate the effect of immunosenescence on ASCs – specifically characterize immunoregulator expression and elucidate if aged ASCs were metabolically different from the younger. *I achieved this* with this study showing that bone marrow-derived ASCs, which are generated throughout a lifespan of an individual, show substantial differences in their gene expression profile in aged as compared to young mice, which may underlie their deteriorating functions. We also showed the increased expression of PD-1 on aged ASCs and its correlation with high levels of cellular ROS levels. This is a link that needs to be studied further.

Discussion:

The aim of this study was to further elucidate the effect of immunosenescence on ASCs. I started by using microarrays to identify age-related changes in ASCs from bone marrow where most long-lived plasma cells reside.

As expected, many genes involved in numerous pathways and functions were differentially expressed between young and aged ASCs. The first one of interest is ATM signaling. Upon aging, cells accumulate DNA mutations, which may lead to structural changes of DNA (28). Our array data show reduced ATM signaling in the aged. The ATM pathway is induced by DNA damage; it prevents cells from progressing through the cell cycle, and can increase DNA repair mechanisms or drive cells to undergo apoptosis (76). ATM regulates apoptosis through p53, a pathway, which was also affected in aged ASCs. Reduced ATM signaling in turn could contribute to increased lasting DNA damage. An analysis of differences in significant biological functions accordingly showed

the most significant differences for cell survival and death and cell growth and proliferation. Although it is assumed that long-lived bone marrow derived plasma cells are terminally differentiated, and thereby incapable of proliferation, recent gene expression array analyses indicate that although bone marrow-derived ASCs are quiescent they are capable of undergoing some replicative cell renewal (77). Reduced proliferation in the aged combined with increased rates of apoptosis could thus explain the marked reduction of numbers of ASCs especially of those residing in the bone marrow.

Ingenuity analysis for significant functions also revealed differences in humoral immune responses. Four of the 13 genes with lower expression in the aged play a role in DNA recombination or generation of antibody diversity confirming previous studies that upon aging antibody responses lose breadth (78).

Another interesting observation was the differential expression of transcripts for the ER stress response. Previous studies showed that the ER stress response also referred to as the unfolded protein response increases in the aged due to loss of chaperone proteins (79). This increases apoptosis and may contribute to the reduced numbers of ASCs. This increased susceptibility for ER stress and unfolded proteins could also contribute to increases in ROS, which can have an effect on the metabolism of the cell.

Aging can impair cellular metabolism in mice in multiple ways. Two reasons are increased resistance to insulin (80) and deleterious alterations of mitochondria caused, for example, by the disorganization of their structure (81). This latter cause is evidenced in our array data by the increase in expression of FIS1, which regulates mitochondrial fission and thereby affects mitochondrial morphology. Upon aging, mutations also

accumulate in mitochondrial DNA4, along with ROS increases (82), which again was recapitulated in our studies. Mitochondria then lose the ability to efficiently generate energy through OXPHOS (83).

This was further investigated by carrying out a functional analysis by Seahorse extracellular flux analyzer, which was conducted with B cell populations as surrogates for ASCs since the paucity of such cells precludes their direct analysis for OCR or ECAR, which measures oxidative phosphorylation and glycolysis respectively. It is worth noting here, that upon stimulation, B cells similar to T cells increase glycolysis, which is relatively inefficient for generating energy but provides building blocks needed for cell division and effector function. However, unlike T cells B cells also increase energy production though OXPHOS. Our experiment showed that aged B cells without further activation appear to be metabolically more active. However, upon stimulation, aged B cells showed increased glycolysis, and this difference over resting B cells was comparable to those observed in young B cells. OCR, in contrast, only increased in young but not old B cells after mitogen-mediated stimulation, demonstrating severe mitochondrial defects, which only became obvious once cells had to increase energy production to allow for cell proliferation and effector function. OXPHOS can be fueled by numerous substrates including carbohydrates, amino acids and lipids, which generate NADH and $FADH_2$ upon degradation in the TCA cycle. In turn, NADH and $FADH_2$ are essential for the conversion of ADP to ATP, the energy currency of life, in the energy transport chain, which according to the array data was significantly altered upon aging.

Previous studies demonstrated that the immunoinhibitor PD-1 is more highly expressed on aged $CD4^+$ T cells (84). In the same token, we found that PD-1 expression increases on aged naïve B cells and on ASCs. PD-1 is typically viewed as an exhaustion marker

on T cells where it blocks activation of the AKT/mTOR pathway by T cell receptor and costimulator ligation (85) and thereby reduces the cells' ability to use glycolysis for energy production (69). Previous data reported that PD-1 is expressed on B cells, where it was shown to inhibit B cell receptor signaling through the recruitment of the tyrosine phosphate SHP-2 (86). It is unknown if PD-1 affects glycolysis in B cells and thereby adds additional stress upon immunosenescence when energy production through OXPHOS becomes impaired.

In summary, the goal of this study was to investigate the effect of immunosenescence on ASCs – specifically study immunoregulator expression and elucidate if aged ASCs were metabolically different from young ASCs. *I achieved this with this study showing that bone marrow-derived ASCs, which are generated throughout a lifespan of an individual show substantial differences in their gene expression profile in aged as compared to young mice, which may explain their deteriorating functions. I also showed the increased expression of PD-1 on aged ASCs and its correlation with high levels of cellular ROS. This is a link that needs to be studied further.*

Materials and methods:

Mice: Mice were obtained from Charles River labs and housed in the Wistar Institute's animal facility. Young mice were 6-8 weeks of age, and aged mice were >17 months of age. All procedures followed approved protocols.

Sample collection: Mice were euthanized by CO_2 asphyxiation, and samples were collected. Bone marrow samples were isolated from the bones of the hind legs of each mouse. Cells within the marrow were flushed out using a 27 ½ G syringe, and resuspended in prewarmed L-15 media (Life technologies, CA). Whole spleens and PBMCs were also -collected. Spleen samples were ground up in L-15 media, and filtered through BD Falcon cell strainers. Cells from both samples were then counted using a haemocytometer, and resuspended in DMEM (Life technologies, CA) media containing 10% Fetal Bovine Serum, Antibiotics (Pennicillin and Streptomycin).

B cell analysis by flow cytometry: The following fluorochrome conjugated antibodies were used. CD3-Pacific Blue, CD14-Pacific Blue, AmCyan Aqua Blue Live/Dead stain, CD19, B220, IgD, PD-1, CD138 and CellRox) Green mitochondrial dye.

For assays with mitochondrial dyes, cells were stained with mitochondrial dyes, diluted in media for 45 minutes at 37^{o} Celsius. Samples were then washed with PBS and then stained with fluorochorome-conjugated antibodies. For assays without the mitochondrial dyes, the previous step was omitted. Samples were stained for 30 minutes at room temperature, washed with PBS and then resuspended in 100µl of CytoFix/CytoPerm (BD Pharmingen) for 30 minutes. Samples were then washed with 100uL of BD PermWash, and stained for intracellular markers with fluorocohrome conjugated antibodies, diluted in PermWash for 30 minutes at 4^{o} Celsius. All antibodies were obtained from BD

Biosciences (San Jose, CA) unless otherwise specified. The stained samples were analyzed in a LSRII flow cytometer (BD Biosciences, San Jose, CA).

Microarrays: Bone marrow resident cells were isolated as described above, and stained for extracellular markers. Cells were then sorted on a FACS ARIA fluorescent cell sorter for ASCs. Total RNA and small RNA were isolated from sorted ASCs using the modified protocol of Ambion RNAqueous -Micro kit (cat #AM1931) to recover the small RNAs. The amount of total RNA was measured by Nanodrop and run on Agilent RNA 6000 pico kit (cat # 5067-1513). Total RNA at 10ng was amplified with NuGEN Ovation PicoSL WTA system (cat # 3310-48) to generate amplified cDNA, which was then labeled with Biotin (NuGEN Encore BiotinIL Module, cat # 4210-48).

Biotin labeled cDNA at 750ng obtained from ASCs of young and aged mice was hybridized to Illumina MouseWG-6 v2 whole genome BeadChips. All arrays were processed in the Wistar Institute Genomics Facility. The data was submitted to GEO database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE72224.

Data Processing and Analysis: Arrays were quantile normalized and the data filtered to remove non-informative probes that were expressed at background levels in the majority of the samples or had a maximum fold change below 1.2 between any two samples. A total of 25,988 probes were retained for further analysis. Replicates available for 2 samples were averaged prior to statistical analysis. A t-test with a p-value cutoff of 0.05 was used to identify differentially expressed genes between the aged and young mice. All preprocessing and microarray data analysis were conducted in MATLAB 8.0.0.783.

Probes with a p-value < 0.01 (unless otherwise noted) and a minimum fold change of 1.4 were selected for functional analysis. Ingenuity Pathway Analysis (IPA) and DAVID were used to identify enriched pathways and biological functions at a p-value cutoff of 0.05 after multiple-test correction using the Benjamini procedure.

Magnetic Separation: Magnetic separation of naïve B cells was carried out using the pan B cell isolation kit from Miltenyi Biotec (CA) as specified in their protocol. Cells were resuspended at a concentration of 10^7 cells per ml in the separation buffer. Amounts of magnetic separation cocktail containing biotinylated antibodies and Streptavidin conjugated magnetic beads were calculated per manufacturer's instructions and added to the cells, and set aside for 10 minutes. The magnetic separation cocktail contained antibodies for T cells, macrophages, and non-B cells. The cells were then sorted using the magnet and resuspended in DMEM media containing 10% Fetal Bovine Serum, and antibiotics. These cells were used for Seahorse ^{TM} Extracellular Flux assays.

B cells stimulation: B cells were stimulated with a mitogen cocktail consisting of *S. Aureus* capsid, PokeWeed Mitogen extract, a CpG oligonucleotide (ODN 2006) and murine-IL-4 (Prepro Biotech) for 24 hours for flux assays. Concentrations of mitogens were determined experimentally.

Extracellular Flux assays: Cells were analyzed according to manufacturer's instructions for the Seahorse^{TM} analyzer, with the Mitochondrial Stress test kit (Seahorse Biosciences, MA). Cells were seeded at a concentration of 1 million cells per well of a 24 well plate of the Seahorse^{TM} analyzer.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

Significance:

There is an imperative need for better vaccines for the aging population especially better vaccines to influenza viruses. In the 2013-14 influenza season, the vaccine effectiveness was lowest among persons > 65 years, at just 39%. This was reflected by almost half the estimated hospitalizations (~188,795) in the year occurring within this age group (87). According to the CDC, in many years more than 90% of influenza deaths occur in this population, reiterating the need for a better vaccine (88).

Creating better vaccines for the aged requires a detailed understanding of immunosenescence, with a focus on defects in B cells and ASCs – the source for neutralizing antibodies. The findings in this thesis help understand this phenomenon, and aim to serve as a stepping stone for future studies that hopefully will eventually result in in better vaccines for the aged.

Conclusions and future directions:

In accordance with the overarching hypothesis, this thesis has shown significant differences in expression of immunoregulators on ASCs and their precursors, differences in metabolism and has identified potential links between these two factors.

In chapter 2, I show that aged ASCs in humans expressed significantly lower levels of CD27 and CD38 on their surface, two markers that are used for identifying human ASCs by techniques like flow cytometry. This finding has considerable implications in immunosenescence – primarily that we need another marker to measure ASCs in aged humans, such as an intracellular stain for immunoglobulins. Secondly, both CD27 and CD38 also play a role in signaling, and the decline in expression of these two surface

markers on ASCs, could indicate a potential loss of signaling, another possible reason for the decreased immune responses seen in the aged.

In chapter 3, I show that BTLA expression on B cells in humans declined significantly upon aging. This finding has several implications. Firstly, this is the first report to indicate a role for BTLA on B cell functions and it's the first time that age-related differences in its expression levels were observed. BTLA expression and its signaling pathway have been studied in T cells, where blocking BTLA with the glycoprotein D of the Herpes Simplex Virus (HSV gD) increases T cell responses to vaccines (58), but its role in B cells is largely unknown. Secondly, the relationship between BTLA expression on mature B cells prior to vaccination, and increases in IgG titers along with concurrent decreases in IgM titers, indicates a potential role for BTLA in shifting the immune response from IgM to IgG or from plasma cell development towards class switching. This could be of vital importance in vaccine design. Finally, the link between high levels of BTLA on switched memory B cells to better VNA responses to the influenza A strains, *in the following year* is fascinating. This suggests a different role for BTLA – as a promoter of ASCs and memory B cells, rather than an inhibitor, as it is currently thought of. This is a hypothesis that needs to be studied further.

Decreased immunoglobulin class switching, and lesser germinal center formation have been previously described in the aged, and have been implicated in the lower quality of humoral responses. The above-mentioned findings of this thesis corroborate this, and offer new insights into these defects. Low expression of CD27 and CD38 on circulating ASCs of the aged might reflect the decreased germinal center formation observed in this population. In addition, the low expression of these molecules could also have an effect on signals received by these ASCs. i.e., both CD27 and CD38 have been shown the

ability to transmit signals. CD27 has been observed to affect survival and proliferation signals, via the NF-KB pathway, while CD38 signaling plays a role in cell adhesion and lymphocyte trafficking and modulates intracellular NAD^+ levels and sirtuins. The findings from chapter 3 also corroborate this. Low BTLA expression on aged mature B cells was also linked to decreased class switching in the aged. Thus loss of these markers upon aging could affect B cell fate decisions, and push them towards a non-GC path.

There are some caveats in the human studies mentioned above that need to be readdressed. All human studies are performed with certain limitations. Our studies were limited to blood, which only allows assessing cells that are circulating. Long-lived ASCs reside primarily in the bone marrow and spleen, and since these specimens cannot be acquired readily in humans especially in highly vulnerable aged individuals, circulating ASCs were used although they are unlikely to be representative of the whole cell population. Another limitation was cell numbers. Metabolic assays – like the ones using the Seahorse^{TM} extracellular flux analyzer – require high cell numbers to generate accurate readouts, and the limited availability of PBMCs prevented the usage of these techniques in the human studies. In addition, we obtained human samples from our collaborator at Duke University. Metabolism changes very rapidly once cells are harvested. The time needed for shipment of the samples to my laboratory precluded accurate assessment of metabolic differences between young and aged human B cells. In the same token gene profiling of B cell subsets, which gives unprecedented information about differences between two cohorts, is sensitive to time delays between removing cells from their physiological environment, i.e., the blood of the human subjects, and isolation and preservation of messenger RNA. I circumvented these caveats by studying B cell immunosenescence in mice, as described in chapter 4.

In chapter 4, I show that ASCs in mice also expressed different levels of immunoregulators upon aging, and they were metabolically different compared to the young. Similar to their human counterparts, aged mice had lower levels of mature B cells and ASCs. However, the most interesting observations came from samples and techniques that couldn't be replicated in humans. Micro-array analysis of bone marrow derived ASCs (which are generated throughout the lifespan of an animal) revealed significant differences in gene expression upon aging. These ASCs were from the primary reservoir of long-lived plasma cells, which are responsible for maintaining levels of neutralizing antibodies in the blood, and are thus vital in the success or failure of vaccinations. Metabolic assays – extracellular flux analysis with the Seahorse^{TM} analyzer – showed the inability of aged B cells to respond to mitogen-mediated stimulation on par with younger B cells; specifically a need for increased energy production following stimulation could not be satisfied by an increase in oxidative phosphorylation but rather had to rely on increases in the less efficient glycolysis pathway upon aging. Furthermore, this study revealed another relationship between immunoregulator expression and metabolism. Specifically, the increased expression of PD-1 on aged naïve B cells and ASCs, and its correlation with high levels of cellular ROS. PD-1 is known to affect many metabolic pathways> It blocks Akt/mTOR signaling and thereby reduces glycolysis. Combined with the observed defects in OXPHOS these results suggest that aged B cells are poorly suited to increase energy production when challenged. The observed correlation between PD-1 and cROS levels is very interesting; also additional studies are needed to trace the source of the ROS. Most ROS is produced by mitochondrial during OXPHOS but ROS can also be produced by oxidative processes within the ER or peroxisomes. It should also be noted that ROS is involved in signaling including activation of Akt, which directly opposes PD-1 signaling (89). Furthermore mitochondrial

ROS has been described to play a role in B cells' fate decisions (90).

his finding also provides new insights into the defective metabolism of aged B cells. Specifically, the increased levels of PD-1 on aged B cells and their correlation with cellular ROS levels point towards an exhausted phenotype. This is a hypothesis that needs to be investigated further, but it is the author's opinion that the combination of high expression of a well-studied exhaustion marker (PD-1) along with the reduced ability to meet energy demands (inability to increase OXPHOS upon stimulation) indicates exhaustion.

Another observation is that while the aged B cells show decreased OXPHOS upon stimulation, they still express increased levels of cellular ROS. Since OXPHOS is one of the major contributors of cellular ROS, this appears to suggest that ROS clearance pathways might be defective in the aged. This is another hypothesis that needs to be investigated further. Another major caveat that needs to be addressed is the apparent lack of congruity between the mouse and human studies. BTLA appears to play a role in human B cell immunosenescence, but not in mice where I found expression levels to be comparable between aged and young B cells. PD-1 on the other hand was differentially expressed in an age-dependent manner in mice but not humans. While both immunoregulators may exhibit links to metabolism, as is thus far only known for PD-1, their signaling pathways are quite different. To address this caveat, we need to understand the differences between these two immune systems. Firstly, while the mouse immune system has been extensively studied, has played an enormous role in our understanding of immunology and is considered by most as an acceptable model for the human immune system, there are still considerable differences between the two species. Hence, it is possible that while both species have a link between immunoregulator

expression and metabolism, the specific nature of that link – the molecules involved for example – could be very different.

In summary, this thesis addresses my hypothesis, that:

a. *Expression levels of immunoregulators on ASCs or their precursor populations change upon aging.*
b. *Aged ASCs or their precursors are metabolically different from their younger counterparts, and*
c. *These two factors are linked.*

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