THE DETRIMENTAL EFFECTS OF MALNUTRITION AND *PLASMODIUM CHABAUDI* INFECTION ON GUT INTEGRITY AND IMMUNITY

Thesis
by
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Abstract

THE DETRIMENTAL EFFECTS OF MALNUTRITION AND *PLASMODIUM CHABAUDI* INFECTION ON GUT INTEGRITY AND IMMUNITY

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Plasmodium falciparum is a protozoan parasite that causes malarial disease in humans, which resulted in approximately 405,000 deaths 2018. *P. falciparum* infections commonly occur in Sub-Saharan Africa, a region with high rates of micronutrient malnutrition due to poverty, poor crop management, and droughts. Micronutrient malnutrition is known to cause wasting and immunological defects, which leaves the host susceptible to infection and disease. The prevalence of endemic malarial disease and micronutrient malnutrition in rural Sub-Saharan Africa has exacerbated morbidity and mortality. Despite their prevalence, there is a disparity in knowledge about the relationship and outcomes associated with malaria and malnutrition, especially in the gut. Therefore, we aimed to elucidate this complex relationship and hypothesized that malnutrition hampers innate mucosal immunity and leads to increased morbidity during malaria infection.

The complex relationship between malaria and malnutrition and their effects on gut immunity and physiology are poorly understood. Thus, we investigated the effects of moderate malnutrition and malaria infection in the guts of mice. We utilized a well-established low protein diet that is deficient in zinc and iron to induce moderate malnutrition and investigated intestinal permeability and mucosal innate immunity in the gut. We first performed macroscopic analyses on the gut tissues of the mice and found that

the moderately malnourished mice had increased yellowing of their gut tissues, which is indicative of lymphangiectasia. This effect was only exacerbated by malaria infection. Upon microscopic evaluation of the small intestine, we observed deformed epithelial mucosal cells in the moderately malnourished mice. Next, we administered FITC-dextran to these mice via oral gavage and found that the moderately malnourished mice also had increased intestinal permeability, which was again exacerbated by malaria infection. We also saw shortening of the small and large intestine. This effect indicates increased intestinal permeability (IP), which occurs due to inflammation and malnutrition.

To determine if these effects impacted mucosal innate immune cells, we investigated the expression of CD11b or CD11c cells in the small intestine, large intestine, and cecum. We found that the malnourished mice had decreased numbers of these cells during malaria infection. To further investigate the functionality of these cells, we delineated them using F4/80, and determined their activation by examining MHCII expression. Surprisingly, an increased proportion and number of $CD11b^{+}F4/80^{+}$ cells expressed MHCII, indicating that more macrophages were activated. Furthermore, there was also an increased proportion and number of $CD11b^{+}F4/80^{+}$ macrophages secreting IFNγ. These data suggest that moderate malnutrition decreases mucosal innate immune cell numbers in the guts of mice during malaria infection. Despite the decrease in mucosal innate immune cells, they are increasingly activated and secreting inflammatory cytokines, which could be causing increased intestinal permeability and shortening.

Acknowledgements

Firstly, I would like to thank my mentor and role model, Dr. Michael Opata, for not only his support and guidance, but also for continually challenging me to be a better scientist. Dr. Opata always encouraged me to think critically, look beyond superficial details, and invest in the pursuit of knowledge. His mentorship prepared me for any challenge that comes my way, and for that I am eternally grateful. I would also like to thank my committee members, Dr. Maryam Ahmed and Dr. Mary Kinkel, for their support in this process and always wanting the best out of me. My lab members and peers have also been an integral part of my experience during my time as a master's student here at Appalachian State University. Current and past lab members: Amari Smith, Annie Murray, Malorie Reuter, Lyndsay Richard, Kadra Ibrahim, and Isaac Ogden have all dedicated their time and labor to invest into my thesis project and I am grateful for them. I would also like to give a special thanks to my mentee, Tyler Olender, for his dedication, interest in learning, and unwavering support during our short time together. Lastly, and perhaps most importantly, I would like to express my never-ending gratitude to my fiancé, Sarah Scrattish, who has been there with me through this entire process. Whether it be bringing me food during my 22-hour experiments, allowing me to vent, or supporting my hard work, she has been my light in all of this.

Dedication

I would like to dedicate this thesis to my late mother, Martina Lynn Murr. I believe it is her presence and those words she always prayed over me that got me to this point. She blessed me with insurmountable will power and a desire to achieve anything I set my mind to. Through my lowest lows and my highest highs, I know she is there, constantly encouraging me to keep going. It is her presence that keeps me going, knowing how proud she must be to see her boy achieving things he never thought possible. May I never doubt my abilities, and may I always attribute them to the one that has always been there for me.

Martina Lynn Murr
1957-2012

Table of Contents

List of Figures

List of Abbreviations

CD: Cluster of differentiation

Ctrl: Control

FITC: Fluorescein isothiocyanate

GALT: Gut associated lymphoid tissue

IFN-γ: Interferon gamma

Ig: Immunoglobulin

IL: Interleukin

Inf: Infected

IP: Intestinal Permeability

iRBC: Infected red blood cells

MAdCAM-1: Mucosal addressin cell adhesion molecule 1

Mal: Moderately Malnourished

MHCII: Major histocompatibility complex II

PAMP: Pathogen associated molecular pattern

PEM: Protein energy malnutrition

PI.: Post infection

POI: Peak of infection

RBC: Red blood cells

SCFA: Short chain fatty acid

SEM: Standard error of the mean

TNF-α: Tumor necrosis factor alpha

Unf: Uninfected

Chapter 1
INTRODUCTION

Malaria is a morbid disease that is caused by infection from the protozoan *Plasmodium* parasite. There are four different strains of *Plasmodium* that can infect humans, *P. vivax, P. ovale, P. malariae,* and *P. falciparum,* the most prevalent and deadly of the four (1, 2). *P. falciparum* was estimated to have evolved to infect humans approximately 10,000-100,000 years ago in early hominids (3). To this day, the parasite has evolved with us, incurring drug resistance and insecticide resistance that allows it to continually cause disease in approximately 229 million people, killing almost half a million people in 2019 (4). Sub-Saharan African is home to 94% of all malaria cases, due to political turmoil, poor health care, lack of education, and malnutrition (5). These issues are compounded by the complexity of the *Plasmodium* life cycle that grant it endemicity, effectively evading vaccine and treatment efforts.

The *Plasmodium* parasite grows intracellularly in two phases, a sexual phase in the carrier, a female *Anopheles* mosquito, and an asexual phase in the human host (6). The life cycle is considered to begin when the mosquito vector ingests gametocytes during a blood meal, resulting in gametogenesis in the midgut of the mosquito, however the factors that regulate formation and initiation are not well understood (7). These gametes develop into ookinetes, which become motile and embed themselves in the midgut lumen of the mosquito and form oocysts (8). Within the oocysts, thousands of sporozoites develop and mature, resulting in the oocyst bursting (9). The sporozoites then travel via hemolymph to the salivary glands of the mosquito, which are then infectious to the mosquito's next blood meal, usually a human (10).

Inoculation of sporozoites within a human host begins the asexual phase of the *Plasmodium* life cycle. When a human is bitten by the mosquito vector, hundreds of sporozoites are released from the salivary gland into the blood stream. Many of the sporozoites will be destroyed by macrophages and other innate immune responders (11). Successful sporozoites will traverse capillary networks and across endothelial barriers to enter the hepatic parenchyma (12). Once inside, the sporozoite will induce proteolytic cleavage and processing of the circumsporozoite protein in order to invade hepatocytes (13). Through this process, known as the exoerythrocytic or liver stage, the parasite undergoes asexual reproduction to form schizonts within parasitophorous vacuoles, protecting it from the immune system (14). Schizogony takes approximately 5-7 days in *P. falciparum* and 6-18 days for other species of *Plasmodium.* After schizogony, the liver cells will rupture, releasing merozoites into the blood stream, beginning the symptomatic or erythrocytic stage of infection (15).

Once in the blood stream, the merozoites utilize four major families of *Plasmodium* invasion ligands (EBL, Rh, MSP, and TRAP) in order to enter the host red blood cells (RBC) (16). This process happens in only one minute, which is advantageous for the parasite in its evasion from the immune response (17). Once inside the red blood cells, the parasite replicates exponentially, depending on anaerobic glycolysis for energy. As the parasite grows, the membrane permeability of the red blood cells and cytosolic composition are modified (18, 19). These modifications allow for the uptake of extracellular nutrients and the excretion of metabolic waste, preserving the integrity of the infected red blood cells. This mechanism allows the merozoites to grow and divide through the ring, trophozoite, and schizont stages. Eventually the red blood cells are lysed, releasing new

merozoites into the blood stream, which can infect more red blood cells and repeat the process (20). As the parasite replicates, it can begin to manifest symptoms in the gastrointestinal system (21, 22).

Malnutrition is another issue that is prevalent in Sub-Saharan Africa (23), contributing to approximately 45% of all deaths in children in developing countries (24). In this sense, the majority of people suffer from both micro and macronutrient undernutrition, commonly called protein-energy malnutrition (PEM) (25). Children in Sub-Saharan African commonly experience two forms of PEM, named kwashiorkor and marasmus. Kwashiorkor is defined by adequate calorie intake with inadequate protein intake, while marasmus is defined by inadequate intake of both protein and calories. While individuals are certainly deficient in macronutrients in Sub-Saharan African, there are also common micronutrient deficiencies, such as zinc and iron (26). This is especially concerning as nutrient deficient malnutrition has been linked to increased predisposition to infectious diseases, such as malaria, especially in children under the age of 5 (27).

Malaria and malnutrition have been studied intensively for years and their relationship and interactions have been demonstrated well. Generally, it has been reported that malnutrition increases the risk of malaria significantly, with greater chance of experiencing severe malaria (28). Although much is understood about malaria and malnutrition, the two still account for millions of deaths every year (29). This is mostly due to poor socioeconomic status, education, hygiene, food scarcity, and political corruption in endemic areas (30).

Malnutrition has profound effects on gut mucosal immunity, which also foment morbidity and drastically impact protection against infection and disease outcomes. A

recent study on micronutrient malnourished children in Uganda and Niger had higher levels of fecal calprotectin and plasma inflammatory cytokines (G-CSF, IL-6, TNFα, IL-1Rα, IL-13, TNFβ, and IL-2) (31). It was also determined that they had lower plasma levels of butyrate and propinoate, which are key short-chan fatty acids (SCFAs) that are responsible for regulating inflammation in the gut. Despite having higher basal inflammatory markers, micronutrient malnutrition has been associated with decreased numbers of immune cells, but increased proportions of activated T and B cells (32). This has been shown to disturb nutrient sensing pathways in the gut, such as AhR and RAR signaling, which directly impact mucosal immunity (33). As a result, innate immunity in the gut is hampered, leaving the individual at higher risk for infection and comorbidities (34, 35).

Protein deficiency, especially in the case of kwashiorkor, has been shown to increase rates of morbidity and mortality in children in Africa who are infected with *Plasmodium falciparum* (36, 37). Children who are deficient in protein and infected by malaria typically present higher rates of wasting and stunting, which is associated with thymic hypoplasia and immunodeficiencies during infection (38). As consequence, it has been found that specific IgG antibody levels towards the *Plasmodium* parasite are significantly lowered in children who are deficient in protein (39). This leaves individuals at risk for chronic infection, as well as higher rates of reinfection (40).

Protein deficiencies have also been shown to have important impacts on host susceptibility to disease, as well as their clinical outcomes. Generally, protein deficiency has been historically associated with decreased white blood cell numbers, hampering the innate immune response to infections in general and in the gut (41, 42). This effect has been attributed to altered B-cell responses and obstructed antigen presentation (43),

impaired haematopoiesis due to bone marrow atrophy (44), and thymic and mesenteric lymph node hypoplasia (45). Leukopenia caused by protein deficiency effectively renders the immune system less capable of fighting infection, leading to increased rates of disease (46). Zinc homeostasis is also vital with regards to white blood cells numbers. Deficiencies in zinc have been linked to altered haematopoiesis, cell maturation and differentiation, cell cycle progression, and proper function (47).

Furthermore, transient loss of zin has been associated with cell signaling failures. More specifically, zinc has been shown to be important during inflammatory responses; without it the acute phase response to infection is dampened (48). As a result, this leaves individuals who are deficient in zinc at higher risk for infection, with an increase in morbidity (49). Cross-sectional studies among young children and pregnant women in Africa have shown inverse correlations between zinc status and *P. falciparum* morbidities (50, 51). Mouse studies have also shown that zinc deficiency results in 40% mortality from the normally nonlethal rodent strain of malaria *P. yoelii* (52). Zinc supplementation has been shown to greatly benefit host outcomes, reducing severe malaria episodes by 69% in acutely infected individuals (53).

Iron is also known to play an important role in immunity, such as cell growth, differentiation and cytokine activity (54). Iron deficiency has been reported to significantly lower levels of circulating IgG, IL-6, IL-4, IL-8, and TNF-α (54, 55), suggesting that it is important for cell functionality. A study by Ekiz *et al*, demonstrated that neutropenia decreased oxidative burst and phagocytic activity due to defects in iron-dependent enzymes (56). This is important because deficiencies in iron have been historically linked to decreased severity and susceptibility to malaria infection (57, 58). However, a more recent

study by Zhang *et al,* has shown that iron deficiency exacerbated malaria infections by means of iron exporter ferroportin, which degrades under deficient conditions and does not transport iron out of red blood cells (59). As a result, the *Plasmodium* parasite has more intracellular resources to thrive on.

Deficiencies are typically associated with anemia, poor neurological development, and lower work capacity, especially in children and pregnant women (60). Despite the known detriments of iron deficiency on human physiology, there is a never ending debate on its possible harmful or therapeutic effects during malaria infection (61, 62). However, we do know that iron deficiency is associated with lower IgG levels, as with protein deficiency, IL-6, phagocytic activity, and oxidative burst by neutrophils (63). This is also true in the case of gut immunity, where iron deficiency has shown to impact commensal microbes, which in turn down regulate the immune response (64).

The gastrointestinal tract is a vitally important, yet often overlooked, part of basic human physiology, homeostasis, and immunity. Containing the largest number of immune cells in the body (65, 66), the gastrointestinal tract is charged with maintaining a very delicate balance between protecting the body from harmful xenobiotic organisms while harboring beneficial bacteria (67). One of first lines of defense against foreign pathogens in the gastrointestinal system, and all systems, is the innate immune response (68), which is divided into three groups of innate lymphoid cells. The first group includes natural killer cells which are responsive for secreting cytokines such as IL-15, IL-12, and IL-18 (69). These cytokines act as polarizing cytokines for adaptive immune cells that secrete IFN-γ and TNF-α, and cytotoxic granules (70). Group 1 innate lymphoid cells are further

categorized by the T-box transcription factor eomesodermin, which has high homology to T-bet, expressed by activated natural killer cells (71).

The second group of innate lymphoid cells in the gastrointestinal system is categorized by all innate natural helper cells, nuocytes, and multipotent progenitor cells (72-74). These cells are classified by their shared production of cytokines IL-5, IL-6, IL-9, and IL-13, which are activated by intraepithelial cell derived IL-33 and IL-25 (75). This makes these cells vitally important in response to helminth infections, which are typically coinfected with the *Plasmodium* parasite (76). These cells are also typically partnered with gut derived myeloid cells and macrophages (77).

Group three innate lymphoid cells are more variable and diverse when compared to groups 1 and 2; they are typically defined by IL-17A and IL-22 production, and play a role in the development and maintenance of lymphoid tissues and the gut microbiome (78). More specifically, they are typically involved in barrier maintenance in the gut lumen and are highly specialized in order to differentiate exogenous antigen from self-antigen (79). Therefore, these cells have an important role in infection and immunity by means of critically regulating inflammation and acting as effectors for the downstream adaptive immune response.

In this study, we are particularly interested in macrophages and dendritic cells as they are proportionately the largest responders during an innate immune response, especially in the gut (68, 80). During a malaria infection, macrophages and dendritic cells effectively function to identify pathogen-associated molecular patterns (PAMPs), which induce cross talk with the adaptive arm of the immune system (81). Through this process, the macrophages and dendritic cells protect the host against infection via NOD-2 mediated

release of cryptidins and proinflammatory cytokines, such as IFN-γ and TNF-α (82). This mechanism is essential to gut homeostasis and regulation of the intestinal microbiota, which have been shown to have implications in infection and immunity (83). When there are defects in these cells and cellular mechanisms, such as those brought about by malnutrition, the consequences can be disastrous.

Collectively, deficiencies in protein, zinc, and iron are all decremental to immunity, with emphasis on protection against infections, as well as mitigating morbidity. More importantly, deficiencies in these nutrients also directly impact gut immunity and physiology (84-87). It has also been shown that the *Plasmodium* parasite can travel to the gut and infect the villi during the symptomatic stage of infection (88, 89). Under the influence of malnutrition, this could allow the *Plasmodium* parasite to infect the gut tissues, increasing harmful gastric symptoms, and increasing the chance of a fatal infection

Malaria has been found to have severely deleterious effects in the guts of mice and men (90-92). Some of these severe effects include gastrointestinal bleeding and intestinal obstruction due to absent peristalsis caused by splanchnic nerve dysfunction (93). Intestinal ischemia is also common, which results from cytoadherence and resetting of iRBCs (94). This effect can lead to destruction of the microvasculature in the intestines, disallowing nutrient and oxygen delivery and ultimately causing tissue necrosis and death (95). Many of the ischemic effects of malaria on the intestine are irreversible, causing permanent damage to the gut mucosal layer (96), intracellular signaling (97), nutrient absorption (98), and mitochondrial activity (99). Malaria infection has also been found to modulate the gut microbiome and induce dysbiosis (100). This can allow opportunistic pathogens to diversify and grow, causing further intestinal symptoms (101, 102)

What is not known is how the guts of mice that are fed a diet with limited protein and deficient in iron and zinc respond to *Plasmodium chabaudi* infection. Our first aim was to investigate changes in gut physiology during moderate malnutrition and malaria infection in these mice. To investigate this, we performed macroscopic analysis of the gut tissues, took their lengths, studied gut leakage with FITC-dextran, and used hematoxylin and eosin (H&E) staining to resolve differences in epithelial disorganization.

Our second aim was to determine the impact of malaria infection on mucosal innate immunity during moderate malnutrition. More specifically, we sought to determine the functionality of the mucosal macrophages based on a model of infection and moderately malnourished conditions in mice.

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 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 17-04). Male mice aged 8-14 weeks were used for experiments with consistency in both the malnourished and control groups.

2.2. Malnutrition and infection

Mice were fed either a moderate malnourished diet (Mal; TD.99075) with 3% protein and deficient in iron and zinc, or a well-nourished diet (Ctrl; TD.99103) with 17% protein and all necessary micronutrients from Envigo/Teklad (Indianapolis, IN). Both diets have similar caloric content, which is made up by extra carbohydrates in the moderate malnourished diet. The diets were administered to each group of mice at 3 grams per mouse daily for approximately 4-5 weeks to induce moderate malnutrition. After 4-5 weeks, both the control and malnourished mice were infected with $1x10^5$ *P. chabaudi* intraperitoneally and other groups of control and malnourished mice were left uninfected to serve as negative controls. 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 and cecum 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 using a 21-gauge needle and 10-mL syringe 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. Type I collagenase (ThermoFisher #17018029) was added at a concentration of 100 U/mL, followed by a one-hour incubation at 37°C 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 4°C 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. Fluorescent antibodies were used to label cells of interest: PE-Cy5-conjugated anti-CD11b (Tonbo 55-0112-U100), FITC-conjugated anti-CD11c (Tonbo 35-0114-U500), and PE-conjugated anti-MHCII (Tonbo 50-5321-U100) 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 an FC500 flow cytometer (Beckman Coulter, Indianapolis, IN).

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 place into a HERAcell 150i incubator set at 37ºC and 5% CO_2 overnight. 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. Fluorescent antibodies were used against surface markers to label cells of interest: PE-Cy5-conjugated anti-CD11b (Tonbo 55-0112-U100) and PE-conjugated F480 (Biolegend 123110) 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 permeabilization buffer (Tonbo Biosciences, San Diego, CA) and stained with IFNγ

(Biolegend 505850). The cells were then resuspended with 200 μL of FACS buffer and filtered.

2.6. Assessment of gut leakage in the intestines

Intestinal permeability and gut leakage were studied using fluorescein isothiocyanate (FITC)-labeled dextran on day 9 post-infection. Mice were first water starved overnight and weighed the next morning. FITC-dextran was prepared at a concentration of 100 mg/mL in sterile PBS and administered to each mouse at 4 mg/g body weight via oral gavage. Four hours after dextran administration, the mice were culled, and 0.5 mL of blood was taken via cardiac puncture. Blood samples were then spun at 1,677 X g for 5 minutes. The sera were collected and then diluted at a 1:1 ratio with sterile PBS. Dextran absorbance was read at 488 nm excitation/530 nm emission on a spectrophotometer. FITC-dextran concentrations in the sera were determined based on the standard curve.

2.7. Histological analysis of epithelial disorganization

Gut tissues were excised from day 9 *P. chabaudi* infected mice, 3 mm sections of the small intestine were cut and cleaned then fixed in 10% phosphate-buffered formalin. Tissues were fixed for approximately 48 hours and embedded in paraffin. Sections 8 μm thick were cut using a microtome and stained with hematoxylin and eosin (Sigma-Aldrich). Sections were viewed under an Olympus IX81/DP80.

2.8. Data analysis

Raw data collected from the FC500 Beckman Coulter flow cytometer were analyzed using FlowJo software (Ashland, OR). Calculations for all raw data were

performed in Microsoft Excel and graphs and statistics were performed in Prism GraphPad (San Diego, CA). Data were considered significantly different with a P value less than 0.05.

Chapter 3

RESULTS

3.1 Malnutrition experiments

Malnutrition Significantly Lowers Parasitemia and Body Weight but Not Body Temperature

The disparities and differences in conclusive results on the impacts of protein, zinc, and especially iron deficiencies during malaria infection led us to investigate the physiological changes under these conditions. To determine this, we fed mice either a control diet or moderately malnourished diet for 4-5 weeks and then infected them with $1x10^5$ *P. chabaudi* intraperitoneally. Blood smears were taken from the tails of all mice at 3, 5, 7, and 9 days post-infection to determine parasitemia. Weights and temperatures were taken the day of and every following day after initial infection until sacrifice. We found that mice fed the moderately malnourished diet had lower percent parasitemia at the peak of infection (POI) (**Figure 1a**), which is consistent with findings in the literature. These mice also had lower percent change in body temperature, indicating that there was less change in their temperature at POI when compared to the control mice (**Figure 1b**). This effect is explained by the lower parasitemia, which indicates they had lower parasite burden, and as a result had less change in body temperature. However, the moderately malnourished mice did lose more weight, but not significantly, at POI compared to the control mice (**Figure 1c**).

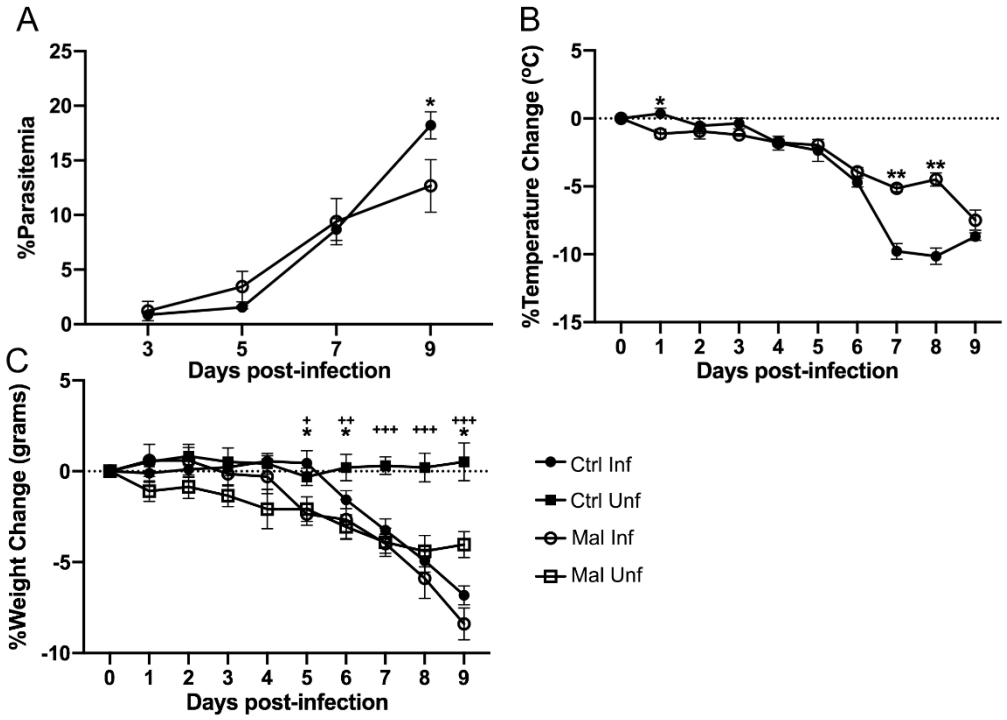
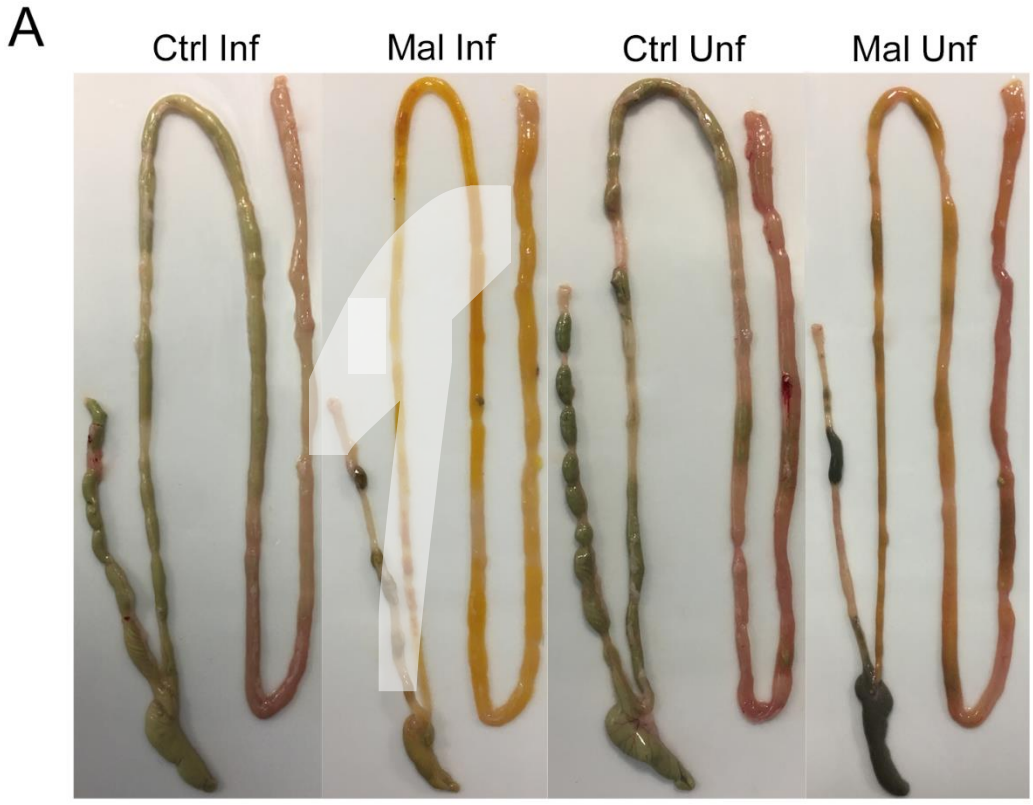


Figure 1. Malnutrition Significantly Lowers Parasitemia and Body Weight but Not Body Temperature. C57BL/6 mice were fed either a control diet or a moderately malnourished diet. Parasite levels were recorded to give (**A**) percent parasitemia. Temperatures were recorded via rectal thermometer to give (**B**) percent temperature change. Weights were recorded to give (**C**) percent weight change. All infected mice were interperitoneally injected with $1x10^5$ iRBCs of *P. chabaudi*. All data are shown as means ± SE, n = 3 male mice per group, representative of 16 independent experiments. Statistical analysis was performed using a two-way ANOVA with Tukey's test. +,* $p < 0.05$; ++,** $p < 0.01$; +++,*** $p < 0.001$, indicates a statistically significant difference between treatments. Ctrl = control diet, Mal = moderately malnourished diet, Inf = infected, Unf = uninfected.

Malnutrition Significantly Impacts Gut Physiology and Lengths During Malaria Infection

Since we know that malaria and malnutrition exacerbate the morbid effects of one another and independently affect the gut based on the literature, we sought to elucidate

their combined effects on gut physiology. We harvested the gut tissue by incising at the pyloric and anal sphincter and took macroscopic images of them and measured the small intestine, large intestine, and cecum. Previously it had been shown that diets deficient in protein induce pathological lymphangiectasia, which can directly affect lymphatic vessels and cause yellow discoloration due to chyle buildup (103). Malaria infection causes constriction of blood venules, which results in paleness of tissues (104). Here we can see that the moderately malnourished mice exhibit a slight yellowing of the tissue and the effect is further exacerbated by malaria infection (**Figure 2a**). It has also been shown that inflammation cause by malaria infection can cause intestinal shortening (88). We also observed a similar effect, but more importantly we found that moderate malnutrition further increased shortening in the small and large intestine (**Figure 2b**).



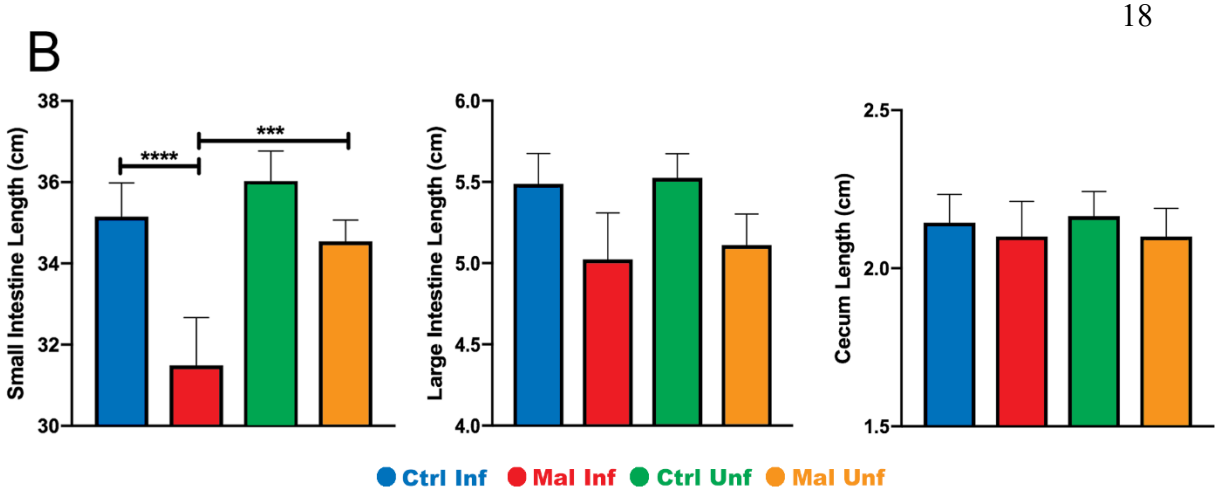


Figure 2. Malnutrition Significantly Impacts Gut Physiology and Lengths During Malaria Infection. C57BL/6 mice were fed either a control diet or a moderately malnourished diet. Gut tissues were harvested, and images were taken for macroscopic analysis (**a**) and lengths were taken (**b**). All infected mice were interperitoneally injected with $1x10^5$ iRBCs of *P. chabaudi*. All data are shown as means ± SE, n = 3 male mice per group, representative of 16 independent experiments. Statistical analysis was performed using a two-way ANVOA with Tukey's test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$: **** $p < 0.0001$, indicates a statistically significant difference between treatments. Ctrl = control diet, Mal = moderately malnourished diet, Inf = infected, Unf = uninfected.

Moderate Malnutrition and Malaria Infection Causes Gut Leakage

Intestinal shortening caused by malaria infection is associated with inflammation that can cause damage to the gut epithelia, which can increase intestinal permeability. Furthermore, inadequate protein intake along with zinc and iron deficiencies have been shown to impact tight junction protein organization, resulting in decreased gut integrity. Therefore, we sought to investigate the effect of both malaria and moderate malnutrition on intestinal permeability by utilizing FITC-dextran, which is normally impermeable to the gut epithelia. In an inflammatory environment, such as malaria infection and/or malnutrition, FITC-dextran can pass through the gut epithelium and leak into the blood stream. Increased serum concentrations of FITC-dextran indicate gut leakage as indicated

by our results (**Figure 3**). These results indicate moderate malnutrition tends to increase gut leakage and this effect is exacerbated by malaria infection.

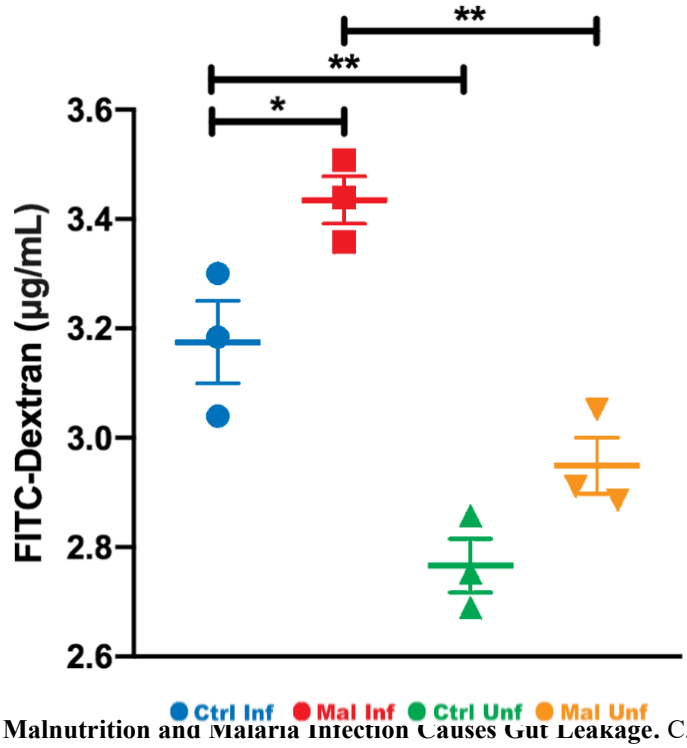


Figure 3. Moderate Malnutrition and Malaria Infection Causes Gut Leakage. C57BL/6 mice were fed either a control diet or a moderately malnourished diet. At POI mice were administered FITC-dextran via oral gavage after a 16 hour fast. Concentrations of FITC-dextran were calculated from a standard curve. All infected mice were interperitoneally injected with $1x10^5$ iRBCs of *P. chabaudi*. All data are shown as means ± SE, n = 3 male mice per group, representative of 1 independent experiment. Statistical analysis was performed using a two-way ANOVA with Tukey's test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, indicates a statistically significant difference between treatments. Ctrl = control diet, Mal = moderately malnourished diet, Inf = infected, Unf = uninfected.

Moderate Malnutrition Tends to Lower Innate Immune Cell Numbers in the Small Intestine During Malaria Infection

Malnutrition has been shown to negatively impact both innate and adaptive immunity, specifically in the small intestines (105, 106). Deficiencies in nutrients are also associated with increased susceptibility and severity of infection by the *Plasmodium*

parasite in the small intestine (107, 108). In order to investigate the impacts of moderate malnutrition and malaria infection on innate mucosal immunity, we performed flow cytometric analyis on the small intestine, large intestine, and cecum of moderately malnourished mice at POI. The tissues were harvested from the mice, cleaned, and suspended into solution. Cells were stained with different fluorphores against surface markers of interest. We found that the moderately malnourished mice tended to have decreased numbers of innate immune cells; macrophages, myeloid cells, and dendritic cells in the small intestines when compared to control mice (**Figure 4**).

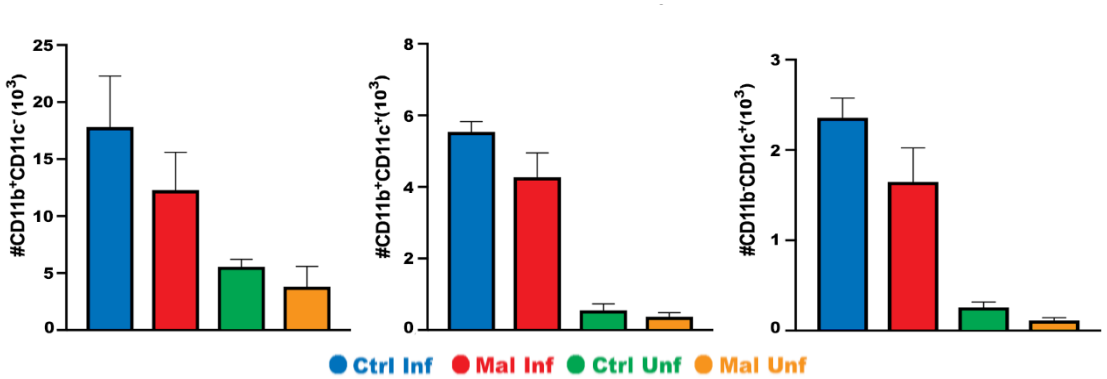


Figure 4. Moderate Malnutrition Tends to Lower Innate Immune Cell Numbers in the Small Intestine During Malaria Infection. C57BL/6 mice were fed either a control diet or a moderately malnourished diet. At POI mice were sacrificed and the small intestines were collected for FACs analysis. All infected mice were interperitoneally injected with $1x10^5$ iRBCs of *P. chabaudi*. All data are shown as means ± SE, n = 3 male mice per group, representative of 16 independent experiments. Statistical analysis was performed using a two-way ANOVA with Tukey's test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, indicates a statistically significant difference between treatments. Ctrl = control diet, Mal = moderately malnourished diet, Inf = infected, Unf = uninfected.

Moderate Malnutrition Significantly Lowers Innate Immune Cell Numbers in the Large Intestine During Malaria Infection

Malnutrition is also associated with immunological and changes in the large intestine. It has been shown that children who are malnourished tend to incur a diet-dependent enteropathy resulting in lower levels of secretory IgA, effectively lowering their defense against xenobiotic organisms and opportunistic pathogens (109). Furthermore, it has been shown that malnutrition can modulate the host immune response, which in turn has also been shown be decremental during malaria infections (110, 111). To further investigate gut mucosal immunity, we investigated the large intestine, the home of the microbiome and a complexity of immunological interactions. We used flow cytometry to investigate the number of macrophages, myeloid cells, and dendritic cells in the large intestine of moderately malnourished mice. Similarly to the small intestine, we examined decreased numbers of these cells in the moderately malnourished mice, indicating a hampered immune response due to dietary inadequecies during malaria infection (**Figure 5**).

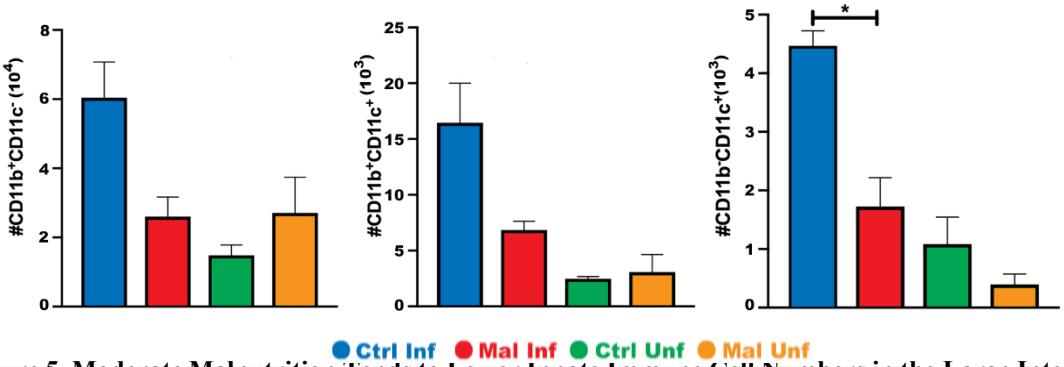


Figure 5. Moderate Malnutrition Tends to Lower Innate Immune Cell Numbers in the Large Intestine During Malaria Infection. C57BL/6 mice were fed either a control diet or a moderately malnourished diet. At POI mice were sacrificed and the large intestines were collected for FACs analysis. All infected mice were interperitoneally injected with $1x10^5$ iRBCs of *P. chabaudi*. All data are shown as means ± SE, n = 3

male mice per group, representative of 16 independent experiments. Statistical analysis was performed using a two-way ANOVA with Tukey's test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ indicates a statistically significant difference between treatments. Ctrl = control diet, Mal = moderately malnourished diet, Inf = infected, Unf = uninfected.

Moderate Malnutrition Tends to Lower Innate Immune Cell Numbers in the Cecum During Malaria Infection

The cecum carries much of the same functional immunological characteristics as the large intestine. However, not much is known about the impacts of malaria and malnutrition on this tissue. But what is known is that the microbial communities of the cecum tend to mimick those in the large intestine, meaning they both communicate with the immune system via their microbial communities (112). It has also been shown that group 3 innate lymphoid cells play an important role in maintaining homeostasis within the cecum and processing cecal content (113). Therefore, we aimed to investigate the effects of malaria and moderate malnutrition on cecal immunity. Given the similarities to the large intestine, we expectedly found a trend toward a decrease in the numbers of macrophages, myeloid cells, and dendritic cells within the tissue, although it was not signicant. (**Figure 6**).

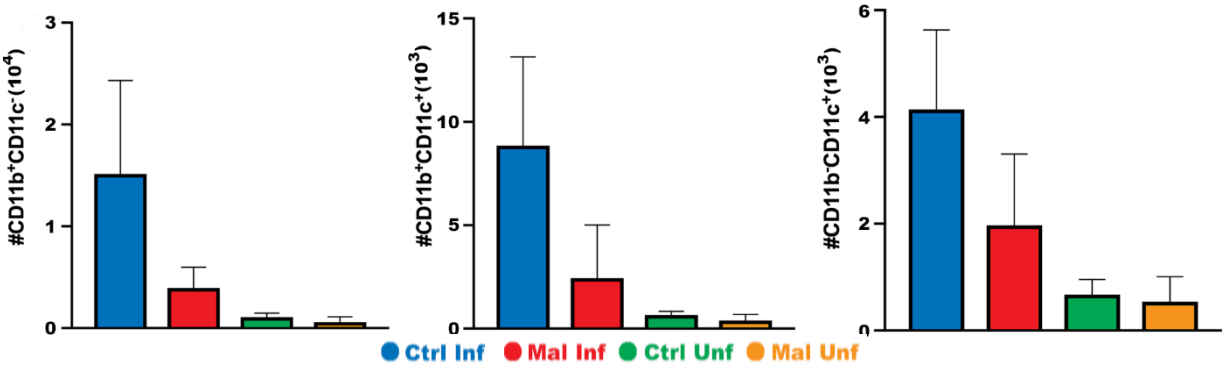


Figure 6. Moderate Malnutrition Tends to Lower Innate Immune Cell Numbers in the Cecum During Malaria Infection. C57BL/6 mice were fed either a control diet or a moderately malnourished diet. At POI

mice were sacrificed and the cecum were collected for FACs analysis. All infected mice were interperitoneally injected with $1x10^5$ iRBCs of *P. chabaudi*. All data are shown as means ± SE, n = 3 male mice per group, representative of 16 independent experiments. Statistical analysis was performed using a two-way ANOVA with Tukey's test. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.001$, indicates a statistically significant difference between treatments. Ctrl = control diet, Mal = moderately malnourished diet, Inf = infected, Unf = uninfected.

Moderate Malnutrition Tends to Lower MAdCAM-1 Proportions and Numbers in the Small Intestine, Large Intestine, and Cecum During Malaria Infection

Mucosal addressin cell adhesion molecule 1 (MAdCAM-1) has been shown to be critically important in leukocyte immunosurveillance, homing, and adhesion in the gut (114). More importantly, MAdCAM-1 expression has been shown to be upregulated during infections, such as malaria (115). However, malnutrition experiments in mice have shown decreased MAdCAM-1 mRNA expression in the Peyer's patches (116). Therefore we sought to investigate the effects of both malaria infection and moderate malnutrition on the proportion and number of cells expressing MAdCAM-1 in the small intestine (**Figure 7a**), large intestine (**Figure 7b**), and cecum (**Figure 7c**) of mice. As we previously saw decreased numbers of macrophages, myeloid cells, and dendritic cells in these tissues, we also found a similar trend with MAdCAM-1 expression. In all tissues, MAdCAM-1 expression trended towards a decrease in moderately malnourished mice during malaria infection.

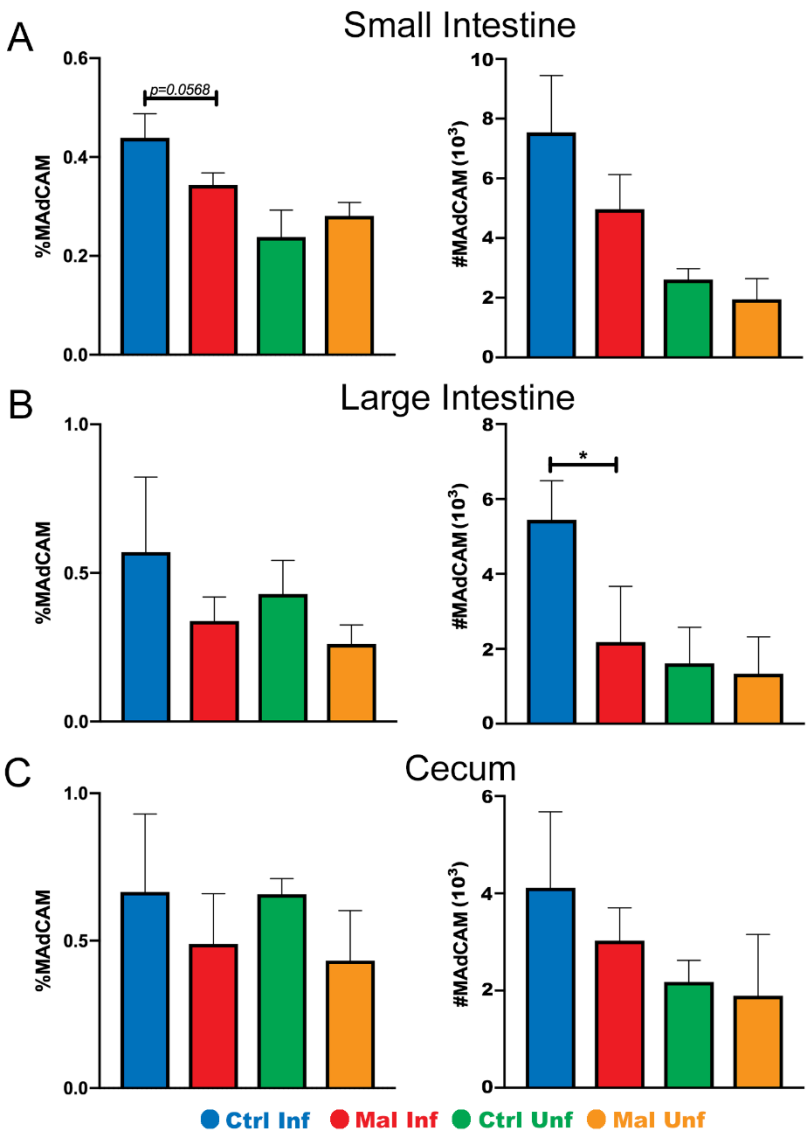


Figure 7. Moderate Malnutrition Tends to Lower MAdCAM-1 Proportions and Numbers in the Small Intestine, Large Intestine, and Cecum During Malaria Infection. C57BL/6 mice were fed either a control diet or a moderately malnourished diet. At POI mice were sacrificed and the small intestine (**a**), large intestine (**b**), and cecum (**c**) were collected for FACs analysis. All infected mice were interperitoneally injected with $1x10^5$ iRBCs of *P. chabaudi*. All data are shown as means ± SE, n = 3 male mice per group, representative of 16 independent experiments. Statistical analysis was performed using a two-way ANOVA with Tukey's test. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.001$, indicates a statistically significant difference between treatments. Ctrl = control diet, Mal = moderately malnourished diet, Inf = infected, Unf = uninfected.

Moderate Malnutrition Increases CD11b$^+$MHCII$^+$ Cell Proportions and Numbers in the Small Intestine, Large Intestine, and Cecum During Malaria Infection

Because we saw an increase in CD11b$^+$ and CD11c$^+$ cell numbers along with increased expression of MAdCAM-1 in the gut of moderately malnourished mice during malaria infection, we wanted to investigate antigen presenting macrophages. These cells are responsible for phagocytosing antigen and communicating with the adaptive immune response. In order to do so we stained for CD11b and MHCII.We found that the moderately malnourished mice had increased proportions and numbers of these cells in their small intestine (**Figure 8a**), large intestine (**Figure 8b**), and cecum (**Figure 8c**). This indicates that the moderately malnourish mice have increased phagocytic activitiy and antigen presentation in their gut tissues. This could possibility indicate an increased inflammatory environment.

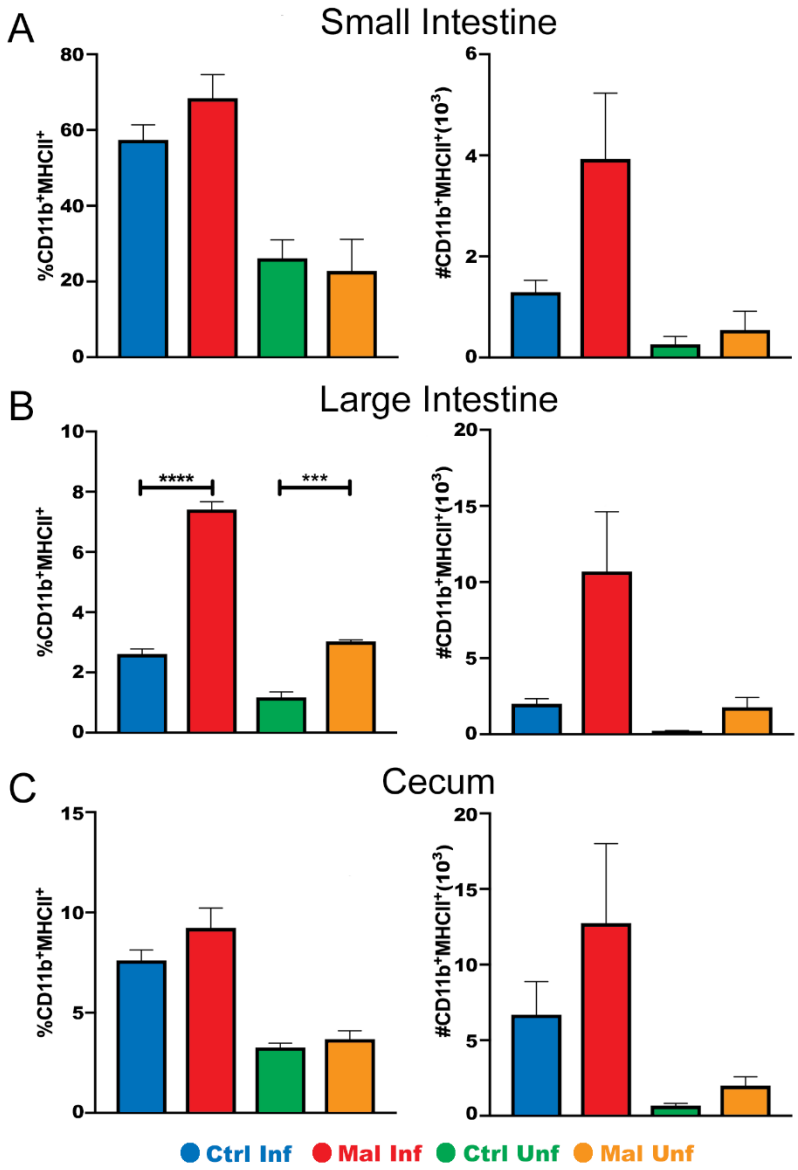


Figure 8. Moderate Malnutrition Increases CD11b+MHCII+ Cell Proportions and Numbers in the Small Intestine, Large Intestine, and Cecum During Malaria Infection C57BL/6 mice were fed either a control diet or a moderately malnourished diet. At POI mice were sacrificed and the small intestine (**a**), large intestine (**b**), and cecum (**c**) were collected for FACs analysis. All infected mice were interperitoneally injected with $1x10^5$ iRBCs of *P. chabaudi*. All data are shown as means ± SE, n = 3 male mice per group, representative of 12 independent experiments. Statistical analysis was performed using a two-way ANOVA with Tukey's test. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.001$; **** $p < 0.0001$, indicates a statistically significant difference between treatments. Ctrl = control diet, Mal = moderately malnourished diet, Inf = infected, Unf = uninfected.

Moderate Malnutrition Increases Cytokine Production by Macrophages in the Small Intestines During Malaria Infection

Since we found an increase in MHCII expression on $CD11b^+$ cells in the small intestine, we hypothesized that the macrophages present would be producing more IFNγ, as IFNγ production correlates with MHCII expression (117). Therefore, we investigated the proportion and number of macrophages secreting IFNγ in the small intestines of moderately malnourished mice. Expectedly, we found that the moderately malnourished mice tend to have higher proportions and numbers of macrophages secreting IFNγ (**Figure 9**). This indicates that these mice have more inflammation in their small intestines during malaria infection.

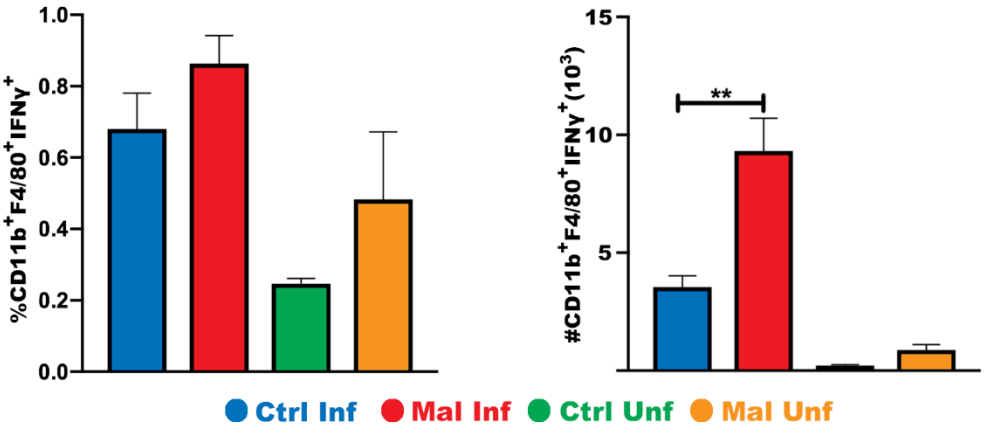


Figure 9. Moderate Malnutrition Increases Cytokine Production by Macrophages in the Small Intestines During Malaria Infection. C57BL/6 mice were fed either a control diet or a moderately malnourished diet. At POI mice were sacrificed and the small intestines were collected for FACs analysis. Cells were stimulated with PMA and ionomycin. All infected mice were interperitoneally injected with $1x10^5$ iRBCs of *P. chabaudi*. All data are shown as means ± SE, n = 3 male mice per group, representative of 1 independent experiment. Statistical analysis was performed using a two-way ANOVA with Tukey's test. * $p < 0.05$; ** $p < 0.001$, indicates a statistically significant difference between treatments. Ctrl = control diet, Mal = moderately malnourished diet, Inf = infected, Unf = uninfected.

Moderate Malnutrition Induces Epithelial Damage in the Small IntestineDuring Malaria Infection

Inflammatory cytokines cause inflammation and recruit other cells at the site of tissue injury and infection (118). One key mediator of intestinal permeability and integrity is IFNγ, which was present in higher number in the small intestine of moderately malnourished mice during infection. Therefore, we hypothesized that increased IFNγ, may cause tissue injury in the small intestine. To investigate this, we took sections of the small intestine from the mice and stained them with hematoxylin and eosin. We found that moderate malnutrition tends to cause epithelial disorganization as illustrated by the black arrows (**Figure 10**). Furthermore, we found that this effect was exacerbated in the moderately malnourished mice that were infected with *P. chabaudi*. This indicated that the increased inflammation caused by IFNγ is causing damage to the epithelium in the small intestines of moderately malnourished mice during malaria infection.

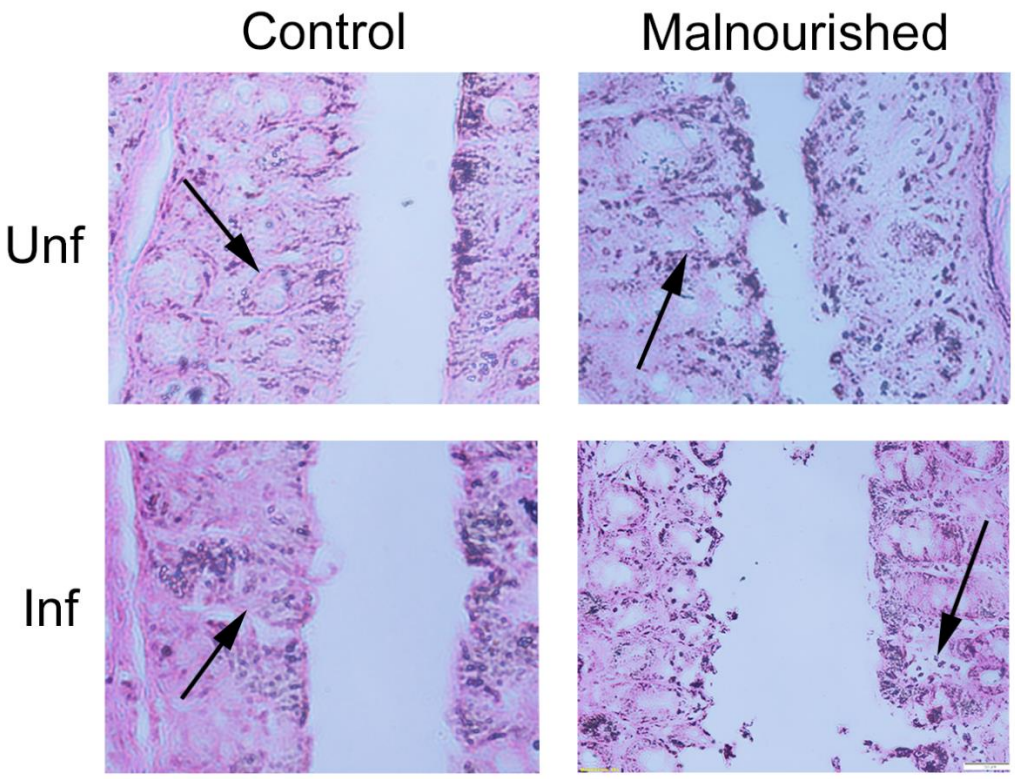


Figure 10. Moderate Malnutrition Induces Epithelial Damage in the Small Intestine During Malaria Infection. C57BL/6 mice were fed either a control diet or a moderately malnourished diet. At POI mice were sacrificed and the small intestines were collected for histological analysis. Tissues were fixed, embedded, and then stained with H&E. All infected mice were interperitoneally injected with $1x10^5$ iRBCs of *P. chabaudi*. Images are representative of 4 independent experiments with 3 mice per group. Ctrl = control diet, Mal = moderately malnourished diet, Inf = infected, Unf = uninfected. Arrows indicate epithelial cells.

Chapter 4

DISCUSSION

Malaria and malnutrition have been occurring in Sub-Saharan Africa for decades (119, 120). Despite this, little is known about the detrimental effects of malaria infection on gut immunity during moderate malnutrition. Here, we have demonstrated that mucosal innate immunity, including early macrophage and dendritic cell progenitors, in the gut tissues are lower in number after *P. chabaudi* infection during moderate malnutrition. Even though there are diminished numbers of $CD11b^+$ and $CD11c^+$ cells in the moderately malnourished mice after infection, there is possible compensatory response by the $CD11b^+MHCII^+$ macrophages in both proportion and number in response to infection. The high activation of macrophages could induce inflammation, which was indicated by increased proportions and numbers of $CD11b^+F480^+$ macrophages producing IFNγ. An inflammatory environment in the gut can cause damage to the epithelium, leading to gut leakage. This was shown by high FITC-dextran concentrations in the sera of the moderately malnourished mice, intestinal shortening, and epithelial disorganization.

In a study by the Melby group, using similar model as our moderate malnutrition diet, it was shown that malnutrition related reduction in macrophages and monocytes was associated with reduced lymph node-resident cells, but not migratory cells in response to infection (121). Earlier studies by Fuss et al., reported that hypoproteinemia could lead to lymphangiectasia because of decreased presence of immune cells in the gut. This condition is caused by inhibitory effects towards CD45RA+ cells migrating to the intestines (122). The guts of the moderately malnourished mice exhibited macroscopic symptoms of lymphangiectasia, as seen by the paleness of the gut. This could be due to the high

expression of $CD11b^{+}F480^{+}$ macrophages producing IFNγ which leads to small intestine damage, hence altering immune cell migration to the gut mucosal surface. Other forms of micronutrient deficiencies have shown similar compensatory response by activated macrophages (123, 124). This compensatory response is associated with increased inflammation, which is meant to maintain gut integrity against infection, as well as diet induced dysbiosis via upregulation of MHCII and production of IFNγ (125, 126). However, the increased inflammation can lead to further damage to the epithelium, rather than maintenance of the gut barrier. It is highly possible that regulatory mechanisms are altered. It has been reported that nutritional factors can modulate Treg plasticity, metabolism, and function (127), but we did not test for Tregs and anti-inflammatory responses, as our study focused on innate immune cells.

In addition to altering regulatory mechanisms in the gut, the compensatory response due to malnutrition can have consequences regarding the integrity of the gut. Kim et. al. showed that dysbiosis in the intestines can polarize macrophages towards an inflammatory phenotype, and this is supported by malnutrition linked dysbiosis that was shown by Kumar et. al. (128, 129). With increased dysbiosis due to malnutrition comes the exacerbated and damaging affect induced by malaria. Taniguchi et. al. showed that malaria infection in mice causes dysbiosis and inflammation (130). Therefore, malaria and malnutrition together can drive macrophages towards an inflammatory phenotype that is characterized by increased MHCII expression and IFNγ secretion, which can cause increased damage to the gut epithelium.

An increased proportion and number of $CD11b^{+}MHCII^{+}$ macrophages were seen in all sections of the gut, with significant differences in the large intestine. This result is supported by Stough et. al. research which highlighted the interaction between the microbiome and malaria infection with emphasis on the large intestine, the home of the microbiome (131). Malnourished individuals experiencing dysbiosis have higher levels of inflammation, and their microbiomes are more susceptible to opportunistic pathogens (132). This could provide a possible explanation for the increased macrophage activation and cytokine secretion seen in the gut, not only during infection, but without infection as well in the moderately malnourished mice. The increased macrophage activation and cytokine secretion compounded by malnutrition could also be causing physical damage to the gut leading to gut leakage (133, 134).

This is important because macrophages are one of the key mediators of malaria infection, charged with phagocytosing the parasite and activating other cells to fight the infection, resulting in inflammation (135). Furthermore, diet linked dysbiosis of the microbial communities in the gut has been linked to increased macrophage activation and secretion of IFNγ (136). While both of these inflammatory events are meant to restore homeostasis and integrity of the gut barrier, they often result in increased disease pathologies such as intestinal damage incurred by infection and malnutrition (137-139). Consistent with this, we found that moderately malnourished mice that were infected with malaria had increased proportions and numbers of $CD11b^{+}F4/80^{+}$ macrophages secreting IFNγ, which can significantly impact intestinal permeability.

A commonly used method to test intestinal permeability is with the administration of FITC-dextran, which does not normally pass through the gut epithelium (140). When

the gut barrier is compromised due to inflammation, FITC-dextran can pass through the epithelial barrier and leak into the blood and can be measured using spectrophotometry. Previous research has showed that malaria and malnutrition can independently increase sera FITC-dextran concentrations (88, 141). The supposed mechanism by which this happens is due to defects in tight junction proteins, such as occludin and claudins. Zinc is an important nutrient involved in maintenance of tight junctions, specifically occludin and claudins. Occludin and claudin structure and organization are significantly affected by zinc depletion, which can result in leaky gut due to loss of integrity (142). Furthermore, protein deficiencies, such as the case of severe acute malnutrition (SAM), can impact gut integrity as well, which has been shown with endoscopic procedures and lactose/mannitol urine tests (143, 144). Similarly to protein and zinc deficiencies, iron reduction has also been linked to increased intestinal permeability by way of tight junction disorganization (145).

Regarding the moderately malnourished diet, we would then expect the moderately malnourished mice to experience increased gut leakage and epithelial disorganization due to tight junction dysfunction. Furthermore, this effect must be exacerbated by malaria infection due to the chronic and inflammatory nature of the infection. Moderately malnourished mice that are infected by *P. chabaudi* experienced the highest degree of intestinal permeability and epithelial disorganization. This is explained by increased concentrations of serum FITC-dextran, intestinal shortening, and epithelial disorganization seen in our study. Supporting the hypothesis that malnutrition exacerbates gut leakage induced by malaria infection, since malaria infection alone leads to deformities in the gut epithelia (146).

To our knowledge, this is the first study to demonstrate the exacerbated effects of moderate malnutrition on gut integrity during malaria infection. Previous studies by Singh and colleagues showed that unwarranted inflammation results from altered nutrition leading to increased intestinal permeability, which was also associated with shortening of the gut tissues (147). Intestinal shortening is also associated with IFNγ levels in the intestines (148), which is a sign of chronic inflammation and tissue fibrosis (149). This goes hand-in-hand with the increased number of macrophages secreting IFNγ seen in our study.

Collectively, these results indicate decremented gut physiology induced by moderate malnutrition, which is exacerbated by malaria infection. Through utilization of a well-established moderate malnutrition mouse model, that mimics malnutrition seen in malaria endemic areas (150), we were able to successfully show the increasingly harmful effects of malaria and malnutrition in the gut. This is shown by the increased proportion and number of activated macrophages secreting inflammatory cytokines, as well as intestinal permeability (**Figure 11**).

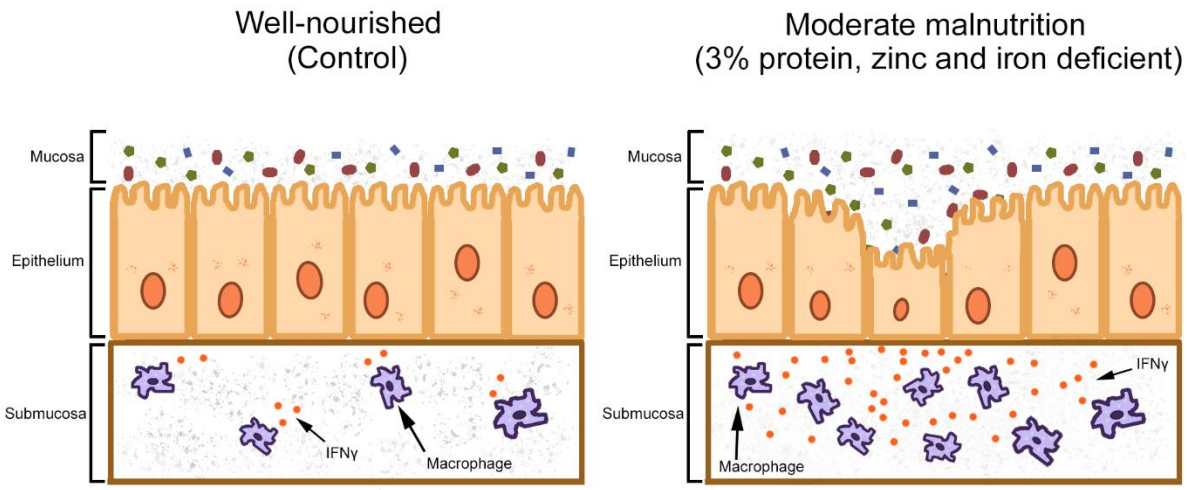


Figure 11. Schematic illustration of gut epithelial damage occurring due to *Plasmodium chabaudi* infection in the gut. Inadequate protein intake and deficiencies in iron and zinc can disrupt the epithelial barrier integrity, which is exacerbated by *P.chabaudi* infection, and can result in inflammation. Macrophages are a key mediator of gut epithelial integrity and response to infection. During moderate malnutrition and *P.chabaudi* infection, macrophages release excess inflammatory IFNγ which causes moderate epithelial disorganization and damage. This results in gut leakage and intestinal shortening.

Furthermore, these results paint an interesting dynamic occurring between malaria and moderate malnutrition. Previously, the immunological effects that malaria has in the intestines was little desired and often overlooked, despite the occurrence of severe gastrointestinal symptoms during infection (151). In this study, we successfully showed the damaging immunological effects that occur, and how malaria infection influences gut innate immunity during moderate malnutrition. Since our diet mimics deficiencies commonly found in parasite endemic regions, these data suggest that individuals in these areas are at high risk for damaging inflammation in their intestines. This can spell disaster, as damaging inflammation can further reduce nutrient adsorption already caused by malnutrition and ultimately lead to death (152). Our research has brought this often-overlooked issue to attention, and highlights the devastating effects of malaria and malnutrition in the gut.

Future studies will aim to elucidate this relationship, increasing scientific and public knowledge for future investigators. Focuses will be on analysis of tight junction proteins with methods such as western blotting and RT-PCR, which will allow us to see if there is dysfunction in tight junction protein transcription and expression. When there is dysfunction in tight junction proteins gut leakage can occur, which we have seen in this study. However, dysfunction can also result in bacterial translocation (153), which can cause harm to other organ systems, such as the liver and kidney (154, 155). Such studies may help define causes of acute liver and kidney damage associated with malaria infection as seen in malaria endemic regions (156).

Secondly, we will aim to identify shifts in microbial communities during malaria and malnutrition. It is known that diet heavily influences the gut microbiome and alters susceptibility to disease and infection (157-159). To investigate these shifts and their impacts on disease severity, DNA extractions from stool samples will be performed. This will allow us to amplify rRNA via PCR, which can be used for sequencing. Differences in microbial communities towards or away from inflammatory phenotypes and genotypes will help to further outline the damaging effects of malaria and malnutrition.

Lastly, we want to determine what nutrient deficiency primarily causes the inflammation and resulting gut leakage we saw during our study. We will utilize new customized diets that are only deficient in either zinc or protein, with or without lowered protein content. This will allow us to correctly identify which nutrient has the greatest implication on morbidity associated with malaria infection. We will also aim to study the effects of severe malnutrition during malaria infection. Diets will mimic kwashiorkor and/or marasmus, which are commonly found in Sub-Saharan Africa (160). This

investigation will enable us to more closely model the most common form of malnutrition that is occurring in malaria endemic regions (161).

Chapter 5

CONCLUSIONS

Malnutrition is implicated to have dire consequences on those affected; this is especially true when considering coinfections and the immunology behind it. Our data suggests that moderate malnutrition tends to lower the numbers of $CD11b^+$, $CD11b^+CD11c^+$, and $CD11c^+$ innate immune cells in the small intestine, large intestine, and cecum. Furthermore, we can conclude that deficiencies in iron and zinc with inadequate protein intake during malaria infection causes increased MHCII expression by macrophages, which results in increased IFNγ secretion. As a result, there is increased yellowing of the gut tissues, gut shortening, intestinal permeability, and epithelial disorganization associated with the inflammatory environment. Moderate malnutrition also caused increased weight and body temperature loss during malaria infection. Collectively, these results indicate that moderate malnutrition plays a deleterious role in physiology and morbidity during malaria infection, which could ultimately lead to increased mortality.

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Vita

Noah Joseph Murr was born in Winston-Salem, North Carolina in 1995. Growing up, he was always interested in the intricacies medicine, physiology, and disease, reading his dad's weekly medical journals that he got in the mail. This fascination originally led him towards an interest in medicine and wanting to work in the health care field. At the age of 16 his mother was diagnosed with terminal pancreatic cancer, the resulting stress caused him to develop ulcerative colitis. His drive to understand how these things happen and how he could make an impact on their outcomes drove him to pursue a master's degree in Cellular and Molecular Biology with a concentration in Immunology at Appalachian State University.

Noah's dedication in the field of immunology proved to be a worthy ambition. Through his hard work he accumulated an ability to critically think about aspects of disease states and the influence of diet, which will eventually lead to his first author publication titled "*Plasmodium chabaudi* infection Alters Gut Morphology and Mucosal Innate Immunity in Moderately Malnourished Mice". He has attended the North Carolina American Society for Microbiology conference in 2019 and 2020 as a travel award winner, poster presenter, and oral presenter. He will also be presenting part of his thesis data at the American Association of Immunologists 2021 annual conference in May. In addition, he has written several competitive university sponsored research grants for funding for his projects. Noah's passion for his work and his desire to mentor and teach others has inspired him to pursue a terminal degree in immunology and/or cancer biology. One day, Noah hopes to lead ground-breaking research in the field of immunology with a focus on the development of autoimmune disease and drug development.

In his free time, believe it or not, Noah enjoys reading immunology papers focused on autoimmunity, with specific interests in ulcerative colitis. He is also an avid body builder who believes proper diet and exercise are essential for a well-balanced life and prevention of disease. Lastly, he also enjoys spending time with his fiancé and close friends, whether it be getting together to cook or go on hikes.