THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF VETERINARY AND BIOMEDICAL SCIENCES

Determining the Impact of B Cell Selenoproteins on the Humoral Immune Response

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SPRING 2021

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Immunology and Infectious Disease
with honors in Immunology and Infectious Disease

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ABSTRACT

Selenium (Se) is an essential micronutrient which exerts its biological effects through its incorporation into proteins via the 21st amino acid, selenocysteine. Se has shown to play a role in promoting the immune response through events such as immune cell signaling, T helper cell differentiation, and phagocytic activity, however, few studies have examined the role of B cell specific selenoproteins. Recently, our lab has found that B cells lacking selenoproteins exhibit a developmental defect and produce lower levels of basal antibodies compared to wild-type B cells. Given the apparent importance of selenoproteins in B cell development and functioning, we hypothesized that B cell selenoproteins contribute to the protective humoral immune response. We assessed the role of B cell selenoproteins the humoral immune response against *Bordetella pertussis* using a transgenic mouse model lacking all 25 selenoproteins in a B cell-specific manner ($Trsp^{B}$). After vaccinating the mice with Tdap and challenging them with *B. pertussis, we found that* $Trsp^{B}$ mice had significantly reduced antibody titers compared to control mice. However, this was not paired with a difference in bacterial clearance. We also performed a study in which mice were fed one of three Se diets: deficient, adequate, or supplemented to determine if Se supplementation could help boost the vaccine-mediated humoral immune response. In this study we did not see differences in either antibody titers or bacterial clearance between diet types. While the impact of Se supplementation is not clear, our studies provide evidence in support of the role of B cell-specific selenoproteins in the humoral immune response.

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ACKNOWLEDGEMENTS

First, I would like to thank Dr. Girish Kirimanjeswara for providing me with the opportunity to work in his lab and for his mentorship. I have thoroughly enjoyed working in this lab and working alongside the other members of the Kirimanjeswara lab. I would like to especially thank Sarah Sumner for her guidance in the lab. Sarah has helped me greatly with my project and I am very grateful for all the time she has spent teaching me techniques in the lab and giving me feedback on my writing. Furthermore, I am grateful to Dr. Bhuvana Katkere, Dr. Katherine Restori, and McKayla Nicol for their support and for always helping me when I had questions.

I would also like to thank the Schreyer Honors College for their support of my research pursuits and for the financial support from the NIH/NIAID (AI123521-05), the NIDDK STEP-UP program, and the Penn State Erickson Discovery Grant. I am also immensely grateful for the Millennium Scholars Program (MSP). MSP introduced me to a career in research and provided me with tremendous guidance and encouragement throughout these four years. The MSP advisors, in particular, Georjanne Williams and Adidi Etim-Hunting, as well as my fellow Millennium Scholars, have become like a family to me and have been one of the best parts of my experiences as a Penn State student. Finally, I am grateful to my family for believing in me and for always encouraging me to “échale ganas”.

ABBREVIATIONS

Se	Selenium
Sec	Selenocysteine
Trsp	The selenocysteine tRNA gene
LMC	Littermate control
ROS	Reactive oxygen species
NOX	NADPH oxidase
NAC	N-Acetyl Cysteine
PTx	Pertussis toxin
FIM	Fimbriae
FHA	Filamentous hemagglutinin
PRN	Pertactin
TDAP	Tetanus, Diphtheria, Pertussis vaccine
ELISA	Enzyme-linked immunosorbent assay
BG	Bordet-Gengou
wP	Whole-cell pertussis
aP	Acellular pertussis

Chapter 1

Introduction to Selenium and Its Role in Human Health

Selenium (Se) is a dietary micronutrient that is essential for human health. Se is mainly obtained through foods such as meat and dairy products, seafood, grains, and nuts. The concentration of Se in these foods is dependent upon factors including the Se content in the soil from which plants were grown and animals were raised,[1] the pH of the soil, and how well plants can take up Se, which depends on the form of Se.[2]

For adults, the recommended daily intake is around 55 micrograms per day.[3] It is estimated that Se deficiency affects up to one billion people worldwide.[3] According to the Centers for Disease Control and Prevention (CDC), Se deficiency in the United States is relatively rare but more common in countries and regions including China and Eastern Europe where Se content in the soil is lower.[4] Deficiencies in Se intake have been implicated in adverse outcomes including suboptimal immune functioning. For example, lower Se levels have a negative effect on T helper cell differentiation and macrophage signaling. Se deficiency has also been shown to correlate with HIV prevalence and poorer outcomes of patients with tuberculosis.[5] Other adverse outcomes associated with Se deficiency include an increased risk of cancer and cardiovascular disease.[6] Evidence in support of the detrimental effects of Se deficiency has also been seen with Keshan-Beck disease (KBD), which is a cardiomyopathy. KBD is mainly seen in regions of China with low Se content in the soil and supplementation with Se has been shown to prevent or reverse the development of KBD.[7]

Se exerts its biological functions through co-translational incorporation of the 21st amino acid, selenocysteine (Sec), into proteins. Proteins that contain at least one Sec are referred to as selenoproteins. Sec is encoded by a UGA codon, which is usually read as a stop codon. In order for Sec incorporation to occur, a tRNA specific to Sec is required. This tRNA must first be charged with a serine. The serine residue is then phosphorylated and converted to selenocysteine. The importance of the Sec tRNA is seen in mice, as the deletion of Sec tRNA gene, designated as *Trsp*, results in embryonic lethality. In addition to the tRNA modifications, in eukaryotes, a stem-loop structure called a Sec Insertion Sequence (SECIS), must be present in the 3' end of the untranslated region, and several other protein factors are also required.[8]

In humans, there are 25 different genes that encode for selenoproteins, and 24 genes in mice. This similarity between humans and mice has made mice a good model system to study selenoproteins. Both the cellular and organ localization and function of the 25 selenoproteins vary widely. However, there exists a hierarchical structure to the localization of selenoproteins. For example, during a time of Se deficiency, preference is given to tissues such as the brain and thyroid gland, whereas tissues including immune cells may experience lower Se bioavailability.

While the functions of selenoproteins vary, most functionally characterized selenoproteins contain Sec in the enzyme active site and serve as antioxidants and regulate redox reactions.[9] This role is important in maintaining homeostatic levels of reactive oxygen species (ROS), which are highly reactive oxygen molecules that have unpaired electrons. ROS can be produced as toxic byproducts that come from the main reactions of mitochondrial respiration. The other main source of ROS is NADPH oxidases (NOXes), which regulate ROS production as their main function and not as byproducts. NOXes produce superoxides which are then reduced to hydrogen peroxide (H_2O_2) by enzymes such as superoxide dismutase. Other examples of common ROS include

superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$). When ROS levels are not properly regulated, cells can experience cytotoxicity and oxidative stress, which can damage nucleic acids, proteins, and other cellular components. Oxidative stress can also trigger apoptosis and is linked to pathologies including cancer, immune dysfunctions, and chronic inflammation.[10]

The antioxidant activity of selenoproteins has been shown to play a critical role in immune functioning, particularly in T cells. In response to stimulation of the T cell receptor (TCR), T cells produce a burst of ROS. T cells that lack selenoproteins ($Trsp^T$) produce higher levels of ROS upon TCR engagement compared to control cells. This is paired with a reduction in proliferation and T-cell dependent antibody production. When $Trsp^T$ cells are treated with the ROS scavenger, N-Acetyl Cysteine (NAC), the control T cell phenotype is rescued. These results demonstrate the importance of selenoprotein-mediated ROS control in the development and activity of T cells.[11]

Immune functioning is also inhibited by a lack of selenoproteins in macrophages ($Trsp^M$). Similar to $Trsp^T$ cells, $Trsp^M$ cells also produce higher levels of ROS compared to control macrophages. Additionally, selenoproteins have been shown to impact the differentiation and survival of macrophages. This impact was mediated in response to parasitic infection through the antioxidant activity of Selenoprotein P.[12] Loss of selenoproteins in macrophages also inhibited their ability to migrate through the extracellular matrix.[13] Furthermore, knockout of Selenoprotein K (SelenoK), which regulates calcium flux upon immune activation, was shown to impair the phagocytic activity of macrophages.[14] This calcium flux is one of the requirements needed to generate an effective oxidative burst for microbial killing.[9] While the impact of selenoproteins in T cells and macrophages has been extensively studied, less is known about the role of selenoproteins in B cells.

Interestingly, ROS are not just toxic molecules. At moderate levels, ROS can also function as secondary messengers to mediate cell signaling and promote events such as lymphocyte proliferation and differentiation. In response to receptor stimulation and activation, immune cells undergo an oxidative burst. In B cells, NOXes are the main source of this oxidative burst, with the production of ROS occurring in two phases. In studies conducted by Wheeler and DeFranco as well as Feng, et. al., treatment of stimulated B cells with NAC during the late phase of oxidative burst completely inhibited B cell proliferation. This result emphasizes the importance of ROS for sustained signaling. Furthermore, sustained ROS production was also found to be important for the phosphorylation of signaling molecules involved in the NF-kB and PI3K pathways. Signaling for these pathways is critical for the survival and proliferation of B cells.[1516]

More specifically, a common ROS that mediates the survival and proliferation of activated B cells is H_2O_2. H_2O_2 acts by specifically oxidizing the cysteine residue of proteins. The specificity of H_2O_2 makes it more of a mild ROS compared to other ROS molecules, which oxidize any molecule they encounter.[9] One major target of H_2O_2 is protein tyrosine phosphatases (PTPs). PTPs such as SHIP-1 and PTEN serve as negative regulators of TCR and B cell receptor (BCR) signaling.[16] Oxidation of PTPs leads to a conformational change in its active site which inhibits its phosphatase activity and enhances TCR or BCR signaling. This is a reversible process, as PTPs can return to the active site through reduction by cytosolic glutathione and the selenoprotein, thioredoxin. Other studies have proposed a model in which selenoproteins such as glutathione peroxidase (GPX) reduce and detoxify H_2O_2 and subsequently oxidize the cysteine residue of PTPs. In this model, H_2O_2 acts indirectly on PTPs.[17] While ROS are important for BCR signaling, ROS levels must still be regulated to prevent cellular damage and stress, and potentially cell death.[9]

Given that selenoproteins regulate ROS levels, which are important in lymphocyte development upon receptor stimulation, the Kirimanjeswara group found it plausible that B cell-specific selenoproteins could impact B cell development and functioning. Our lab has generated a transgenic mouse model in which all 25 selenoproteins were knocked out in a B cell specific manner (*Trsp*^{B}) using a lox-cre system. As expected, the numbers and functions of B cells from *Trsp*^{B} mice were impacted. Spleens from *Trsp*^{B} mice exhibited reduced numbers of total B cells as well as reduced numbers of different splenic B cell populations compared to littermate control (LMC) mice. These reduced numbers are believed to be due to a developmental defect seen between the late pro-B/large pre-B cell and small pre-B cell stage. The *Trsp*^{B} B cells also exhibited reduced homeostatic levels of the majority of immunoglobulins. Given these defects seen in *Trsp*^{B} cells, we sought to determine the impact of B cell-specific selenoproteins on the humoral immune response *in vivo*.

Chapter 2

Humoral Immune Response to *Bordetella pertussis*

The adaptive immune response is composed of two arms: cell-mediated immunity and humoral immunity. The main effector cells of the humoral immune response are B cells, which differentiate into plasma cells and are then responsible for the production of antibodies. One function of antibodies is to provide protection against infectious diseases through the neutralization of pathogens and toxins to prevent infectivity and toxin-induced damage. Antibodies also have functions that are dependent upon effector cells and molecules. These functions include opsonization of bacteria to promote phagocytosis by neutrophils or macrophages and the activation of the classical complement pathway, which leads to bacterial cell lysis.[18] In addition to antibody-mediated functions, B cells can also promote the activity of T cells through antigen presentation, costimulation, and cytokine-stimulated activation.[19]

Beyond regulating ROS levels, one B cell function that could be impacted by a lack of selenoproteins is antibody secretion and functioning. Selenoprotein F (Selenof) is involved in regulating the secretion of disulfide-rich glycoproteins, such as antibodies. Selenof is an endoplasmic reticulum (ER)-resident protein that interacts with and promotes that activity of UDP-glucose: glycoprotein glycosyltransferase (UGGT). UGGT is responsible for recognizing improperly folding glycoproteins and promoting their reglycosylation to ensure the proper quality of proteins exiting the ER to the golgi. Mice that lack Selenof have been shown to secrete higher titers of IgM compared to control mice, However, these antibodies are nonfunctional, which supports Selenof's role in enhancing UGGT activity. Additionally, Selenof deficient mice express lower levels of proteins involved in antigen presentation and vesicle transport, further implicating Selenof in B cell functioning.[20]

In order to understand how B cell-specific selenoproteins impact the humoral immune response, we used *Bordetella pertussis* as the model pathogen. *B. pertussis* is the causative agent of the acute respiratory infection, whooping cough, which is an important public health concern. Whooping cough is a highly contagious disease that is spread by airborne respiratory droplets[21] and can cause serious illness or even death in infants.[22] Worldwide, it is estimated that there are around 24.1 million cases and 160,700 deaths in children under the age of 5 each year from whooping cough. A large percentage of these cases stem from developing countries, however, whooping cough is still a prevalent concern in the United States.[23] While adults may become infected with *B. pertussis*, the severity of the disease is milder. However, infected adults may act as reservoirs for *B. pertussis* and spread the disease to children who lack immunity.[24]

B. pertussis, is a T-dependent gram-negative bacterium that causes localized infection in the lower respiratory tract. The pathogenesis of *B. pertussis* is aided by the secretion of toxins which act as virulence factors. Some of these toxins include the pertussis toxin (PTx), fimbriae (FIM), filamentous hemagglutinin (FHA), and pertactin (PRN). These specific toxins are all components of the current acellular pertussis vaccine and are targeted by antibodies and T cells. The functions of these toxins include adhesion to lung epithelial cells, immunomodulation, and damage to respiratory tract tissue. Notably, PTx is particularly involved in blocking the effector functions of the humoral immune response. The activity of PTx disrupts G protein signaling, which can lead to lymphocytosis[25] and also interferes with the neutrophil-mediated clearance of antibody-bound *B. pertussis*.[28] PTx can also inhibit antigen presentation and lymphatic migration of antigen-presenting cells.[26]

The humoral immune response plays a crucial role in clearing infection by *B. pertussis*, which is exemplified in mice that lack B cells, as they are unable to clear infection.[27] The

importance of B cells in clearing *B. pertussis* infection makes *B. pertussis* a good model system to understand the role of B cell-specific proteins in the humoral immune response. Many studies have shown the importance of antibodies in protection against *B. pertussis*. Serum antibodies, specifically IgG, are important for opsonizing *B. pertussis*, and recruiting neutrophils for phagocytosis.[28] Antibodies are also important in neutralizing toxins secreted by *B. pertussis* and inhibiting bacterial binding to epithelial cells.[26]

In this study, we explored the role of B cell-specific selenoproteins in the vaccine-induced humoral immune response. Given the developmental defects seen in $Trsp^{B}$ cells, we hypothesized that $Trsp^{B}$ mice would have an impaired humoral immune response compared to LMC mice. We approached this question by vaccinating and boosting mice with the acellular pertussis vaccine, Tdap, and subsequently challenging mice with B. pertussis (Figure 1). We found that $Trsp^{B}$ mice had lower titers of IgM and IgG. Although we hypothesized that lower antibody titers would track with reduced bacterial clearance, no significant differences were seen. Ultimately, this study provides insight into the impact of selenoproteins on the memory response and functions of humoral immunity.

Materials and Methods

Generation of *Trsp*^{B} and *Trsp*$^{fl/fl}$ Mice

The mouse mutants were generated through Cre-Lox recombination[29] using mice that were a generous gift from Bradley Carlson. The *Trsp* gene was floxed and the cre recombinase was under the control of the CD19 promoter. Mice that were either *Trsp*$^{fl/fl}$ CD19$^{WT/WT}$ or *Trsp*$^{fl/fl}$ CD19$^{WT/Cre}$ were kept as used as littermate controls (LMC) or B cell-specific selenoprotein knockout mice (*Trsp*^{B}), respectively. The breeding scheme was as follows:

$$F_0\text{: CD19}^{WT/WT}\ Trsp^{fl/fl}\ \text{x}\ \text{CD19}^{cre/cre}\ Trsp^{WT/WT}$$

$$F_1\text{: CD19}^{Cre/WT}\ Trsp^{fl/wt}\ \text{x CD19}^{Cre/WT}\ Trsp^{fl/WT}$$

$$F_2\text{: CD19}^{Cre/WT}\ Trsp^{fl/fl}\ \text{x CD19}^{WT/WT}\ Trsp^{fl/fl}$$

Animal Experiments- *Trsp*^{B} and *Trsp*$^{fl/fl}$ Mice- Experiment 1

All experiments with mice were performed in accordance with institutional review board guidelines. Mice were vaccinated through intraperitoneal injection of 200 µL of the tetanus diphtheria attenuated pertussis (TDAP) vaccine, Adacel, from Sanofi Pasteur. Ten mice per genotype were vaccinated with TDAP. Four mice per genotype were injected with PBS instead of TDAP as a control. Blood was collected on days 0, 7, 14, 28 post-vaccination, and days 3 and 7 post-inoculation. A booster vaccination of 200 µL was administered two weeks after the initial vaccination. Four weeks after the initial vaccination, the mice were sedated with isoflurane and inoculated intranasally with 50 µL of phosphate-buffered saline (PBS) containing 5 x 10^5 CFU of B. *pertussis*. A group of mice were sacrificed on days 3 and 7 post-inoculation. Colonization of the lungs was determined by homogenizing the lungs in 1 ml of PBS. The lung homogenate was

plated on Bordet-Gengou (BG) agar containing 10% sheep's blood. Following three-day incubation at 37ºC, colonies were counted and CFU enumerated for bacterial burden.

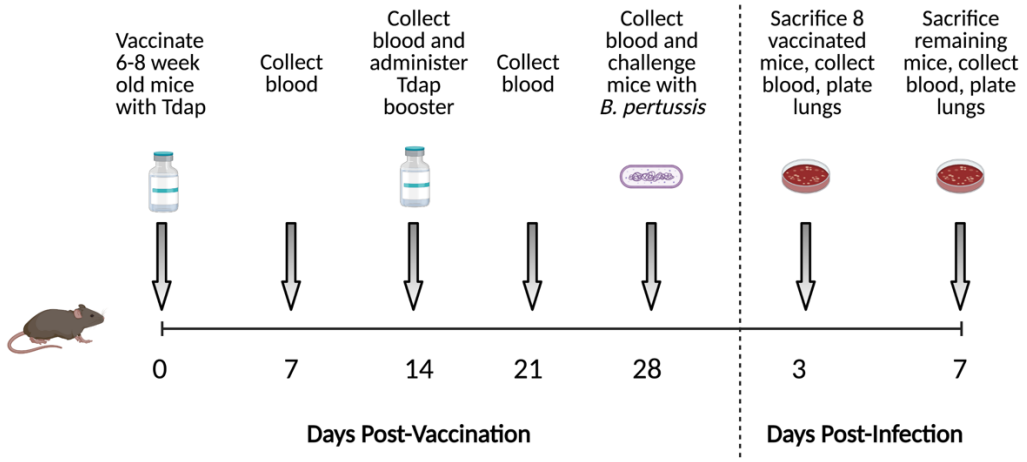

Figure 1. Schematic diagram of mouse experimental protocol

Animal Experiments- *Trsp*^{B} and *Trsp*$^{fl/fl}$ Mice- Experiment 2

The procedures outlined in experiment 1 were followed with the following exceptions: blood was collected at days 7, 14, 28 post-vaccination, instead of the days mentioned in experiment 1. Mice were challenged with a streptomycin-resistant strain of *B. pertussis*. Experiment 2 lung homogenates were plated on 20 µg of streptomycin per ml in addition to the BG agar and 10% sheep's blood.

Enzyme-linked immunosorbent assay (ELISA)

Anti-*B. pertussis* antibody titers in sera were determined using ELISAs. Plates were coated at a CFU of 5 x 10^5 of B. *pertussis* using carbonate buffer. Plates were incubated for 2 hours at 37°C and then kept at 4°C overnight. Plates were washed three times with 0.05% Tween-20 in PBS in between steps. Next, plates were blocked with PBS with 10% fetal bovine serum for 1 hour and

serum samples were added for 1.5 hours at the following dilutions in 1x PBS: 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200. Secondary biotinylated Anti-mouse IgM or IgG was added in a 1:500 dilution in 1xPBS for 1 hour followed by the addition of Streptavidin-HRP in a 1:500 dilution in 1xPBS for 20 minutes. The substrate buffer and solution were added and the OD of each well was read at 405nm. Differences in antibody titers were analyzed at the 1:200 dilution throughout this experiment.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 9. Differences in antibody levels were determined by t test, grouped according to dilution. The standard deviation of the samples is shown in the graphs.

Results- Experiment 1

In order to determine if there were any differences in antibody levels between *Trsp*^{B} mice and LMC mice, blood was collected weekly, starting after the initial vaccination. Antibodies from the serum of this blood were specific to *B. pertussis*. At day 7 post-vaccination, there were no significant differences in IgM titers at any dilution (Figure 2). However, the lack of a significant difference may have been due to the small sample size of two mice per group. While we vaccinated eight mice per genotype, we had difficulty with blood collection at day 7, which resulted in only two serum samples per group. At day 14 post-vaccination, we began to see significant differences in IgM titers between LMC and *Trsp*^{B} mice. The IgM levels of *Trsp*^{B} mice were about 50% lower than LMC mice at day 14 (Figure 3).

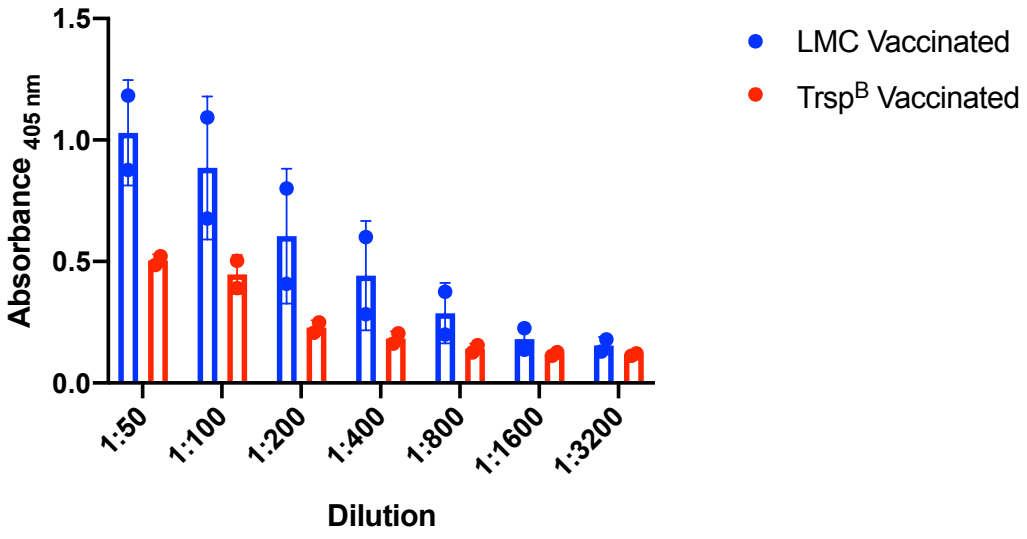

Figure 2. Day 7 Post-Vaccination IgM Titers

LMC n=2, *Trsp*^{B} n=2

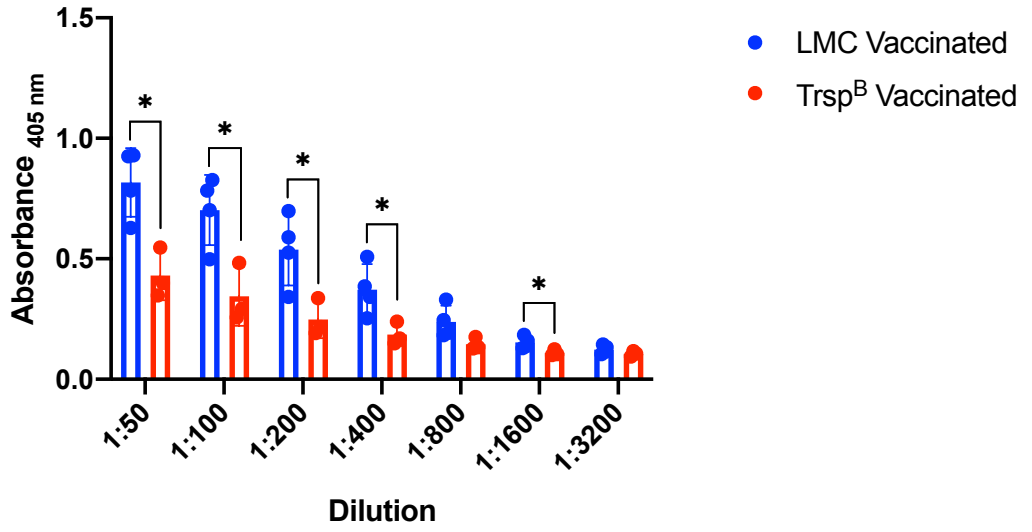

Figure 3. Day 14 Post-Vaccination IgM Titers

*p<0.05, LMC n=3, *Trsp*^{B} n=4

The trend of lower antibody titers in *Trsp*^{B} mice compared to LMC mice continued for the rest of the time points. At day 21, *Trsp*^{B} mice had 60% IgM titers compared to LMC mice (Figure 4). Similarly, at day 28, *Trsp*^{B} IgM antibody titers were 55% lower than those of LMC mice (Figure 5).

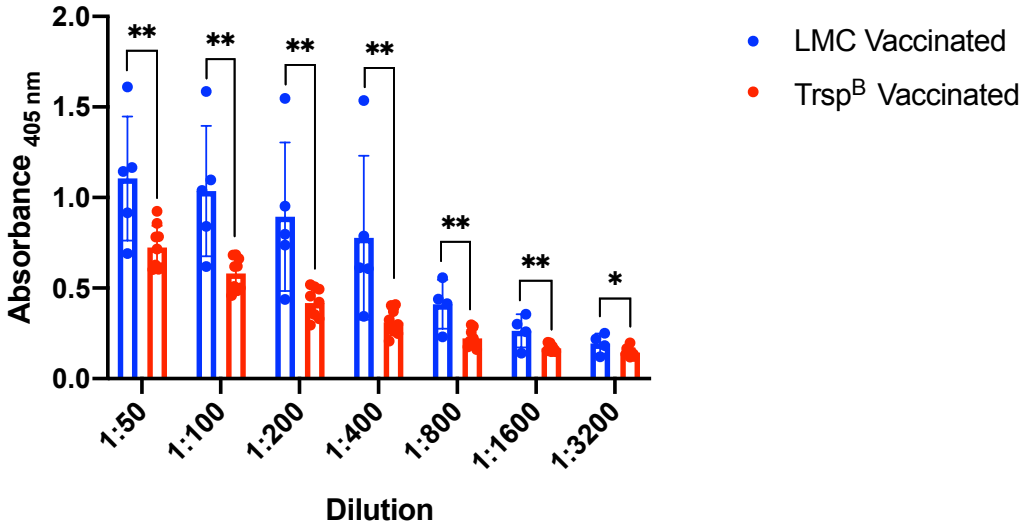

Figure 4. Day 21 Post-Vaccination IgM Titers

*p<0.05, **p<0.01, LMC n=5, *Trsp*^{B} n=10

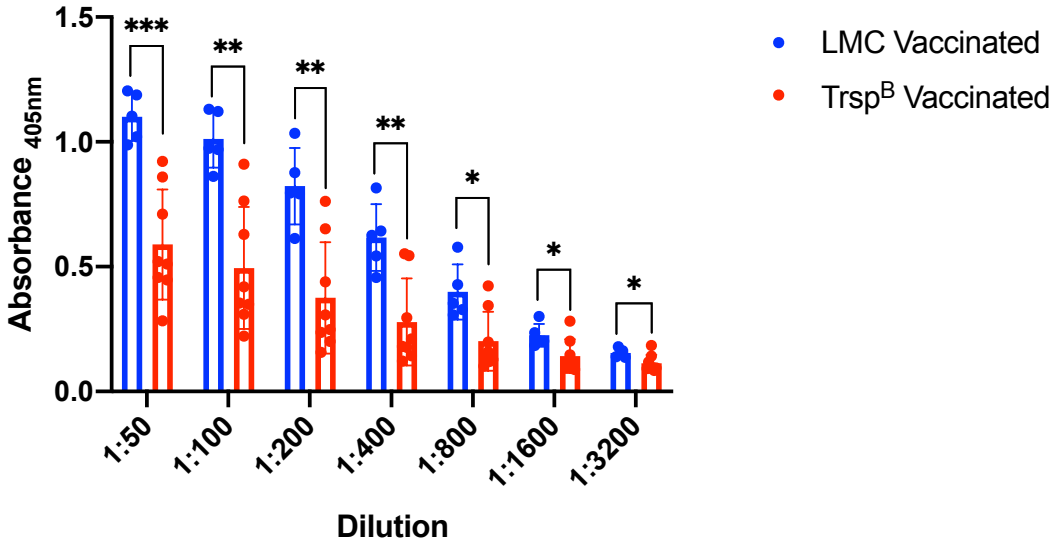

Figure 5. Day 28 Post-Vaccination IgM Titers

*p<0.05, **p<0.01, ***p<0.001, LMC n=5, *Trsp*^{B} n=8

At 28 days post-vaccination, we challenged the mice with 5 x 10^5 CFU of *B. pertussis*. Three days after infection, we sacrificed eight vaccinated mice (four per genotype) to enumerate lung bacterial burden. Blood was collected at this time point as well for antibody level analysis. Again, we found that *Trsp*^{B} IgM titers were about 50% lower than those of LMC mice (Figure 6). However, due issues with contamination, were not able to properly enumerate *B. pertussis* colony counts in the lungs. There were only three plates from *Trsp*^{B} mice and one plate from an LMC mouse that were countable (data not shown). Thus, significant differences could not be calculated.

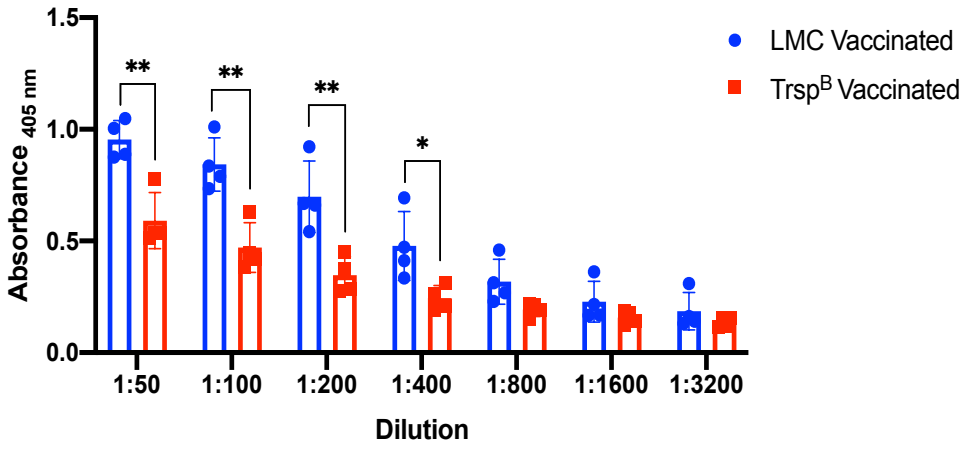

Figure 6. Day 3 Post-Infection IgM Titers

*p<0.05, **p<0.01, LMC n=4, *Trsp*^{B} n=4

At seven days post-infection, blood samples were collected and the remaining mice were sacrificed for bacterial enumeration in the lungs. The greatest difference in IgM titers between LMC and *Trsp*^{B} mice was seen at this time point, at which *Trsp*^{B} IgM titers were 66% lower than those of LMC mice. (Figure 7). Again, we came across issues with contamination and could not see significant differences between vaccinated LMC and *Trsp*^{B} mice as only one LMC sample was countable (Figure 8). Interestingly, vaccination of *Trsp*^{B} mice did not seem to improve bacterial clearance as the control *Trsp*^{B} mice did not have significantly higher bacterial load. However, this data should be interpreted with caution as the sample size (n=3) for each of the *Trsp*^{B} groups was low due to constraints caused by contamination.

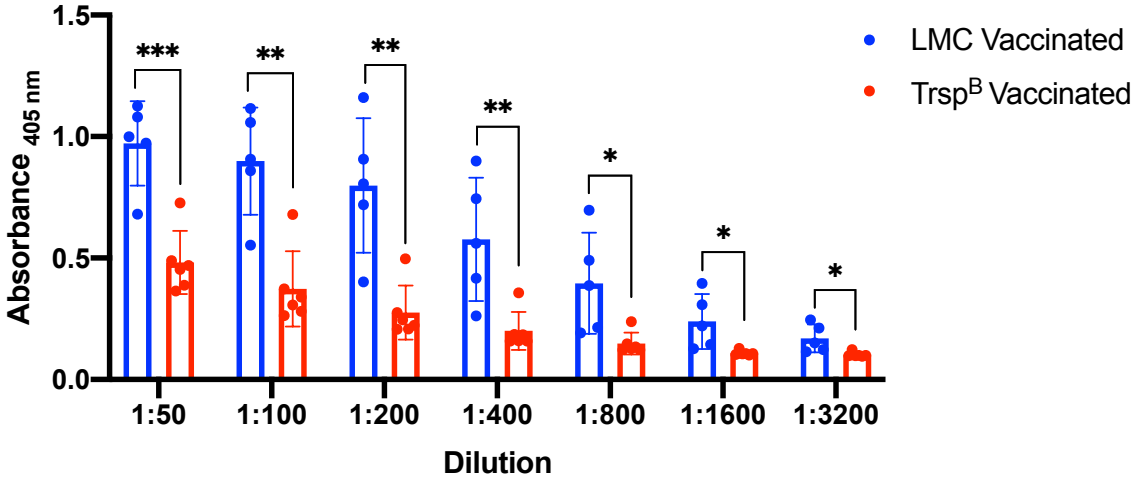

Figure 7. Day 7 Post-Infection IgM Titers

*p<0.05, **p<0.01, *p<0.001, LMC n=5, *Trsp*^{B} n=6

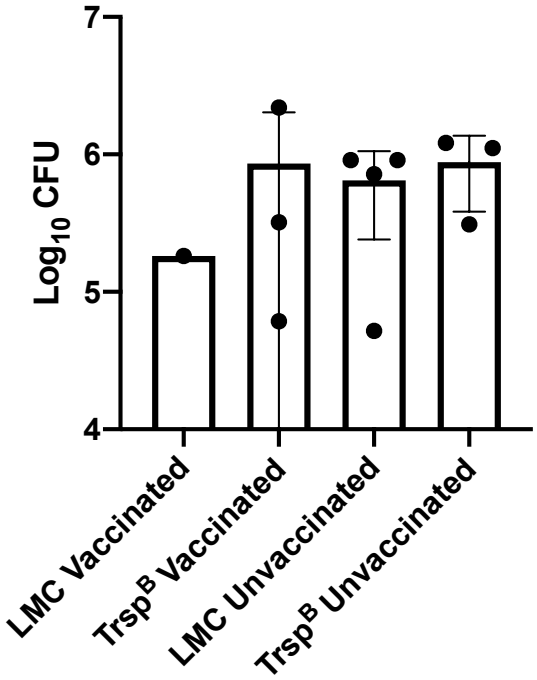

Figure 8. Day 7 Post-Infection Lung Bacterial Burden

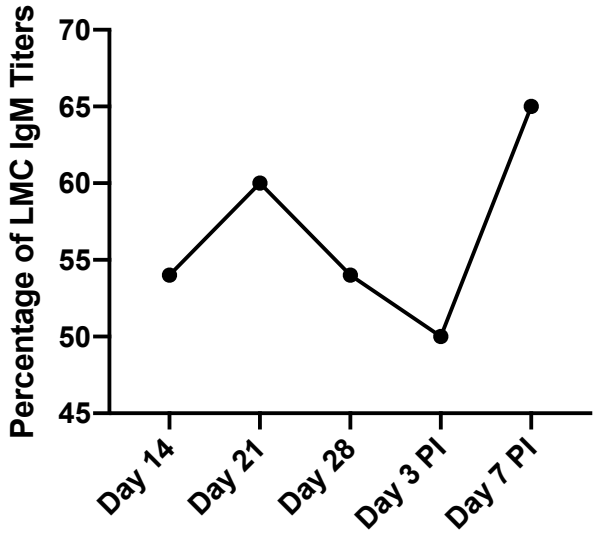

Figure 9. Summary of *Trsp*^{B} IgM Titers Compared to LMC IgM Titers

PI represents post-infection

Results- Experiment 2

In order to measure difference in IgG titers and be able to better measure lung bacterial burden, we repeated the previous experiment. Similar to experiment 1, there were clear differences in antibody titers between LMC and *Trsp*^{B} mice. Starting at day 7 post-vaccination, IgM titers from *Trsp*^{B} mice were 30% lower than that of LMC mice (Figure 10). At day 14 we began measuring IgG levels, as class-switching should have occurred by this time point. We observed 60% lower IgG titers in *Trsp*^{B} mice compared to LMC mice (Figure 11). As with day 14 post-vaccination, at day 28 post-vaccination we observed nearly 60% lower IgG levels in *Trsp*^{B} mice (Figure 12).

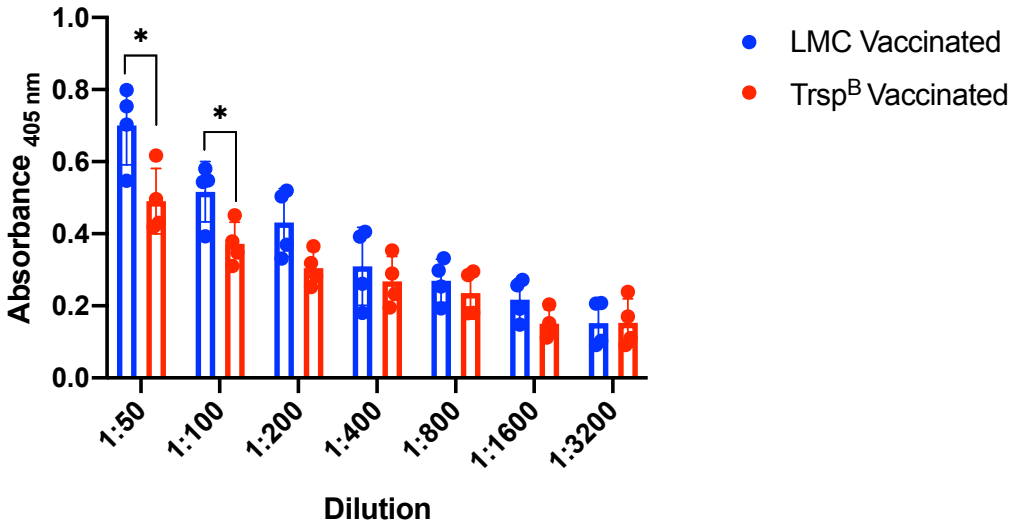

Figure 10. Day 7 Post-Vaccination IgM Titers

*p<0.05, LMC n=4, *Trsp*^{B} n=4

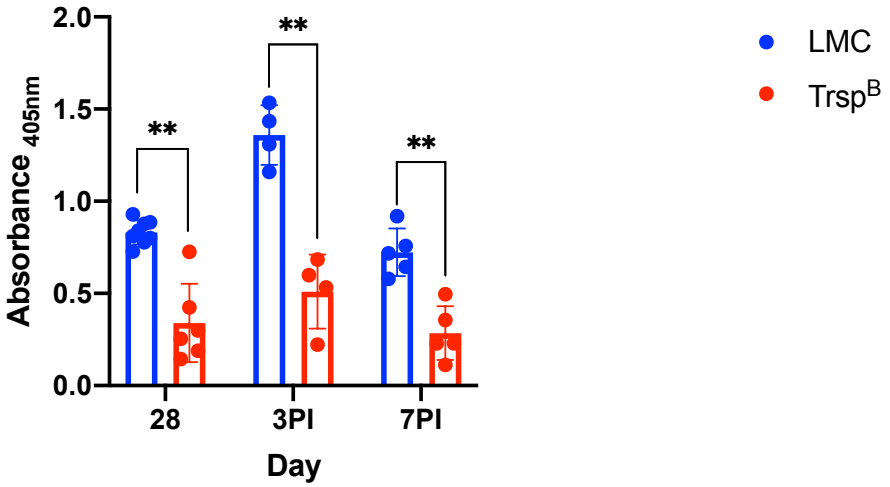

Figure 11. Day 14 Post- Vaccination IgM Titers

*p<0.05, **p<0.01

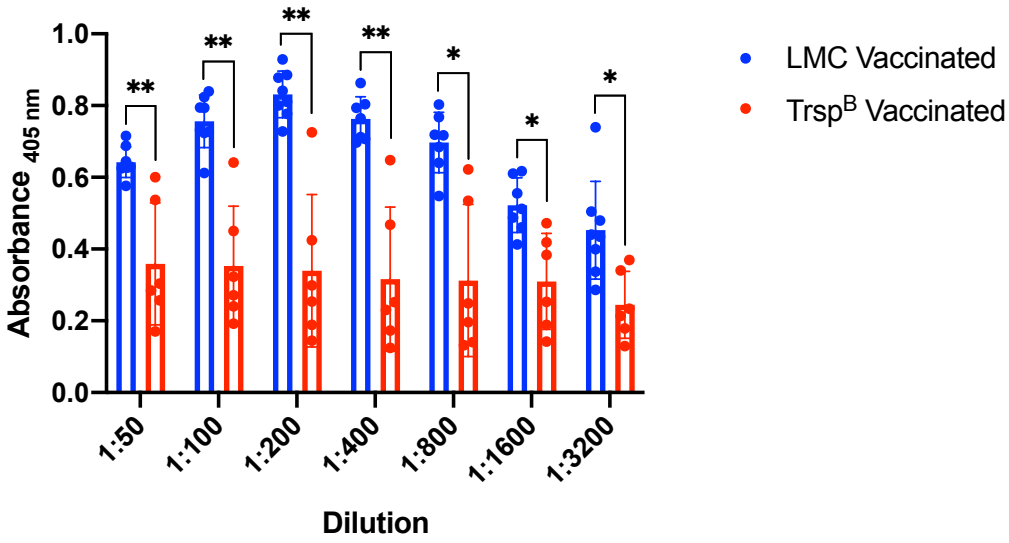

Figure 12. Day 28 Post-Vaccination IgG Titers

*p<0.01, **p<0.001, LMC n=8, *Trsp*^{B} n=6

Just as was done with the first replicate of this experiment, we challenged the mice with *B. pertussis* at day 28 post-vaccination. At three days post-infection, we sacrificed eight vaccinated mice, four of each phenotype. After collecting serum from these mice, again we saw around 60% lower IgG level in *Trsp*^{B} mice (Figure 13), and this percentage remained the same on day 7 post-

infection (Figure 15). Despite using a streptomycin-resistant *B. pertussis* strain and using plates containing streptomycin, we still encountered difficulty enumerating lung bacterial burden due to contamination. At day 3 post-infection, CFU counts from only four vaccinated mice (two of each genotype) were obtained. We did not see any significant differences in lung bacterial burden (Figure 14). Again, we note the small sample size in this comparison.

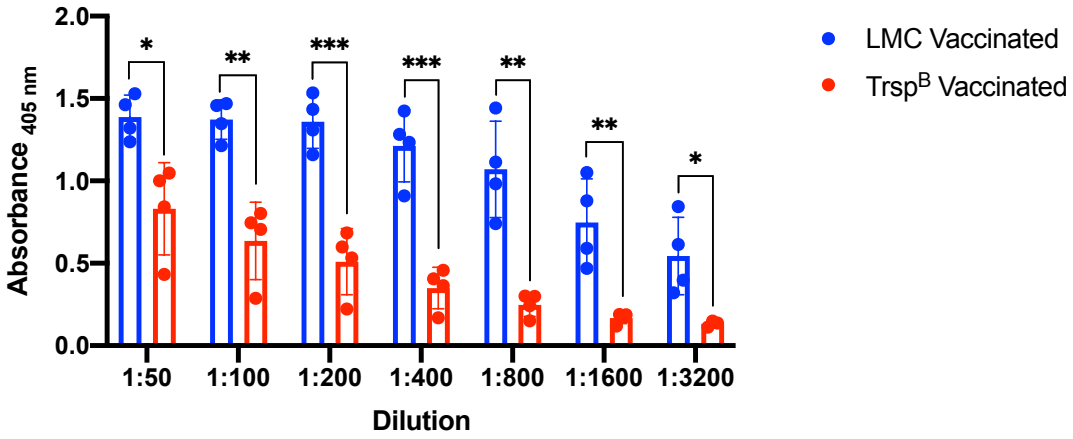

Figure 13. Day 3 Post-Infection IgG Titers

*p<0.05, **p<0.01, ***p<0.001, LMC n=4, *Trsp*^{B} n=4

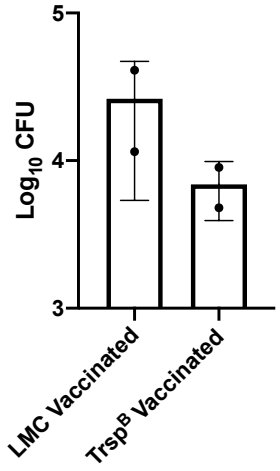

Figure 14. Day 3 Post-Infection Lung Bacterial Burden

At seven days post-infection, the remaining mice were sacrificed. We were not able to compared the lung bacterial burden between LMC and *Trsp*^{B} mice as no plates from LMC mice were suitable for counting.

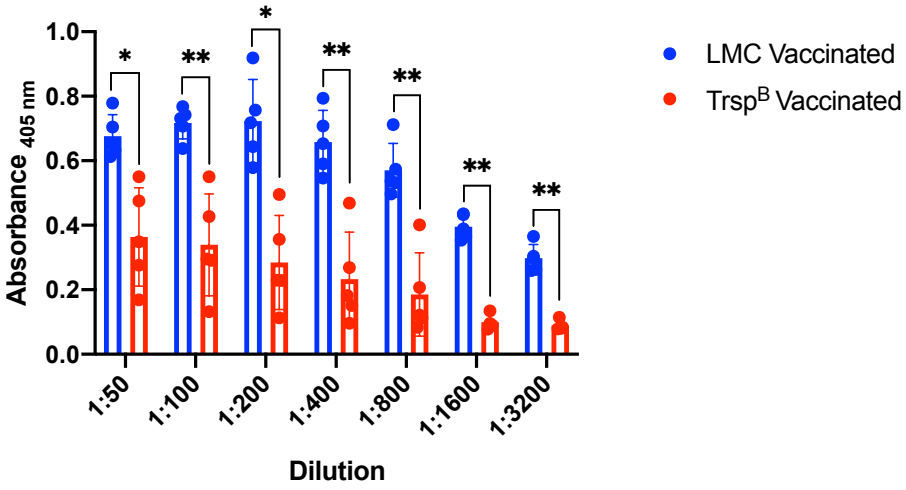

Figure 15. Day 7 Post-Infection IgG Titers

*p<0.01, **p<0.001, LMC n=5, *Trsp*^{B} n=5

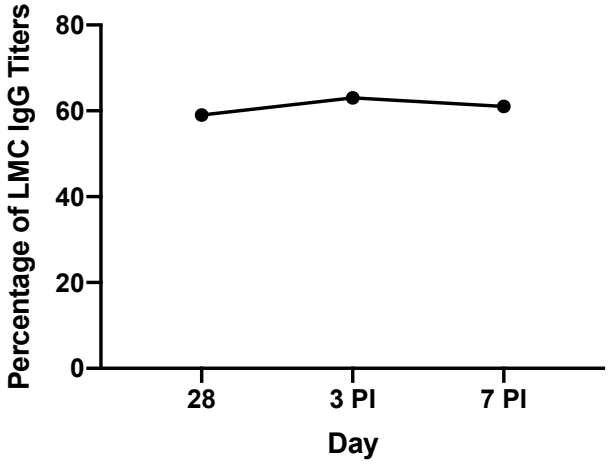

Figure 16. Summary of *Trsp*^{B} IgG Titers Compared to LMC IgG Titers

PI represents Post-Infection

Discussion

In this study we knocked out selenoproteins in a B cell-specific manner. After vaccinating mice with Tdap and challenging them with B. pertussis, we found that *Trsp*^{B} mice had significantly lower IgM and IgG titers compared to control mice. At almost all time points, *Trsp*^{B} mice had between a 50-65% reduction in either IgM or IgG titers. Despite this reduction in immunoglobulins, bacterial clearance in *Trsp*^{B} mice did not appear to be impacted. This lack of a difference could be due to the fact that the reduction in antibody levels is not sufficient to impact bacterial clearance. It is also possible that there was a difference in bacterial clearance, but it was masked by contamination on the plates. Instead of plating lung homogenates to measure bacterial burden, in the future we could potentially quantify *B. pertussis* in the lungs using qPCR. This could help eliminate the problems we faced with contamination.

While we did see differences in antibody titers between *Trsp*^{B} and LMC mice, the cause of these differences is not clear. Given that *Trsp*^{B} mice have reduced populations of B cells in lymphoid tissue and exhibit a developmental defect, it is possible that the reduced antibody titers in *Trsp*^{B} mice is due to reduced B cell numbers, and not differences in secretion. Furthermore, differences in IgG titers could be caused by a defect in class switching, but it could also be caused by lower initial titers of IgM in *Trsp*^{B} mice compared to LMC mice. Additional studies should be conducted to explain the cause of antibody titer differences. It would also be interesting to see if the reduced antibody titers in *Trsp*^{B} mice are paired with reduced functionality as was seen with the Selenof knockout.

In our model we knocked out all selenoproteins in B cells so our next step would be to identify the specific B cell selenoproteins that are important for the humoral immune response, and particularly antibody production. We could approach this by looking at which B cell

selenoproteins are more highly expressed upon B cell activation. Another future step would be to determine how other functions of B cells, such as antigen presentation, are impacted by selenoprotein deficiency. While our lab has seen that Se-supplementation enhances BCR-mediated endocytosis, our understanding of how a B cell specific knockout of selenoproteins impacts antigen presentation is not clear.

Chapter 3

Se Dietary Mouse Model

The first pertussis vaccine was introduced in the 1950s, which was a whole-cell (wP) vaccine composed of inactivated *B. pertussis*. The wP vaccine demonstrated strong efficacy, as it reduced pertussis incidence and mortality by at least 90%.[30] Despite its strong efficacy, its usage has been discontinued in most developed countries due to its adverse effects. In the 1980s the wP vaccine was replaced with the acellular vaccine (aP). Since then, there has been a reemergence of pertussis. Strikingly, in 2012 the United States reported the highest number of cases within the last 50 years. [31]

The reason behind this dramatic increase in incidence is multifold. First, there are poor correlates of protection for the pertussis vaccine. While antibodies are essential for *B. pertussis* clearance, antibody titers do not correlate well with protection against *B. pertussis*.[22] Additionally, while the antibody response is important for protecting against severe disease, it likely does not prevent subclinical infection. This means that individuals with subclinical infection can spread pertussis to those who lack immunity.[28] Lastly, there is a limited duration of protection (~5 years) provided by the existing vaccines, which primarily rely on B cell-mediated antibody responses.[32] Repeated booster shots are often required for complete protection and compliance for such an aggressive vaccine regimen is often poor. Thus, there is a need to optimize existing vaccines to improve B cell-mediated immune responses that lasts longer and limits the spread of infectious agents. Given the need to improve the pertussis vaccine, we were interested in determining if Se

supplementation could function as an endogenous metabolic adjuvant to boost the vaccine-mediated humoral immune response.

Thus far, the beneficial effects of Se intake on the immune response have mainly been observed in studies comparing Se-deficient and Se-adequate groups. We were interested in determining whether supranutritional intake of Se could enhance the immune response beyond what is observed with Se-adequate intake. This could be possible given that recommended dietary allowances (RDAs) are not necessarily based on maximal functioning. Rather, RDAs are based on levels that prevent the failure of functions or signs of deficiency.[33] Furthermore, as mentioned previously, the allotment of selenoproteins is a hierarchical process. With a Se-supplemented diet, there would be enough Se for priority tissues such as the brain, which could lead to more Se availability for immune cells.

While the impact of supranutritional levels of Se intake is not certain, there is some evidence to suggest that Se intake above adequate levels can have beneficial effects. For example, there are some studies that suggest that supranutritional levels of Se intake can reduce the risk of certain types of cancers, such as lung, prostate, and colorectal cancers. However, these results also depend on factors such as age, gender, and genetics. Other studies refute these findings and suggest that supranutritional levels of Se intake do not reduce the risk of cancer.[34]

In regards to immune functioning, our lab has shown that splenocytes isolated from mice that are maintained on a Se-supplemented diet have enhanced BCR-mediated endocytosis and calcium flux.[35] Additionally, one study has shown that the lymphocytes from Se supplemented individuals had an increased proliferative capacity compared to individuals with a low Se diet. This was paired with a slightly higher secondary antibody response in Se supplemented individuals, however, this difference was not significant.[36] Further studies are needed to determine

if there is a difference in the immune response between individuals with a Se adequate and Se supplemented nutritional status. It is important to note that while there are potentially beneficial effects of Se supplementation, intake that is too high could result in toxicity. Toxic effects can be seen in doses above 400 μg per day to 700 μg per day.[37] However, Se toxicity is not common, and the estimated daily intake, which is between 83 μg to 120 μg per day, is far below these toxic levels.[38]

The differences in antibody titers between *Trsp*^{B} mice and control mice suggest that B cell-specific selenoproteins play a role in the humoral immune response functions. After seeing these differences, we were motivated to determine if similar differences would be seen in a dietary model in which mice were fed one of three Se diets: deficient, adequate, or supplemented, which correlate to RDAs. This question and approach have high translational value as a dietary model is a more realistic version of what would be seen in a human population rather than a genetic deletion/mutation. We believed if we saw higher antibody titers and reduced bacterial burden in the Se supplemented mice compared to the Se adequate or Se deficient mice, this study could help inform the potential for Se to be used as an exogenous metabolic adjuvant to enhance the durability of protection of the *B. pertussis* vaccine. However, unlike the genetic knockout model, we failed to see differences in antibody titers. This was paired with a lack of significant differences in lung bacterial burden.

Materials and Methods

Animal Experiments- Se Diet Mice

Four-week old C57BL/6 mice were maintained on one of three Se diets: supplemented (0.4 parts per million (ppm)), adequate-low (0.1 ppm), or deficient (0.008 ppm) for 12 weeks before vaccination, and for the duration of the experiment. Mice were treated with Milli-Q water. Mice were vaccinated through intraperitoneal injection of 200 µL of the TDAP vaccine, Adacel, from Sanofi Pasteur. Eight mice per diet type were vaccinated. Blood was collected on days 14 and 28 post-vaccination, and days 3 and 7 post-inoculation. A booster vaccination of 200 µL was administered two weeks after the initial vaccination. Four weeks after the initial vaccination, the mice were sedated with isoflurane and inoculated intranasally with 50 µL of PBS containing 5 x 10^5 CFU of a streptomycin-resistant strain of B. *pertussis*. Four mice from each diet group were vaccinated on day 3 post-inoculation. The remaining mice were sacrificed on day 7 post-inoculation. Colonization of the lungs was determined by homogenizing the lungs in 1 ml of PBS. The lung homogenate was plated on BG agar containing 10% sheep's blood and 20 µg of streptomycin per ml. Following three-day incubation at 37ºC, colonies were counted and CFU enumerated for bacterial burden.

Enzyme-linked immunosorbent assay (ELISA)

Anti-*B. pertussis* antibody titers in sera were determined using ELISAs. Plates were coated at a CFU of 5 x 10^5 of B. *pertussis* using carbonate buffer. Plates were incubated for 2 hours at 37°C and then kept at 4°C overnight. Plates were washed three times with 0.05% Tween-20 in PBS in between steps. Next, plates were blocked with PBS with 10% fetal bovine serum for 1 hour and serum samples were added for 1.5 hours at the following dilutions in 1x PBS: 1:50, 1:100, 1:200,

1:400, 1:800, 1:1600, 1:3200. Secondary biotinylated Anti-mouse IgM or IgG was added in a 1:500 dilution in 1xPBS for 1 hour followed by the addition of Streptavidin-HRP in a 1:500 dilution in 1xPBS for 20 minutes. The substrate buffer and solution were added and the OD of each well was read at 405nm. Differences in antibody titers were analyzed at the 1:200 dilution throughout this experiment.

Results

After 12 weeks on their respective diets, wild-type mice were vaccinated with Tdap and received a booster two weeks later. Serum was collected at days 14 and 28 post-initial vaccination as well as days 3 and 7 post-infection. As seen in figures 17, 18, 19, and 20, there were no significant differences in IgG titers at any dilution. At days 3 and 7 post-infection we sacrificed mice and plated the lungs to assess bacterial burden. However, similar to the B cell-specific knockout experiments, we came across issues with contamination and were only able to count samples from two mice at each time point (data not shown). Thus, we were not able to determine if there were any significant differences in bacterial clearance between the Se diet types.

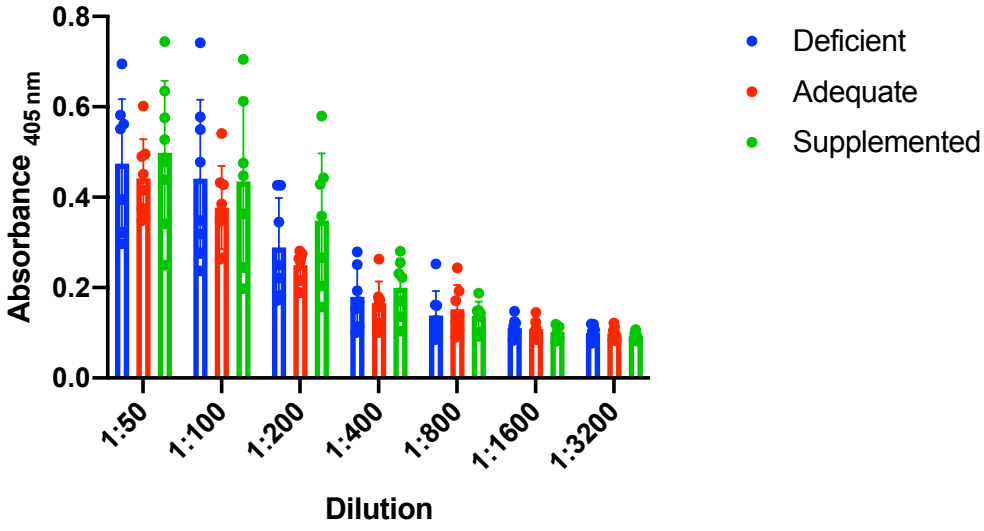

Figure 17. Day 14 Post-Vaccination IgG Titers

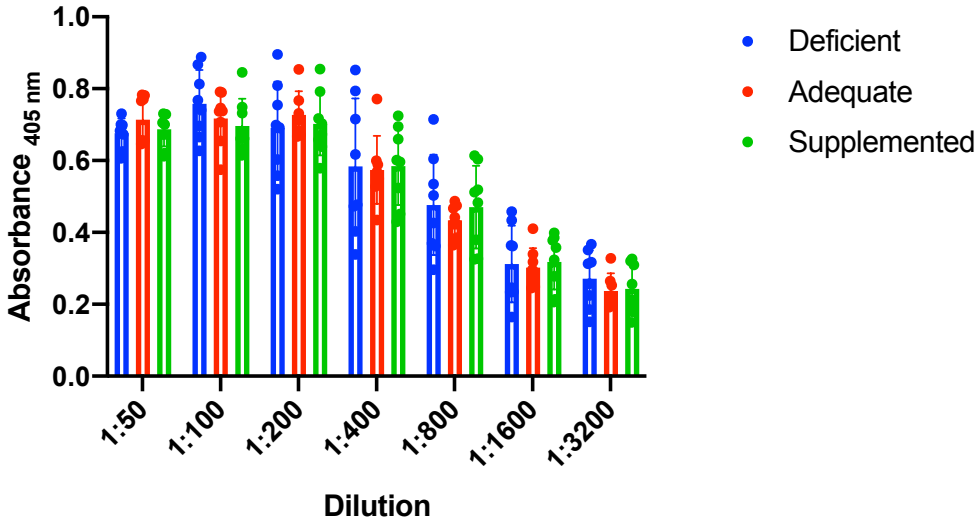

Figure 18. Day 28 Post-Vaccination IgG Titers

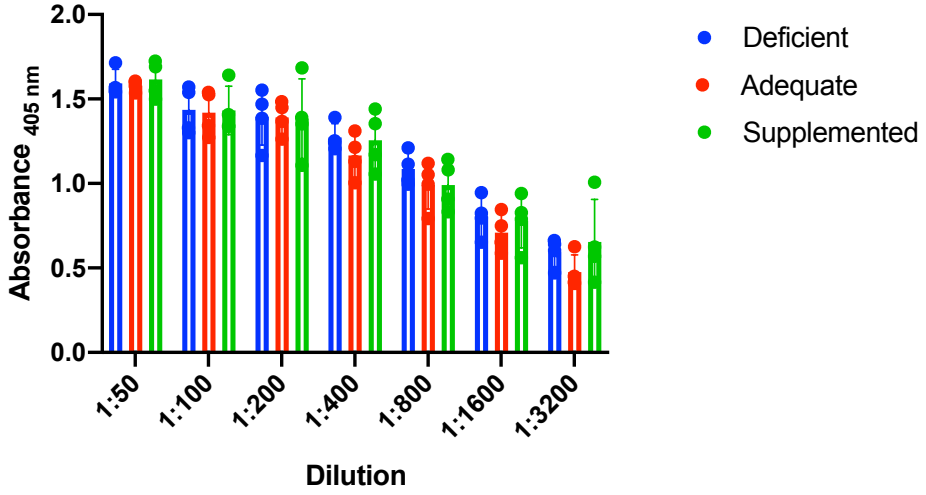

Figure 19. Day 3 Post-Infection IgG Titers

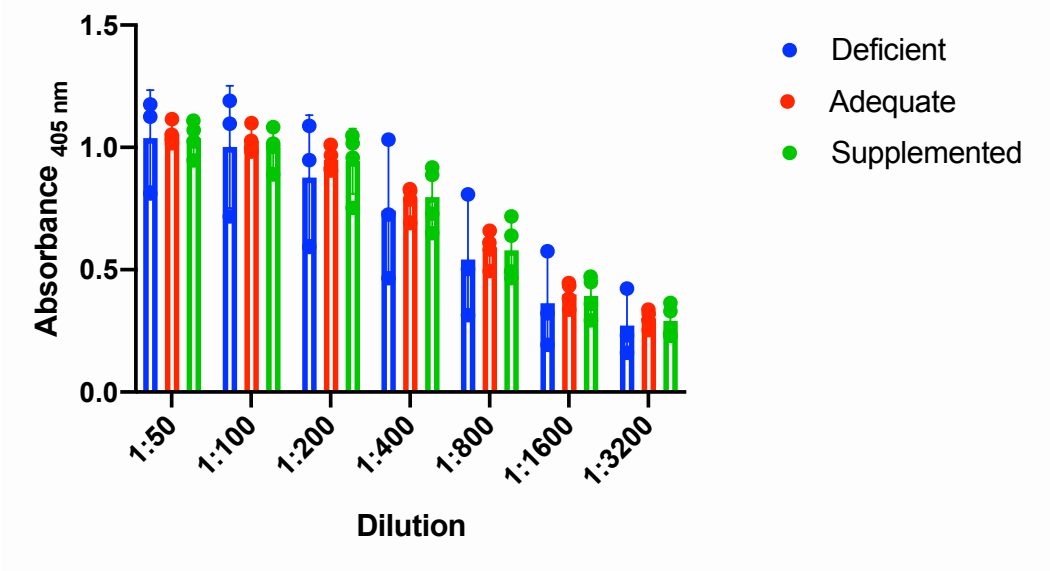

Figure 20. Day 7 Post-Infection IgG Titers

Discussion

Given that B cell-specific selenoproteins play a role in functions of the humoral immune response, we hypothesized that we would observe greater antibody titers in Se-supplemented mice compared to Se-deficient and -adequate mice. We also predicted that Se supplementation would enhance bacterial clearance from the lungs, as antibodies are essential components of the immune response against *B. pertussis*. Neither of these predictions were supported by our results, as there were no significant differences in antibody titers or bacterial burden between any diet type at any time point.

One potential explanation for why we did not see differences in antibody titers or bacterial burden between Se-supplemented and -adequate mice is that supplemented intake may not enhance selenoprotein expression or functions. This could be because immune expression of selenoproteins and selenoprotein functions are already optimized at Se-adequate levels. However, given all the studies demonstrating the negative impact of Se deficiency on immune functioning, we were surprised that there were not significant differences in bacterial burden and antibody titers between Se-deficient mice and the other two diets. Perhaps this could be due to a greater impact of Se deficiency on immune cells other than B cells. Another possibility is that there were compensatory effects with Se deficiency. For example, to compensate for reduced B cell selenoprotein expression, there could have been an increase in IL-4 secretion to help stimulate B cell activation and proliferation. Finally, the lack of differences in antibody titers between any of the three diet types could be explained by the immunogenicity of the vaccine that we used. High immunogenicity could lead to high antibody production regardless of the Se diet.

It is worth repeating this study to see if there are differences in bacterial burden, as there are some drawbacks to our study which prevented accurate bacterial enumeration. One possible

explanation for why we did not see differences in bacterial burden was because of contamination with commensal microbes. This contamination occurred even after using a streptomycin-resistant *B. pertussis* strain and using plates containing streptomycin. Another possibility is that *B. pertussis* is not the best model system for this experiment. Some mice appeared to have cleared the infection quickly, which made it difficult to see differences in bacterial burden between diet types. Additionally, mice that are infected with B. *pertussis* do not show apparent symptoms, which eliminates the possible of scoring disease severity using symptoms. A pathogen that is lethal to mice or takes longer to clear would make it easier to see differences in bacterial burden. Beyond measuring bacterial burden, a lethal pathogen would also allow us to quantify survival and disease severity as a measure of the effectiveness of the humoral immune response.

Despite not seeing differences in antibody titers or bacterial burden, it is still possible that the Se diet could impact bacterial clearance. These potential differences could be impacted by impaired functioning of cellular immunity, which is closely tied to the antigen presentation and signaling roles of B cells. In future studies it would be interesting to measure how antigen presentation, cytokine secretion, and T cell differentiation and functions are impacted by Se status. Furthermore, while there were not differences in antibody titers, this does not necessarily mean that antibody functionality was not impacted. Future studies should explore if the neutralization potential and opsonization functions of antibodies are impacted by Se status.

Another point to note about our Se diet study is that the diet model results in Se deficiency in all cell types whereas in $Trsp^{B}$ mice, all cell types are Se-supplemented except for B cells. Thus, with the diet model it is difficult to determine if any differences in bacterial clearance are due to the functions of B cells being impacted or an impact on other cell types. Despite not seeing differences in antibody titers or bacterial clearance in our experiment, it would be interesting to

see if the results would be different if another model pathogen or vaccine were used. Ultimately, our work establishes a role for selenoproteins in mediating antibody production and future work should continue to explore how Se status impacts vaccine efficacy.

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