EFFECTS OF MORINGA OLEIFERA ON MUCOSAL IMMUNITY DURING MALNUTRTION IN PLASMODIUM CHABAUDI INFECTED MICE

AThesis
by
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Submitted to the Graduate School at
Appalachian State University
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

December 2022
Department of Biology

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ABSTRACT

EFFECTS OF MORINGA OLEIFERA ON MUCOSAL IMMUNITY DURING MALNUTRTION IN PLASMODIUM CHABAUDI INFECTED MICE

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Plasmodium falciparum, the most lethal species of the *Plasmodium* parasites, is commonly found in the Sub-Saharan African region, where more cases of malnutrition occur due to food insecurity. While it's known that malaria affects gut microbiota, the contribution of malnutrition to this effect and mucosal immunity is not well studied. Due to drug resistance and sometimes limited resources, use of natural plant products for treatment of malaria is encouraged in these areas. One of the commonly used plants is *Moringa oleifera*, which has been used for hundreds of years as tea or in curries and other foods. Despite its widespread use, little is known about the beneficial effect of this plant to the immune system. We hypothesized that food containing Moringa would increase functionally activated immune cells during moderate malnutrition. Therefore, we investigated the effects of Moringa on gut immunity during malaria infection in a moderate malnourished murine model. We utilized a food-restriction diet to induce moderate malnutrition and investigated the adaptive immune response in the gut mucosal tissues. We observed that malnourished mice receiving moringa supplementation had increased expression of effector T cells, germinal center B cells, and slight increase in secretory IgA in the small intestine. Our data suggests that Moringa supplementation may have immune benefits during moderate malnutrition.

Acknowledgements

I would first like to thank my mentor Dr. Opata for not only pushing me to think critically about the work that we do and for keeping me on track to achieve my goals during my time here, but for also being a patient and understanding individual. Him pushing me toward the pursuit of knowledge is what will carry me through my future goals within the healthcare field and enable me to bring critical thinking skills, data analysis, and aspects of immunology to the table. I would also like to thank my committee members, Dr. Rachel Bleich, Dr. Mark Robinson, and Dr. Mark Venable for their support and great feedback that have guided me throughout this journey. My lab mates and fellow department members both past and present have also been an integral part of my time here as a master's student at Appalachian State University: James Erny, Robert Onjiko, Clay L, Brittany, and Paige. In different ways they have dedicated time and labor to aiding in my thesis project and I am very grateful for that. I would finally like to thank my lab mate/ peer mentor Tyler Olender. He has guided and taught me various skills throughout my time in the lab as a master's student. During our numerous, lengthy, and challenging experiments and time in the lab, we have both accomplished and learned so much. His constant pursuit for knowledge and dedication to hard work are inspiring and I am forever grateful for his support and help in my thesis project.

Dedication

I would like to dedicate this thesis to my parents, MaryAnn Catherine Warnick and Phillip Tyler Warnick. I believe that it is their constant support and encouragement that has been the driving force behind me achieving what I set forth to accomplish. They have been my pillars of strength, my confidants, and best friends. I give all of my gratitude to them. May I continue to push forward towards the goals I intend to obtain, and never forget who got me there.

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List of Abbreviations

APC: Antigen presenting cell

CD: Cluster of differentiation

FDC: Follicular dendritic cell

FITC: Fluorescein isothiocyanate

GALT: Gut associated lymphoid tissue

IFN-γ: Interferon gamma

Ig: Immunoglobulin

IL: Interleukin

iRBC: Infected red blood cells

MHCII: Major histocompatibility complex II

PI.: Post infection

POI: Peak of infection

RBC: Red blood cells

TNF-α: Tumor necrosis factor alpha

Chapter 1: Introduction

1.1 Malaria Disease

The causative agents of malaria disease are unicellular protozoan parasites belonging to the genus *Plasmodium.* Species of *Plasmodium* that are known to infect humans are *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi* (1). These *Plasmodium* parasites reside in erythrocytes and can reach host tissues via the circulatory system. The interactions between infected erythrocytes and host tissue are limited to locally specific tissue sites such as the brain, lung, liver, kidney, and intestines. Malaria disease can be classified as acute or chronic based on the specific tissues that are affected by the *Plasmodium* infection (2).

Acute or chronic malaria infection leads to a different array of symptoms. Acute illnesses generally develop suddenly and last for only a few days or weeks. On the other hand, chronic infection develops slowly and may worsen over an extended period of time lasting months to years (3). With an acute infection, symptoms can range from recurrent fever, headache, muscle and joint pain, vomiting, jaundice and anemia to severe complications such as acidosis, respiratory distress, severe anemia, kidney failure and cerebral malaria. With a chronic infection, also known as a long-term infection, symptoms can vary ranging from absence of fever to the typical acute symptoms, such as anemia, predisposition to other infections, as well as complications during pregnancy (4). Sequestration of infected red blood cells (iRBCs) within vasculature is also correlated to disease severity. Other areas that iRBCs are known to sequester are in the gastrointestinal tract, lungs, kidneys, skin, and even the brain blood vessels or retinas as seen for cerebral malaria cases (5).

Plasmodium parasites in the form of merozoites, are released into the bloodstream from liver hepatocytes within specialized vesicles called merosomes. This cycle, always referred to as

malaria blood stage cycle, continues with repetitive erythrocyte-invasion in the blood tissue and results in erythrocyte remodeling, dysfunction, and cell lysis. In order to infect hepatocytes for further development, sporozoites need to traverse into the dermis. Many of these will either get trapped in the skin or transported to draining lymph nodes where an adaptive immune response can take place. If a sporozoite does enter the bloodstream and is carried to the liver, sinusoids can establish the infection within a few hours. *Plasmodium* sporozoites that infect hepatocytes undergo multiple rounds of proliferation to ultimately generate the asexual erythrocytic stage of the parasite, which is known as a merozoite (6). These merozoites are released into the bloodstream, which is followed by continuous infection cycles of erythrocytes that cause disease symptoms. Then some of the merozoites commit to develop into the sexual parasite stages. Infected erythrocytes become sequestered in the bone marrow and even re-enter the circulation. The cycle can then start over for transmission once more if a mosquito comes along to take another blood-meal (6).

The blood stage of malaria causes anemia, toxic haem release, immune activation, and cytokine/chemokine storms. Within blood tissue rosetting, where *Plasmodium*-infected red blood cells bind to uninfected red blood cells, interaction with immune cell populations or endothelial cells of blood vessels is diminished (4). This can lead to RBC sequestration, microhemorrhage, microcirculatory dysfunction, ischemia, and inflammation. Cytoadhesion, or sequestration, of the parasite has been shown to occur mainly in the liver and lung and less in the brain, kidneys, and gut during *Plasmodium chabaudi* infections. Histopathological changes occur during infection, but they are not limited to areas where sequestering occurs (4, 7).

Activated leukocytes can adhere to endothelial cells of blood vessels which have the ability to act as antigen-presenting cells (APCs). The endothelial cells can then present malarial

antigens to CD8+ T cells, which will destroy both the antigen and the endothelial cells. During this process, there is secretion of IFNγ and perforin and disruption of the blood-brain barrier. Looking at the vascular system, blood flow is slowest in capillaries and postcapillary venules. Capillary beds within this system are found near lymphatic vessels, where materials coming from the blood vessels drain into the lymph nodes and return to veins. This indicates that infected red blood cells (iRBCs) could have a great impact on smaller vascular beds in each organ (8).

1.2 Immunity to Malaria

The adaptive immune cells comprising both B and T cells are essentially important for protection against chronic infections such as malaria (9). Several investigators have demonstrated that both CD4+ T cells (helper T cells) and CD8+ T cells (killer T cells) can inhibit the development of parasites and halt the progression of malaria pathogenesis. CD4+ T cells are known to prime phagocytic cells that capture and kill these *Plasmodium* parasites, while also helping B cells to produce anti-parasitic antibodies (9,10). Helper T cells have a whole range of other functions including activation of the cells of the innate immune system, cytotoxic T cells, as well as non-immune cells. As the inflammation due to disease increases, a subset of CD4 helper cells called Treg cells can regulate the response, thus suppressing the immune reactions (11).

B cells on the other hand protect from disease by producing specific antibodies. It has been shown that immunization with attenuated *P. berghei* sporozoites led to production of infectivity neutralized antibodies (9). Antibodies interfere with the development of the sporozoite from the moment the sporozoites are injected into the skin until the infection of hepatocytes occurs. However, the extent to which naturally occurring humoral immunity can

mediate protection against the pre-erythrocytic parasite stage is unknown. In fact, different antibodies are produced at different stages of malaria disease. At the pre-erythrocytic stage antibodies are released to aid in preventing infection, while at the asexual erythrocytic stage antibodies prevent the disease from developing further. Then at the sexual erythrocytic stage antibodies can be released to prevent the transmission of the disease to others.

In the gut, specific antibodies of the Immunoglobulin A (IgA) subtype are secreted as they are important for mucosal immunity (12). Secretory IgA has been shown to provide protection against antigens, including malaria, and toxins while also binding to and coating commensal bacteria within the gut (13). B cells are also found within germinal centers. Germinal centers are organelles that develop within the B cell follicles in secondary lymphoid tissue (such as the mesenteric lymph nodes). They can also be found in tertiary lymphoid tissues, such as the gut-associated lymphoid tissue (GALT) that includes Peyer's patches and the lamina propria (14). Germinal centers form as B cells mature and become specific for particular antigens. Clonal expansion occurs within these germinal centers where long-lived antibody secreting plasma cells and memory B cells are produced. Within the mature germinal centers there are dark and light zones. Proliferating areas occupy the dark zone while the light zone contains a network of follicular dendritic cells (FDCs) which pick up antigens for potential presentation to the adaptive immune cells (15). Within the light zone, B cells carrying high-affinity antibodies receive preferential signals from helper T cells. This promotes their proliferation and differentiation (16). During the process of specific antibody production by B cells, there is increased somatic hypermutation and class switch recombination that occur in the germinal centers (15).

Peyer's patches found beneath the M cells, a type of enterocyte that have MHC class II expressed on their surface, compose the immune system within the gut and identify antigens,

which are presented to B lymphocytes that mature to produce antibodies (17). The B lymphocytes found in germinal centers can be identified by their expression of the GL7 molecule, which indicates activated germinal center B cells. Due to their high specificity, the germinal center B cells are essential for both antibody production and antigen presentation (18).

1.3 Malnutrition

A major issue globally, typically seen in malaria endemic areas is malnutrition. This has been defined as a deficiency, excess or imbalance of a wide range of nutrients, resulting in a measurable adverse effect on body composition, function and clinical outcome (19). Malnourished individuals can either be undernourished or over nourished. There are different types of malnutrition, which include wasting, stunting, undernutrition, micronutrient deficiency, protein malnutrition, and over-nutrition (obesity) (19). Common deficiencies of micronutrients commonly seen in areas also impacted by malaria are a lack of vitamin A, vitamin B, folate, iodide, iron, and zinc leading to micronutrient malnutrition (7). Malnutrition can occur as a result of food insecurity, high absorption, reduced absorption, metabolism, increased exertion, or the result of a certain disease (19).

1.4 Effect of Malnutrition on Gut Immunity During *Plasmodium* Infection

Malnutrition of any form has a significant impact on the gut microbiota. This can result in dysbiosis, a disruption of the homeostasis of the microbiota that is caused by an imbalance of microflora, changes in functional composition and metabolic activities, or even a shift in the distribution of the microbiota. These changes alter the functionality of both innate and adaptive immune responses within the gut (20). The defects on mucosal immunity in the gut caused by

malnutrition can be reversed by the provision of supplements including prescribed medication or natural plant products.

In one study, it was shown that infection with *Plasmodium yoelii* in Swiss Webster and C57BL/6 (B6) mice was associated with a transient dysbiosis not observed in control mice inoculated with uninfected blood (21). These groups showed a reduction in bacteria normally seen within the gut 10 days post-infection, which returned to normal after 30 days. These same mice were found to be more susceptible to harmful bacteria, such as Proteobacteria *Escherichia coli* and nontyphoidal *Salmonella* spp. This indicates that patients suffering from *Plasmodium* infection, could be more susceptible to secondary infections of harmful bacteria within the gut due to dysbiosis. Indeed human autopsies from patients who died from severe malaria, showed that iRBCs sequestered in the gastrointestinal tract. This could suggest that gastrointestinal bleeding occurs during a *Plasmodium* infection. Pathological changes that could occur from this mucosal damage are detachment of epithelia, increased intestinal permeability, shortening of villi and the colon, and dysbiosis within the gut microbiome (6,22).

It has also been suggested that gut microbial communities and their composition can aid in protection against *Plasmodium* infection (23, 24, 25). One study performed with isogenic mice from two different vendors showed less severe disease when infected with *Plasmodium*, when compared with other vendors. The authors concluded that this difference in malaria disease severity between vendors was due to differences in bacterial enterotypes. This effect was seen across various species of *Plasmodium*, such as *P. berghe*i, *P. chabaudi*, and *P. yoelii*. Fecal transplants were then performed from the seemingly protected mice into those experiencing more severe disease, and the protective effects against the disease were transferred, indicating *Plasmodium* protective potential by gut microbiota (24).

The presence of certain bacteria within the gut has been shown to be associated with lower parasitemia levels during infection. One of these was identified as *Lactobacillus,* a bacteria genus typically known for its beneficial effects. On the other hand, unspecified *Enterobacteriaceae* have been associated with higher parasitemia levels and more severe disease (25). One characteristic of *Lactobacillus* that makes it so favorable is its ability to immunomodulate, meaning it can either stimulate or suppress the immune responses. This bacterial group is able to initiate pro-inflammatory and anti-inflammatory interleukin responses, increase phagocytosis, secrete lysosomal enzymes, induce T cell activation, and reduce intestinal permeability (26). *Lactobacillus* exhibits activity that also fights against luminally localized bacterial pathogens attached to the brush border or internalized intestinal epithelial cells. The brush border is a complex part of the intestinal tract that is made up of microvilli that is responsible for nutrient absorption and homeostasis (27). This is something that is ideal for maintaining the balance within the gut microbiome (28).

It has also been shown that with certain bacteria morbidity and mortality is increased. One study showed that individuals infected with malaria and some gram-negative bacteria infection had disrupted iron metabolism. Accompanying this were elevated plasma heme levels and increased mitochondrial reactive oxygen species (ROS) production by phagocytes. Compared to individuals with purely single infections who were able to effectively control the disease, co-infections resulted in higher mortality rates. The cellular/molecular mechanism by which gram-negative bacteria drive higher mortality rates remains unclear (29).

1.5 Benefits of Moringa

Many effective malaria medications including quinine and its derivative and the current combination therapy artemisinin-artesunate are plant derived. Despite many years of research on malaria the parasite consistently becomes resistant leading more people in malaria endemic regions to resort to alternative herbs (30, 31). In recent years, many scientists have explored bioactive components of these plants to help communities fight malaria disease. With a lack of effective vaccines and insufficient supply of anti-malarial drugs to socio-economic demands, investigations into the bioactive methods may be essential to combat malaria. Among these methods is an herbal supplement known as Moringa, specifically, *Moringa oleifera* (MO). There are 14 known species of the Moringa genus: *M. arborea*; *M. longituba*; *M. borziana*, *M. pygmaea*; *M. hildebrandtii*; *M. drouhardii*; *M. longituba*; *M. peregrina*; *M. stenopetala*; *M. rivae*; *M. ruspoliana*; *M. Ovalifolia*; *M. Concanensis* and *M. oleífera* (32).

The Moringa tree is commonly found in malaria endemic regions due to desirable temperatures for the plant to grow. Indeed, Moringa has been found to be a rich source of digestible proteins, fiber, vitamin A and C, calcium, carotenoids, and iron. Moringa can be used as a nutritional supplement or to treat a variety of ailments including scurvy, purgation, headaches, fevers, otitis, sore throat, bronchitis, and eye infections (33). Studies have shown that Moringa provides more than 10X the amount of Vitamin A contained in milk, 7x the amount of Vitamin C in oranges, 9x the amount of protein in yogurt, 15x the amount of potassium found in bananas, and 25x the amount of iron in spinach (34). Human studies have shown that 8 grams of Moringa leaf powder can provide a toddler with 14% of protein, 23% of iron, and 40% of calcium recommended daily (35).

In addition to treating malaria, Moringa has been used to treat various ailments including

inflammation, various infectious diseases, cardiovascular, gastro-intestinal, hematological and hepatorenal disorders in areas such as South Asia (36). The leaves can be dried, cooked, or even consumed raw while the moringa seeds can be roasted, ground up, or eaten while green. Since growing in popularity, different parts of the Moringa tree have been shown to be nutritious and medically valuable, such as leaves, roots, seeds, flowers, and pods. A common way Moringa is prepared for usage is either ground up in powder form to add to meals or steeped in hot water to make tea (36).

Because malnutrition of any form has a significant impact on the gut microbiota, which leads to dysbiosis, a disruption of the homeostasis of the microbiota that is caused by an imbalance of microflora, changes in functional composition and metabolic activities, or even a shift in the distribution of the microbiota. These changes alter the capability of both the innate and adaptive immunity within the gut (35). By providing these varying nutrients, Moringa could prevent dysbiosis from occurring as well as aid in gut mucosal immunity as has been shown by several researchers using different models (33, 37).

Specific Aim 1: Determine the influence of moderate malnutrition on mucosal adaptive immunity at the peak of malaria infection.

Specific Aim 2: Investigate the effect of moringa supplementation on mucosal immunity during malaria infection in moderately malnourished animals.

Hypothesis: *Moringa oleifera* will increase the adaptive immune response within the gut mucosal surface in mice infected with *Plasmodium chabaudi* that are moderately malnourished.

Chapter 2: Materials and Methods

2.1. Mice and parasite

Adult C57BL/6 mice were obtained from Harlan labs and a breeding colony maintained in our animal facility. The rodent strain of malaria, *P. chabaudi* was received as a gift from Dr. Robin Stephens at the University of Texas Medical Branch at Galveston. Authorization to use the parasite was given by Dr. Jean Langhorne from the Francis and Crick Institute, UK. All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at Appalachian State University (Protocol 20-10). Female mice aged 8-14 weeks were used for experiments with consistency in both the moderate malnourished and control groups.

2.2 Preparation of Supplementation Pellets

Control pellets for nutritional supplementation

Control supplementation pellets were made every 3 days using 20.10 g of cassava flour and sterile distilled water to form pellets and 4 g of peanut butter mixed in for flavor enhancement. Twelve pellets were formed and allowed to air dry. Mice were given four pellets daily along with standard mouse chow for 3 days.

Moringa pellets for nutritional supplementation

Moringa pellets were made every 3 days using 20.10 g of cassava flour and 500 mg of Moringa leaf powder per mouse. These were mixed with sterile distilled water to form pellets and ~ 4 g of peanut butter mixed in for flavor enhancement. Twelve pellets were formed and allowed to air dry. Mice were given four pellets daily along with standard mouse chow for 3 days.

2.2. Malnutrition and infection

Mice were either moderately malnourished via food restriction for 4-hours by weight (3g of chow per mouse) or a well-nourished diet (receiving ad libitum access to a regular chow diet). One group of the moderate malnourished mice were supplemented with pellets containing Moringa, while the second group received control pellets without moringa supplementation. The diets were administered to each group for approximately 4-5 weeks to induce moderate malnutrition. After the induction of moderate malnutrition, a set of both the control and malnourished mice were infected with $1x10^5$ *P. chabaudi* intraperitoneally. At 9 days post infection, all mice were culled via cervical dislocation with adherence to our approved IACUC protocol 20-10. The gut tissues including the small and large intestines were harvested and placed in ice cold PBS supplemented with 2% FBS and 0.02% EDTA (Atlanta Biologicals S11150H, Flowery Branch, GA) in preparation for cleaning.

2.3. Preparation of gut tissues for flow cytometry

The gut tissues were cleaned by manually removing all residual adipose residues with scissors and forceps. The tissues were then cut longitudinally and flushed with ice cold PBS supplemented with 0.02% EDTA to remove fecal matter. Any residual fecal matter was removed by scraping with forceps and rinsing with more ice-cold PBS + 0.02% EDTA buffer. The tissues were then placed into 6-well plates with ISCOVES culture media (Corning #10-016-CV) supplemented with 2mM L-glutamine (Atlanta Biologicals B21210), 5mM sodium pyruvate (Gibco 11360-070), non-essential amino acids (MEM NEAA) (Gibco 11140-050), 10mM HEPES (Gibco 15630-080), 100 U/ml penicillin, 100 U/ml streptomycin and 2e-5 M of 2-mercaptoethanol (Gibco 21985-023) then minced with scissors into small pieces. Type I

collagenase (ThermoFisher #17018029) was added at a concentration of 100 U/mL, followed by a one-hour incubation at 37ºC and 5% CO_2 for extraction of lamina propria cells. Cells were agitated every 15 minutes to ensure homogeneity.

2.4. Flow cytometry (surface staining)

After one-hour incubation, the 6-well plates were removed from the incubator and the solid tissues were mashed through 70-μm nylon filters and lysed with 1X RBC lysis buffer for 1 minute at room temperature. The RBC lysis was stopped by adding 5 mL ice-cold PBS and spun at 1200 rpm at 4ºC for 5 minutes. The suspensions were then placed on ice in 5-mL round bottom tubes and cells were counted at a 1:10 dilution with trypan exclusion on a hemocytometer. After determining cell numbers, an aliquot of cells was taken and resuspended in cold FACS buffer (PBS, 2% FBS, and 0.1% NaN3 sodium azide) in new 5-mL round-bottom polystyrene tubes. The cells were incubated with Fc block in the dark at 4ºC for 20 minutes to ensure specific binding of the antibodies. Fluorescent antibodies were used to label cells of interest: PerCP-Cy5.5-conjugated anti-CD4 (BioLegend GK1.5), FITC- conjugated anti-CD27 (BioLegend O323), PerCP-Cy5.5-conjugated CD62L (BioLegend MEL-14), APC Cy7-conjugated anti-CD8, PE-Cy7-conjugated anti-CD19 (BioLegend 6D5), and APC-conjugated anti-GL7 (BioLegend GL7) at 4ºC in the dark for 40 minutes. Fluorescently stained cells were washed in FACS buffer, resuspended in 300 μL of FACS buffer, filtered and collected on Attune NXT flow cytometer.

2.5. Flow cytometry (intracellular cytokine staining)

Aliquots of cells were transferred into a sterile 24-well plate with 1 mL of ISCOVES culture media supplemented with 2mM L-glutamine, 5mM sodium pyruvate, non-essential amino acids (MEM NEAA), 10mM HEPES, 100 U/ml penicillin, 100 U/ml streptomycin and 2e-5 M of 2-mercaptoethanol. The cells were stimulated *in vitro* with 1 µL of cell stimulation cocktail. The 24-well plate was then placed into a HERA cell 150i incubator set at 37ºC and 5% CO_2 for 4-5 hours. After incubation, the cells were resuspended in 5-mL round-bottom polystyrene tubes and spun at 1200 rpm at 4ºC for 5 minutes. The cells were incubated with Fc block in the dark at 4ºC for 20 minutes to ensure specific binding of the antibodies. Fluorescent antibodies were used against surface markers to label cells of interest: PerCP-Cy5.5-conjugated anti-CD4 (BioLegend GK1.5), FITC- conjugated anti-CD27 (BioLegend O323), PerCP-Cy5.5-conjugated CD62L (BioLegend MEL-14), APC Cy7-conjugated anti-CD8, PE-Cy7-conjugated anti-CD19 (BioLegend 6D5), and APC-conjugated anti-GL7 (BioLegend GL7) at 4ºC in the dark for 40 minutes. After incubation cells were fixed with 300 µL of 2% paraformaldehyde. The cells were then permeabilized using a permeabilization buffer (Tonbo Biosciences, San Diego, CA) and stained with BV421-conjugated IFNγ (BioLegend XMG1.2), PE-Cy7-conjugated TNFα (BioLegend MAb11), FITC-conjugated IL-10 (BioLegend JES5-16E3). The cells were then resuspended with 200 µL of FACS buffer and filtered for flow cytometry analysis.

2.6. ELISA Assay

Serum was collected from the blood at the time of sacrifice by spinning clotted blood at 13,000 rpm for 30 minutes and frozen at -80ºC for analysis using ELISA. A 24 well plate coated with capture antibody (IgA) was used according to the manufacturer's recommendation (RayBio® Mouse IgA ELISA Kit). 100 µL of each sample was added to each well, followed by a 2.5 hour incubation at room temperature. 100 µl of prepared biotin antibody was then added to each well and incubated for 1 hour at room temperature. 100 µl of prepared Streptavidin solution was added to each well and incubated at room temperature for 45 minutes. For detection, 100 µl of TMB One-Step Reagent was added to each well and incubated for 30 minutes at room temperature, followed by addition of 50 µl Stop Solution. The plates were read at 450 nm immediately using spectrophotometry.

2.7. Data analysis

Raw data collected from the Attune NXT flow cytometer were analyzed using FlowJo software (Ashland, OR). Calculations of all raw data were performed in Microsoft Excel and graphs and statistics were performed in Prism GraphPad version 9.1 (San Diego, CA). Data were considered significantly different with a P value less than 0.05.

Chapter 3: Results

3.1 *All moderately malnourished mice have a lower average body weight.*

With malnutrition due to undernutrition being a common occurrence in areas endemic to *Plasmodium* infection, we wanted to induce moderate malnutrition in mice and explore whether Moringa supplementation would counteract the disparities caused by undernutrition. To determine this, we fed the mice either a well-nourished, solely moderately malnourished, moderately malnourished with control supplementation, or moderately malnourished with Moringa supplementation diet for 4-5 weeks. Weights were taken each day before the mice were fed. As shown in **Figure 1** the mice on the control well-nourished diet, ad libitum, had a higher average body weight consistently for the entirety of the diet. All other malnourished groups had a consistently lower average body weight each week. Supplementation, whether a control pellet or a moringa pellet, did not increase average body weight by the end of the diet.

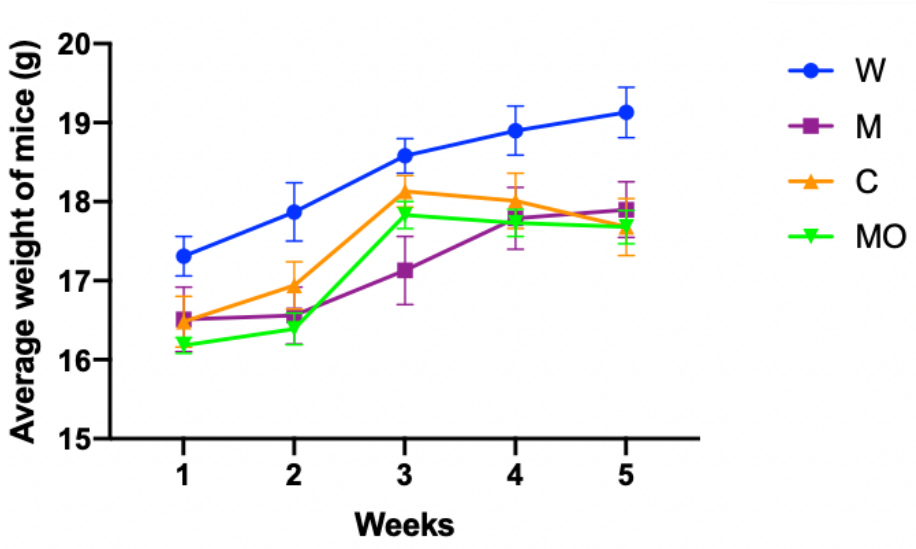

Figure 1. Moringa supplementation has no effect on average body weight.
C57BL/6 mice were fed either a control diet or a moderately malnourished diet. Weights were recorded to give average change in weight. All data are shown as means, n = 4 female mice per group, representative of 14 independent experiments. Statistical analysis was performed using a one-way ANOVA with Tukey's posthoc test. +,* $p < 0.05$; ++,** $p < 0.01$; +++,*** $p < 0.001$, indicates a statistically significant difference between treatments. W= well-nourished, M=malnourished, C= malnourished with control supplementation, MO= malnourished with Moringa supplementation.

3.2 ***Moringa supplementation has no significant effect on the CD4+ T cell response.***

CD4+ T cells prime phagocytic cells to capture and kill the malaria parasites, and also help B cells to mature and produce anti-parasitic antibodies but their functionality may be altered due to malnutrition. Therefore, we wanted to explore how Moringa supplementation would impact this response to a *P. chabaudi* infection. To determine this, we fed mice either a well-nourished diet which was normal lab chow ad libitum (X), a moderate malnourished diet which was food limitation for a 4 hour period (M), a moderate malnourished diet limited to a 4 hour period with a control pellet for 20 hours (C), or a moderate malnourished diet limited to a 4 hour period with a Moringa supplementation pellet for 20 hours (MO). These diets were maintained for 4-5 weeks and mice were then infected with *P. chabaudi* intraperitoneally. We harvested the gut tissue day 9 post-infection (p.i.) and performed flow cytometric analysis on the small intestine for all the four groups. Cells were stained with different fluorescent labeled antibodies against surface markers of interest (CD4, CD27, and CD62L). As shown in **Figure 2A**, there was no difference in the frequency of CD4+ T cells in the small intestine of all the groups, whether fed on constant diet or malnutrition supplemented with Moringa pellets. Effector cells, seen in **Figure 2B**, had a similar trend where there was not a significant difference between the groups, but the mice that were supplemented with Moringa had a slight increase in the frequency of activated effector T cells at a similar level as the mice on constant diet.

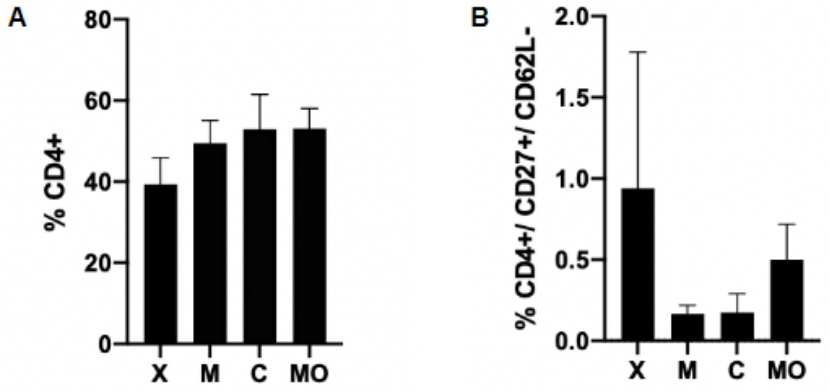

Figure 2. Effect of Moringa supplementation on CD4+ T cell response.
C57BL/6 mice were fed either a control diet or a moderately malnourished diet. All infected mice were intraperitoneally infected with $1x10^5$ iRBCs of *P. chabaudi* and sacrificed at day 9p.i.. (A) Percent CD4 T cells and (B) Activated CD4+ T effector cells. All data are shown as means ± SD, n = 4 female mice per group, representative of 14 independent experiments. Statistical analysis was performed using a one-way ANOVA with Tukey's test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, indicates a statistically significant difference between treatments. W= well-nourished, M=malnourished, C= malnourished with control supplementation, MO= malnourished with Moringa supplementation.

3.3 *Moringa supplementation increases secretion of IFNγ, but not TNFα by CD4+ T cells in moderate malnourished mice*

Since CD4 T cells secrete cytokines to facilitate immune protection, we next investigated IFNγ production by the CD4 T cells in the gut mucosa. This can play a critical role in the blood stage of murine *Plasmodium* infections and can distribute systemically during malaria disease to appropriately balance pro- and anti-inflammatory immune responses. Since Moringa is used for treatment and has been shown to induce inflammation, we explored its effect on the ability of CD4 T cells to secrete IFN-γ. Using a cell stimulation cocktail to block cytokine secretion and intracellular staining technique followed by flow cytometry, we observed increased IFN-γ secretion in the moderate malnourished mice receiving moringa supplementation for both frequency and actual cell numbers **(Figure 3 A & B)**.

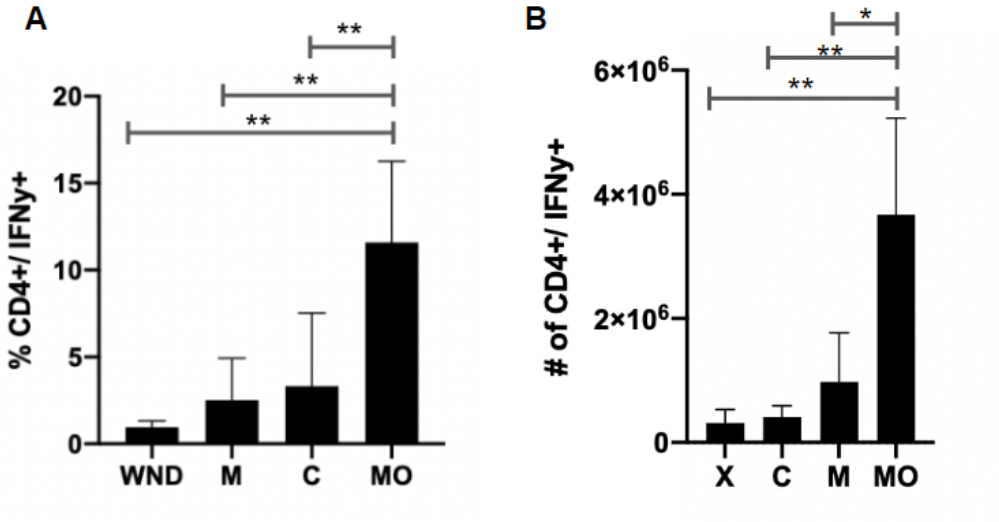

Figure 3. Moringa supplementation increases CD4+ T cell secretion of IFNγ in moderately malnourished mice

C57BL/6 mice were fed either a control diet or a moderately malnourished diet. All infected mice were intraperitoneally injected with $1x10^5$ iRBCs of *P. chabaudi* and sacrificed at day 9.p.i.. (A) Percent of IFNγ producing CD4+ T cells and (B) Numbers of IFNγ producing CD4+ T cells. All data are shown as means ± SD, n = 4 female mice per group, representative of 14 independent experiments. Statistical analysis was performed using a one-way ANOVA with Tukey's test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, indicates a statistically significant difference between treatments. W= well-nourished, M=malnourished, C= malnourished with control supplementation, MO= malnourished with Moringa supplementation.

TNFa, is a pleiotropic cytokine known to have both beneficial and detrimental effects during malaria infection. High levels TNFα are associated with increased fever during malaria, but TNFα also has anti-parasitic activity. We investigated how Moringa supplementation would influence TNFα secretion by the CD4+ T cells during *P. chabaudi* infection as well as moderate malnutrition. As shown in **Figure 4** there was no significant differences between all the groups in the ability of CD4 T cells to produce TNFα, however, the moderate malnourished mice receiving Moringa supplementation appear to have a slightly lower secretion of TNFα by the CD4+ T cells both in frequency and absolute cell numbers.

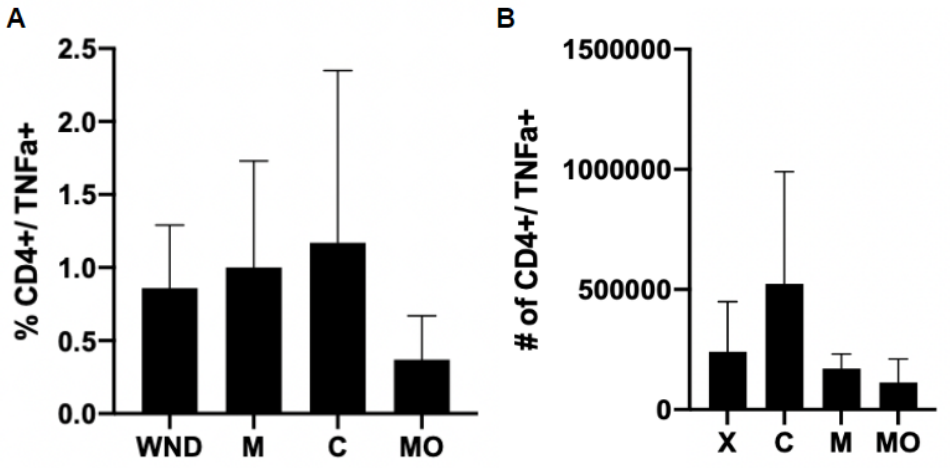

Figure 4. Moringa supplementation has no effect on CD4+ T cell secretion of TNFa in moderately malnourished mice
C57BL/6 mice were fed either a control diet or a moderately malnourished diet. All infected mice were intraperitoneally injected with $1x10^5$ iRBCs of *P. chabaudi* and sacrificed at day 9.p.i.. (A) Percent of TNFα producing CD4+ T cells and (B) Numbers of TNFα producing CD4+ T cells. All data are shown as means ± SD, n = 4 female mice per group, representative of 14 independent experiments. All data are shown as means ± SD, n = 4 female mice per group, representative of 14 independent experiments. Statistical analysis was performed using a one-way ANOVA with Tukey's test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, indicates a statistically significant difference between treatments. W= well-nourished, M=malnourished, C= malnourished with control supplementation, MO= malnourished with Moringa supplementation.

3.4 *Moringa supplementation increases CD4+ T cell secretion of IL-10 in the moderate malnourished mice*

The regulatory cytokine, IL-10, is known for its role in the blood-stage of murine malaria infections. Blocking IL-10 activity can promote anti-parasitic immunity by enhancing APC functions and associated T cell activity. This could also result in concomitant tissue pathology and related disease (38). It has been reported that a deficiency in IL-10 leads to a lower parasite burden and increased inflammatory processes. Specifically, the ratio of IL-10 inhibiting proinflammatory cytokines and tumor necrosis factor (TNF) can determine the effectiveness of parasite clearance. We next investigated if Moringa supplementation would have an effect on IL-10 secretion by the CD4+ T cells in the presence of malnutrition. As shown in **Figure 5**, there was no significant difference between the groups in the frequency IL-10 secreting CD4+ T cells.

When looking at the absolute cell numbers, Moringa supplementation significantly increased IL-10 secretion by CD4+ T cells. Taken together with previous data, these data suggest that Moringa may enhance production of malaria protective cytokines, specifically IFNγ and IL-10 by the CD4+ T cells in the gut.

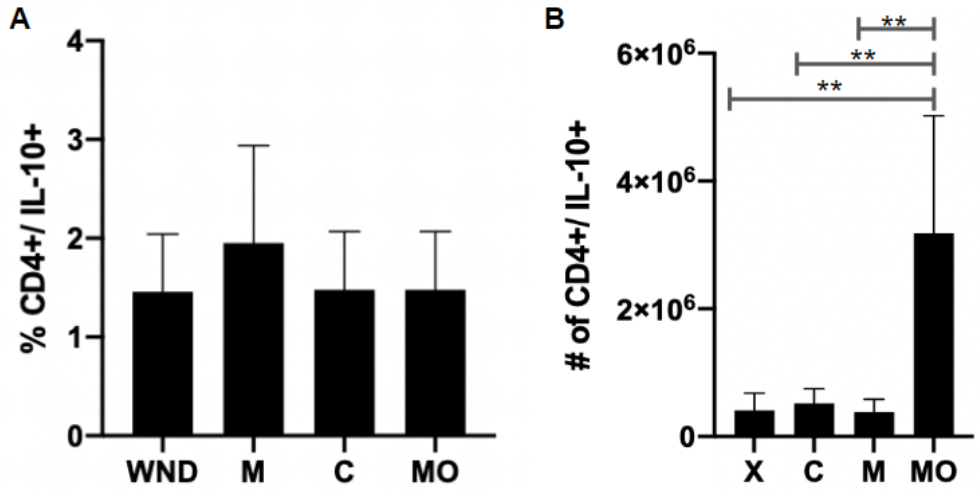

Figure 5. Effect of Moringa supplementation on CD4+ T cell secretion of IL-10 in moderately malnourished mice.
C57BL/6 mice were fed either a control diet or a moderately malnourished diet. All infected mice were intraperitoneally injected with $1x10^5$ iRBCs of *P. chabaudi* and sacrificed at day 9.p.i.. (A) Percent of IL-10 producing CD4+ T cells and (B) Numbers of IL-10 producing CD4+ T cells. All data are shown as means ± SD, n = 4 female mice per group, representative of 14 independent experiments. All data are shown as means ± SD, n = 4 female mice per group, representative of 14 independent experiments. Statistical analysis was performed using a one-way ANOVA with Tukey's test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, indicates a statistically significant difference between treatments. W= well-nourished, M=malnourished, C= malnourished with control supplementation, MO= malnourished with Moringa supplementation.

3.5 *Moringa supplementation increased CD8+ T cell secretion of IFNγ in the moderate malnourished mice*

CD8+ T cells are known for slowing the progression of parasite development by directly interacting with MHCI to eliminate cells. While doing so they release a cytokine known as IFNy. This cytokine can go on to activate cells such as macrophages to eliminate iRBCs. We wanted to explore whether malnutrition and Moringa supplementation will affect the secretion of IFNy by

CD8+ T cells. Similar to the results observed for the CD4+ T cells, Moringa supplementation significantly increased the proportions of IFNy secretion by CD8+ T cells while there is no significant difference between the other groups as shown in **Figure 6A** & **B**.

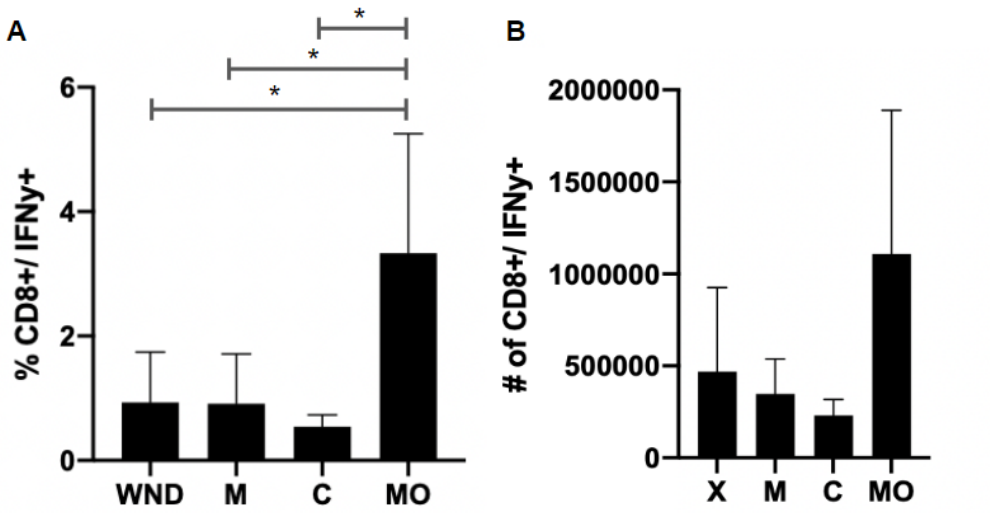

Figure 6. Moringa supplementation increased CD8+ T cell secretion of IFNy in moderately malnourished mice

C57BL/6 mice were fed either a control diet or a moderately malnourished diet. All infected mice were intraperitoneally injected with $1x10^5$ iRBCs of *P. chabaudi* and sacrificed at day 9. p.i.. (A) Percent of IFNγ producing CD8+ T cells and (B) Numbers of IFNγ producing CD8+ T cells. All data are shown as means ± SD, n = 4 female mice per group, representative of 14 independent experiments. All data are shown as means ± SD, n = 4 female mice per group, representative of 14 independent experiments. Statistical analysis was performed using a one-way ANOVA with Tukey's test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, indicates a statistically significant difference between treatments. W= well-nourished, M=malnourished, C= malnourished with control supplementation, MO= malnourished with Moringa supplementation.

3.6 *Moringa supplementation slightly increases germinal center B cell populations in the moderate malnourished mice*

Activated B cells are known for presenting antigens to CD4 helper T cells as well as producing antibodies to help fight off infections. These B cells can be found within germinal centers in Peyer's patches within the gut. Because of the high specificity of germinal center B cells, we investigated the impact of moderate malnutrition and/or Moringa supplementation on these germinal center B cells during *Plasmodium* infection. As shown in **Figure 7,** there was no significant difference between the groups. However, groups receiving Moringa supplementation

had slightly higher frequencies and absolute cell numbers of germinal center B cells compared to the other groups.

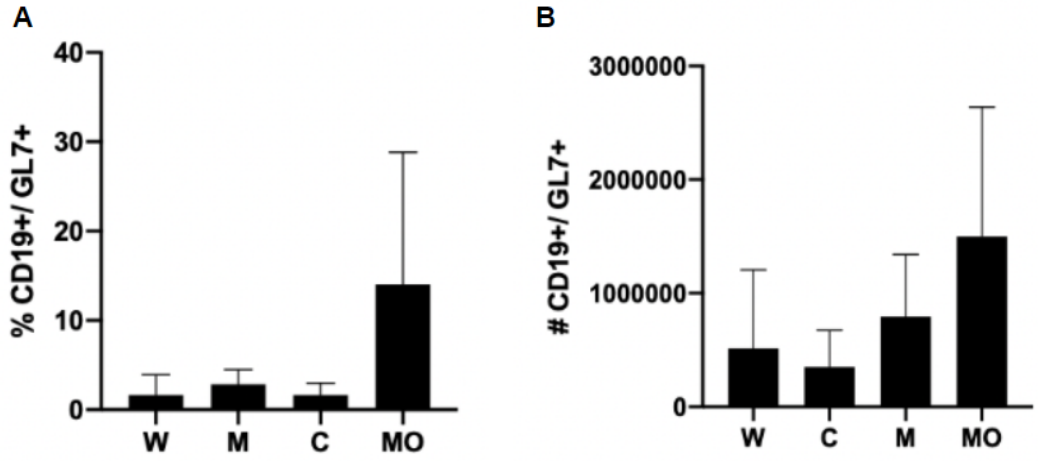

Figure 7. Moringa supplementation slightly increases germinal center B cells populations in moderately malnourished mice
C57BL/6 mice were fed either a control diet or a moderately malnourished diet. All infected mice were intraperitoneally injected with $1x10^5$ iRBCs of *P. chabaudi* and sacrificed at 9 days p.i.. (A) Percent of CD19+ germinal center B cells and (B) Numbers of CD19+ germinal center B cells. All data are shown as means ± SD, n = 4 female mice per group, representative of 14 independent experiments. All data are shown as means ± SD, n = 4 female mice per group, representative of 14 independent experiments. Statistical analysis was performed using a one-way ANOVA with Tukey's test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, indicates a statistically significant difference between treatments. W= well-nourished, M=malnourished, C= malnourished with control supplementation, MO= malnourished with Moringa supplementation.

3.7 *Moringa supplementation does not affect the level of serum IgA*

Because B cells produce IgA, an important antibody for mucosal protection, we suspected mucosal antibodies would be affected due to malaria infection and or malnutrition. With damage that could occur during moderate malnutrition it was suspected that microbial leakage could occur within the gut and leak into the blood. Therefore, we explored the influence of these two parameters on the gut and if Moringa would have a significant effect on any deformities. Using serum collected from blood samples of the control and moderate malnourished mice including those supplemented with Moringa, we performed an IgA ELISA.

This was then converted to concentration (ng/ml) and the resulting values are seen below in **Figure 8**. It was seen that there were no significant differences between the groups for secretion of IgA.

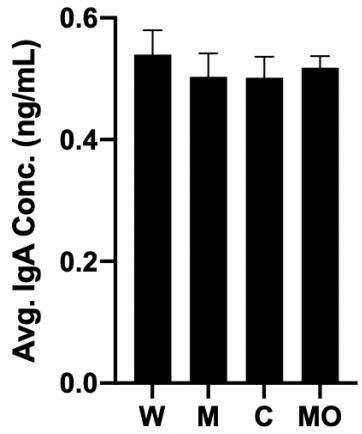

Figure 8. Effect of Moringa supplementation on secretion of IgA into the blood
C57BL/6 mice were fed either a control diet or a moderately malnourished diet. All infected mice were intraperitoneally injected with $1x10^5$ iRBCs of *P. chabaudi* and sacrificed at day 9 p.i.. Average IgA concentration (ng/mL) in each blood sample from each treatment group shown. All data are shown as means ± SD, n = 4 female mice per group, representative of 2 independent experiments with 4 replicates per group. Statistical analysis was performed using a one-way ANOVA with Tukey's test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, indicates a statistically significant difference between treatments. W= well-nourished, M=malnourished, C= malnourished with control supplementation, MO= malnourished with Moringa supplementation.

Chapter 4: Discussion

Our goal for setting up the various groups of mice within each experiment was to make four groups: well-nourished, malnourished, malnourished and receiving a control pellet, and malnourished receiving a moringa pellet. These weights were observed over the course of 4-5 weeks and as shown in **Figure 1**, we demonstrate how the malnourished groups had reduced weight that stayed consistent between those receiving supplementation and those not. This shows that neither Moringa supplementation nor the control pellets caused the mice to regain weight to the degree of being well nourished once again. These data demonstrate we were in fact able to induce malnutrition in these particular groups in order to continue our experiments.

Within adaptive immunity, we have B and T cells that play a significant role and are much more specific to the antigen that initiates the response as opposed to innate immunity. This adaptive immune response is carried out by white blood cells known as lymphocytes that compose either an antibody response or a cell mediated response. B cells are a part of the antibody response, as they secrete antibodies known as immunoglobulins. T cells are a part of the cell mediated response and can either signal other cells via cytokine production to eliminate the antigen or they can eliminate it directly. This is dependent upon whether it is a helper T cell or cytotoxic T cell (39).

CD4+ T cells, also known as helper T cells, have the ability to reduce *P. chabaudi* infection by priming phagocytic cells to capture and kill the malaria parasites, helping B cells to produce anti-parasitic antibodies, and activate innate immune cells (9, 10, 11). With this wide range of tasks the CD4+ T cells can accomplish, we explored how diet, nourishment, and Moringa supplementation would affect abundance of CD4+ T cells within the small intestine. The small intestine is lined with Peyer's Patches where we would typically find immune cells

within the gut. We found in **Figure 2A** that there was not a significant difference in the total population of CD4+ T cells across all four groups, indicating that the malnourishment and supplementation may not make a difference in the total population of these T cell populations.

Within these CD4+ T cells there are different subsets, one of them being effector T cells. Effector T cells play an integral role in directing the immune response and can be both immune promoting or regulatory. There are typically 3 stages that occur when a naive T cell works towards becoming a memory cell. The first stage, the 'expansion' phase, is initiated in the lymphoid tissues, where encounter with antigen induces naive T cells to clonally expand and differentiate into effector T cells — known as T helper (TH) cells or cytotoxic T lymphocytes (CTLs) for CD4+ and CD8+ T cells, respectively. Through the combined abilities of CD4+ and CD8+ effector T cells to secrete inflammatory cytokines and kill infected cells, a typical acute infection can be resolved within days (40). On the other hand, chronic infections take a little longer to eliminate. Several types of effector T cells have been documented with distinct functions, having different transcription factors and releasing different cytokines. Effector T cells such as Th1, Th2, Th17, Tfh and Th9 cells are involved in inflammatory responses while regulatory T cells (Treg) engage in immune suppression (41). In **Figure 2B** we looked at activated effector T cell phenotype using the markers CD4+/ CD27+/ CD62L- for T cells. We found overall that there was no significant difference between the four groups, however it does appear that there is a slight elevation in both the well-nourished group and the moderate malnourished group receiving Moringa supplementation. This could suggest that Moringa supplementation is making up for the malnourishment, decreasing the effector T cells and raising these levels back up to where they would be in a well-nourished environment. Similar

observations have been seen in an in vitro model where Moringa treatment increases immune CD4 T cell numbers (42).

Since CD4+ T cells secrete cytokine to facilitate protection against infections, we investigated IFNγ production by CD4+ T cells in the gut mucosa. Cytokines are proteins that have an effect on both interactions and communication between cells (43). The cytokine IFN-y has been shown to activate macrophages (44). These macrophages can then go on to phagocytose iRBCs and aid in clearance of parasite (45). Examining the Th1 effector cells known to secrete IFN-γ (40), we found in **Figure 3** that mice receiving Moringa supplementation had a significant increase in CD4+ T cells secreting IFNγ compared to the other three groups in both the frequency and actual cell numbers. High production of IFNγ by the CD4 T cells after Moringa supplementation indicates a push to a stronger inflammatory response after *P. chabaudi* infection. Indeed, other studies have shown that Moringa can increase secretion of cytokines in other models (46) as seen in our current results.

Since many components of this plant are edible, each component has different beneficial uses. By testing the different components of the plant, using methods such as HPLC-DAD-electrospray mass spectrometry, it has been discovered that each part contains a different isolate. These extracts/ isolations include flavonoids (found in the leaves), glucosinolate and isothiocyanate (distributed in the leaves), phenolic acid (all distributed in the leaves), alkaloids and sterols (found in the leaves, roots, and seeds), and terpene (all distributed in the pods) (47, 48). Moringa extracts rich in isothiocyanates or ethyl acetate tend to have anti-inflammatory effects while other extracts that are rich in polysaccharides MOP-2 and MOP-3 (components of an RNA-dependent DNA methylation pathway), and *Moringa oleifera* seed resistant protein (MSRP), have also been shown to increase the expression of inflammatory cytokines, secretion

of reactive oxidative species (ROS), and stimulate proliferation and activation of splenocytes and lymphocytes (49, 50). It is unknown which specific extracts are biologically active in our study, but this could be a direction for future studies.

Naive T cells differentiate into various types of effector T cells based on the presence of specific polarizing, typically IL-12, IFN-γ, TNFα, etc. (40, 51). Therefore, cells secreting TNFα are most likely Th1 effector T cells. While there was not significant differences between the groups of TNFα producers, both the frequency and cell numbers of those receiving Moringa supplementation appears to be slightly decreased in CD4+ T cells secreting TNFα. This indicates the opposite case of **Figure 3A** and **B** that showed an increase in the inflammatory cytokine IFNγ for those receiving Moringa supplementation. This could mean that Moringa only increases certain inflammatory cytokines as opposed to others.

One regulatory cytokine secreted by helper T cells, IL-10, plays a critical role during the blood-stage of murine malaria infections. It has been reported that a deficiency in IL-10 leads to a lower parasite burden and increased inflammatory processes. Specifically, the ratio of IL-10 proinflammatory inhibitors and tumor necrosis factor (TNF) can determine the effectiveness of parasite clearance. IL-10 could play a regulatory role within non lymphoid organs such as the liver, lung, and brain and can determine the outcome of tissue-damaging inflammation. However, the cellular source of IL-10 has so far only been explored in the spleen and blood. It has been indicated though that CD4+ IFN-γ+ IL-10+ T cells will distribute systemically during malaria infection to appropriately balance pro- and anti-inflammatory immune responses. IL-10 remains a big part of the adaptive immune system as CD4+ T cells are the predominant source of IL-10.

One study showed CD4+IFN-γ T cells exposed to the antigen, in this case *Plasmodium yoelii*, are the dominant source of IL-10 in both lymphoid and nonlymphoid tissue (52).

IL-10 production by Th1 cells has been shown to relieve inflammation in those who have contracted malaria. It does this by suppressing pro-inflammatory cytokines and chemokine production, downregulating expression of MHC II and co-stimulatory molecules, and by increasing expression of immune checkpoint molecules such as PD-1, CTLA-4, LAG-3, and CD4+ FoxP3+ regulatory T (Treg) cells. Although type I regulatory T (Tr1) cells can protect from this inflammation, they can also suppress Th1 cell-mediated immunity and can allow an infection to persist further (38). In our study CD4+ T cells secreting IL-10 were significantly increased after Moringa supplementation indicating that Moringa has the ability to protect from inflammation during this *Plasmodium* infection.

Other molecules such as IL-27 and ICOS have been found to control maintenance and programming of these T cells expressing IL-10 (52). Studies have suggested that IL-27 inhibits inflammatory Th17 lineage development as well as IL27-IL27R interaction inducing Th1 responses (53). In the same study, it was shown that blockade of IL-27R significantly reduced IL-10 expressing CD4+ T cells in the spleen, liver, lung, and blood but not Peyer's patches (52). This could show that IL-27 and IL-27R either play different roles within the gut. Even though both TGF-B and IL-10 are immunoregulatory, blockade of TGF-β increases the production of IL-10 by CD4+ T cells in the liver, lung, and Peyer’s patches. This could be suggesting that TGF-β inhibits IL-10 production by repressing the strength of activation of the CD4+ T cells. If there is a deficiency in IL-10 this could lead to increased inflammatory response and result in more tissue damage (52). This could be an avenue to explore further when looking into secretion of IL-10 with Moringa supplementation.

Killer T cells, CD8 T cells, mediate clearance of infected red blood cells by initiating apoptosis of those infected. A protective CD8 T cell response is dependent upon the presence of CD4+ T cells to prime them and how the sporozoite antigen persists through the infection (54). When naïve CD8 T cells become activated, they take one of three different pathways: they can secrete TNF-α and IFN-γ, and secrete cytotoxic granules (40). The secretion of the inflammatory cytokine, IFN-γ, can play both a protective and pathological role (55). In **Figure 6** we saw Moringa supplementation led to an increase in IFNγ frequencies as opposed to all other groups present. Looking at **Figure 6B** there was not a significant difference between these treatment groups, however they did follow the same trend. This indicates that Moringa promotes a larger CD8 T cell response to the *P. chabaudi* infection, releasing more IFNγ, activating more macrophages, and in turn clearing parasite infected RBCs from the body, and alleviating those suffering from malaria.

B cells produce antibodies, present antigens, distinguish self from non-self, and form memory to recall antigens they have interacted with previously. These cells can be found within germinal centers in the Peyer's patches of the gut, marked by GL7. The germinal center B cells have been shown to have higher functionality for both producing antibodies and presenting antigens (18). The slightly elevated B cell response seen in groups receiving Moringa supplementation, **Figure 7**, could indicate that Moringa promotes high maturation and specificity of B cells. Even though this increase was not significant, this could be based on the Moringa supplementation dosage used in our study, which was a little lower.

An antibody typically found within mucosal membranes, including the gut, is IgA. Secretory IgA has been shown to provide protection against antigens, including malaria, and toxins while also binding to and coating commensal bacteria that may become harmful within the

gut. In fact, deficiency of IgA has been found to correlate with a predisposition for multiple diseases (13). In **Figure 8** we saw that there are no differences between all the experimental groups. While more IgA would typically be a sign of a good response to infection, this antibody is not typically found within the blood and is more commonly present within mucosal membranes, indicating that a larger presence within the blood could be due to mucosal damage and microbial leakage. Being that there is no difference in secretion of IgA within the blood between the groups, this could indicate we are not seeing mucosal damage as we expected for those that were moderately malnourished.

Chapter 5: Conclusion

Our data shows that we successfully induced malnutrition within the models made for these experiments via food restriction. This suggests that during moderate malnutrition the cytokines released during the CD4+ T cell response, IFNγ and IL-10, were increased during Moringa supplementation while there was no effect on the secretion of TNFα. During the CD8+ T cell response, secretion of IFNy was also increased with supplementation of Moringa. Within the B cell response, it was found that germinal center B cell frequency and cell numbers within the small intestine, were slightly increased with supplementation of Moringa. Examining levels of IgA found within the bloodstream it was determined that there was no significant difference between treatment groups. Collectively these results indicate that Moringa supplementation may increase an inflammatory adaptive immune response within the gut during moderate malnutrition and *P. chabaudi* infections as well as a regulatory adaptive immune response. This in turn could suggest that Moringa provides a balance for the immune system between inflammatory and regulatory cytokines.

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Vita

Nicole Catherine Warnick was born in West Point, New York in the year 1999. Growing up in a military family she had the opportunity to travel the country and meet extraordinary people along the way that helped her develop a passion for not only science but her love for the human experience as well. These passions combined led her towards an interest in medicine. During her time in both high school and college, she developed her love for the field by volunteering at local hospitals as well as entering the emergency medicine field by starting a job as an EMT through Caldwell County Emergency Services. Through her undergraduate degree she knew she wanted to continue to pursue her love of medicine but also develop her interest and skills in research before going on to a medical career. Her drive to become a more well-rounded medical provider led her towards pursuing a masters' degree in Cellular and Molecular Biology with a concentration in Immunology at Appalachian State University. During her time pursuing this graduate degree, Nicole developed her knowledge of immunology and infectious disease that she hopes will strengthen her path forward to becoming a well-equipped physician. One day she hopes to specialize in either emergency medicine, cardiology, trauma, or infectious disease and make a difference for not only patients, but their families.

In her free time Nicole enjoys hiking, rock climbing, skateboarding, and exploring in and around the High Country. When she is not in the lab working on her graduate research, she is either teaching general biology labs, working as a server at The Cardinal (a restaurant in town), or working as an EMT. When not on the go she continues to enjoy and expand her extensive vinyl music collection she holds dearly to her heart. Overall though she looks forward to spending time with the people she loves and continuing to enjoy that human experience that brought her to this field and research in the first place.