MODERATE MALNUTRITION INCREASES NK CELL POPULATION AND INFLAMMATORY ACTIVATION IN THE SPLEEN

Thesis by:

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Abstract

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Malaria is a parasitic disease caused by organisms of the genus *Plasmodium.* The most recent world malaria report detailed 241 million cases of *Plasmodium* infection with 627,000 deaths in 2020. The success of the parasite is tied to its evolving drug resistance in addition to anthropogenic effects related to underdeveloped nations, such as political turmoil, lack of medical access, and malnutrition. Malnutrition significantly impacts the immune system through a variety of mechanisms, with most specifics unknown. The severity of malaria disease is drastically affected by malnutrition, increasing disease severity and morbidity. Despite large geographic overlap, the specific effects of immunodeficiency due to malnutrition during the malaria defense are still unknown. Since NK cells are vital to a functional early malaria response along with an increasing role in parasite defense, I sought to understand the effects of malnutrition on NK cells during *Plasmodium* infection. I hypothesized that NK cell percentage and functionality would be diminished in malnourished mice during *P. chabaudi* infection.

For these studies, I focused on moderate malnutrition, which more closely mimics the diet in sub–Saharan Africa and is attributed to more deaths than severe malnutrition. C57BL/6 mice were fed a diet consisting of 3% protein deficient in iron and zinc, and moderate malnutrition was confirmed by an approximate weight loss of 10%, compared to age-matched

well-nourished controls fed a 17% protein content diet supplemented with iron and zinc over 4 weeks.

To investigate the response of NK cells to infection with *Plasmodium chabaudi* under conditions of moderate malnutrition, circulating blood leukocytes and splenic lymphocytes were collected. NK cells were identified by the expression of NK1.1 and analyzed for maturity by the presence of CD27 and CD11b. Functionality of NK cells was measured by the production of inflammatory cytokines IFN-γ and TNF-α and the cytotoxic granules perforin and granzyme B. I observed that during *P. chabaudi* infection, NK cell maturation was elevated in the moderate malnourished mice, as indicated by increased expression of CD11b and IFN-γ. NK cells in moderate malnourished mice also produced more perforin, but granzyme B levels were unaffected by diet. Furthermore, consistent with NK cell results, B and T cells from moderate malnourished mice were more activated than in well-nourished. This elevation in activation due to diet across all splenic lymphocyte groups in the malnourished mice indicate that the nutritional status has a significant impact activation. This elevated activation could be due to intrinsic effects on the cells due to diet; however, it could also be due to increased bacterial translocation form the gut which has been found to happen in this diet. This heightened activation could play a role in the elevated rates of severe malaria cases in malnourished areas.

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I would like to thank my mentor Dr. Michel Opata, who took me into his lab after others had said no. His unwavering guidance over the course of my graduate program, encouragement, dedication to my success, and belief in my abilities empowered me to successfully reach this stage in my academic career. I would also like to thank Dr. Maryam Ahmed for her expert guidance over the past semester. I would like to thank my other committee members Dr. Andrew Bellemer and Dr. Kevin Zwetsloot for their support and belief in me throughout this experience. I would like to thank my graduate mentors Noah Murr and Emily Xiong for molding me into a cell biologist. I would also like to thank my fellow lab mates who made the 20 hours experiments much more enjoyable: Tyler Olender, Nicole Warnick, and Robert Onjiko. Most importantly, I would like to thank my family and friends without whom I would be nothing. Joining Team Malaria was one of the best decisions of my life. Thank you.

Dedication

Ridley J. Gros (6/9/1941-8/16/2013)

James A. Erny (8/29/1943-10/2/2021)

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CD: cluster of differentiation

cPDD: protein deficient diet/ moderately malnourished uninfected

CSP: circumsporozoite protein

Ctrl.: control

cWND: well-nourished uninfected

EAEC: enteroaggregative *Escherichia coli*

ICS: Intracellular cytokine staining

IFN-γ: interferon gamma

IL: interleukin

ILC: innate lymphoid cell

Mal.: malnourished

MHC II: major histocompatibility complex II

NK: natural killer

PBMCs: peripheral blood mononuclear cells

PDD: protein deficient diet/moderately malnourished infected

PEM: protein energy malnutrition

P.i.: post-infection

SEM: standard error mean

sFGL2: soluble fibrinogen-like protein 2

STAT: signal transducer and activator of transcription

TNF-α: tumor necrosis factor alpha

Treg: regulatory T cells

WND: well-nourished infected

CHAPTER 1

Introduction

Malaria

Malaria is a parasitic disease caused by organisms of the genus *Plasmodium.* Of this genus, only 4 species are known to infect humans: *P. ovale, P. malariae, P. vivax,* and *P. falciparum,* which accounts for over 90% of cases (1, 2). Malaria disease in humans is estimated to go back at least 10,000 years (3), with documented records dating back to 2700 B.C. in China (4). The most recent world malaria report detailed 241 million cases of *Plasmodium* infection with 627,000 deaths in 2020 (5), which are respective 5% and 53% increases from 2019. *Plasmodium*'s success is tied to its evolving drug resistance in addition to anthropogenic effects related to underdeveloped nations such as political turmoil, lack of medical access, lack of education, and malnutrition (2, 5).

The *Plasmodium* lifecycle occurs in 2 distinct phases, sexually in the genus *Anopheles* mosquitos then asexually in the mammalian host (6). When a female *Anopheles* mosquito takes up a blood meal from a *Plasmodium*-infected host, *Plasmodium* gametocytes are also taken up as byproduct. Once in the mosquito midgut, the gametocytes form gametes, leading to the creation of a zygote (7). Following zygote formation, meiosis and genetic recombination occur leading to the transition to an ookinete (8). The parasite is now motile as an ookinete and invades the midgut epithelium of the mosquito in order to transform into an oocyst (6). Over the course of 10-12 days, thousands of sporozoites develop within the oocyst which eventually bursts with the sporozoites traveling to the salivary glands of the mosquito via the hemolymph (6, 9). Once in

the salivary gland, the sporozoites are ready to infect a human host during the mosquito's next blood meal.

The asexual phase of the *Plasmodium* lifecycle begins once inside the human host. On average, a single blood meal leads to the inoculation of slightly under 100 individual sporozoites (10). Sporozoite inoculation tends to last a few hours. Once in-host, the sporozoites must migrate to blood vessels, avoid the immune system, and reach the hepatocytes to continue development (11). Of the initial inoculation, approximately 20% is drained to the lymph nodes allowing for the induction of an immune response, with only ~5% reaching the hepatocytes while the remaining die off (11, 12). Sporozoites are able to traverse the host tissues and capillary network then reach the liver and invade the hepatic parenchyma, where they eventually invade individual hepatocytes (13). This invasion is carried out via the proteolytic cleavage of the circumsporozoite protein (CSP). A single sporozoite reaching the hepatic parenchyma and managing to invade a hepatocyte can lead to full malaria disease (14). Now in the liver stage of infection, the sporozoite begins asexual reproduction with schizogony, which is nuclear division without cell division (13). These schizonts develop within a parasitophorous vacuole, protecting it from the host immune system (15). In *P. yoelii* infection, a single inoculation can result in over 29,000 merozoites in slightly over 2 days (16).

Following schizogony, the hepatocytes will rupture leading to the invasion of the bloodstream by the merozoites, this begins the blood-stage portion of the asexual phase (17). Following contact with an erythrocyte, a merozoite will bind to the surface with low affinity, followed by reorientation of the merozoite in relation to the erythrocyte in order to facilitate invasion (18, 19). Once properly oriented, the merozoite forms a tight junction with the erythrocyte membrane and begins to invade, powered by the parasite's actin-myosin motor (20).

As the parasite invades the erythrocyte, it sheds its merozoite coat and creates a parasitophorous vacuole (19). This begins the ring stage of the parasite in which the parasite seems to pause for a few hours (21). Approximately 15 hours following invasion, the metabolic and biosynthetic activity in the cell increases as the parasite grows into a trophozoite. At this point the parasite is fully reliant upon glycolysis for energy and grows to occupy approximately one third of the cell (22). Once grown, the parasite enters the schizont stage, producing 20-30 merozoites before rupturing to infect more erythrocytes. The invasion to rupture process of the erythrocyte takes 48 hours and is responsible for the cyclical nature of malaria symptoms (21). Following rupture from erythrocytes, some of the merozoites differentiate into gametocytes which have no more function in the mammalian host, but can be picked up during a blood meal and continue the lifecycle in the mosquito (23).

Malnutrition

Malnutrition is defined by the World Health Organization (WHO) as "the cellular imbalance between the supply and demand for nutrients and energy by the body to ensure growth, maintenance, and specific functions" (24). Malnutrition is considered to be the largest acquired immunodeficiency in the world and contributes to many infection-related deaths (25). Protein energy malnutrition (PEM) is a type of malnutrition lacking in protein and calories and is a broad term used to describe multiple subtypes of other malnutritions (25) . Nutrient deficiencies often accompany malnutrition, primarily iron and zinc; however, specific assessment of nutrient deficiencies for each case is rarely performed (25). Malnutrition is scored upon a group of factors such as weight-for-height (WFH), WFH z score (WHZ), or height-for-age (HFA), and HFA z score (HAZ). Based on these scores, an individual can be classified as severely, moderately, or mildly malnourished if below the standard value. Moderate malnutrition is defined in an

individual who is between 2-3 standard deviations below the expected value scores (24). Malnutrition has been found to increase mortality, with severe malnutrition having the highest mortality rate of the three classifications (12 times more compared to normal) (26).

A 2010 study determined that over 925 million people suffer from undernourishment in the world and approximately one-third of disease burden could be eliminated if this was addressed (27). Mortality and malnutrition have been linked and data estimates attribute undernutrition as a significant contributor to 45% of deaths in individuals under the age of five (28). However, the WHO estimates 83% of all malnutrition-associated deaths are found to occur in individuals with moderate-to-mild malnutrition since infections are the primary cause of death in these cases and susceptible individuals succumb before becoming severely malnourished (29). The relationship between infection and malnutrition has been termed "bi-directional" (25) as each condition is able to influence the other. For example, the innate arm of the immune system responds to inflammation (infection or other cause) with the acute-phase response. The response leads to increased energy expenditure due to high fever accompanied with low appetite, netting a negative energy balance (30, 31). This leads to a negative feedback loop for malnourished individuals in which malnourishment increases disease severity and in turn the infection causes appetite suppression leading to increased malnourishment.

Micronutrient deficiencies lead to moderate malnutrition, and each nutrient plays a significant role in maintaining the host defense, but also is required by the infectious agent to proliferate and continue the infection. My study focuses on iron and zinc deficiencies which are the two most prevalent micronutrient deficiencies in the world, accompanied by low protein dietary content (25).

Iron is the most prominent micro-nutrient deficiency and the leading cause of anemia in the world (32-34). Iron has significant roles in immune cell growth, differentiation, activation, and cytokine function (35). There are conflicting reports on the effects of iron supplementation on infection as iron deficiency increases susceptibility to infection (33), but iron supplementation has been found to also increase susceptibility to both malaria (36) and tuberculosis (37). While iron supplementation has been shown to increase malaria susceptibility, iron deficiency has been found to do the same due to degradation of ferroportins. Loss of ferroportins traps more nutrients for the parasite in the erythrocyte, allowing it to thrive (38). Deprivation of iron from microbes is a part of the innate defense, therefore over supplementing iron can allow for this defense to fail and give chance to the pathogen to thrive (33).

While not as prevalent as iron deficiency, zinc deficiency is responsible for more deaths annually (27, 39). Zinc is vital for many cellular processes including stabilizing DNA, RNA, and ribosomal structure as well as regulating T cell maturation (25, 39). Zinc deficient mice have depleted numbers of both immature and mature B cells in the bone marrow (40). Decreases in thymic hormones which regulate maturation of T cells were also found in zinc deficient mice (41, 42). In addition, zinc deficiency has been linked to thymic atrophy, lymphopenia, and reduced CD4/CD8 ratio (25). Mice deficient in zinc and infected with enteroaggregative *Escherichia coli* (EAEC) had increased virulence and impairment of the host inflammatory response (43). These mice also had an expansion of IL-12-producing ILC2s, leading to an increased protection from helminths (44).

Protein deficiencies have been shown to cause increased thymocyte apoptosis (45, 46). Autopsies of malnourished children who died of infection showed significant thymus atrophy (47). Mechanisms for the increased apoptosis have been attributed to reduced leptin levels, as

leptin has been found to have a critical role in thymus mass maintenance (46), and elevated glucocorticoids (48) associated with malnutrition. These findings coincide with data showing increased mortality and morbidity to *Plasmodium falciparum* infection in children (49, 50). Protein deficiency caused a decrease in spleen size in mice as well as reductions in all lymphocyte numbers (51). These changes were correlated with increases in IL-10 and STAT-3 protein expression and deceases in IL-2 and STAT-1 expression. Mice on a low protein diet and infected with influenza virus had larger viral burdens and mortality resulting from impaired T cell and B cell responses (52). Using an adoptive transfer model, some scientists have shown that virus specific $CD8^+$ T cells primed in protein deficient mice function better when transferred to well-nourished mice, indicating that a malnourished environment contributes to the impaired functionality of the T cells rather than an intrinsic change due to malnutrition (53).

To understand how moderate malnutrition impacts immune cell populations during a chronic infection, an appropriate infection model must be chosen. Due to the geographic endemic overlap with moderate malnutrition, as well as its significant disease burden, malaria was chosen to be used in these investigations. While malaria pathogenesis has been extensively studied, the influence of moderate malnutrition on immunity to the disease has not been well explored, making this infection a good model to study the effects of micronutrient moderate malnutrition on innate lymphocytes during an active immune response.

Immunity to malaria and malnutrition

There have been some studies on the effects of malnutrition and *Plasmodium* infection, but most of these have not sought to determine causality of malnutrition on malaria disease exacerbation (54). As with most diseases, malnutrition increases the chance of severe malaria (55). Repeated *Plasmodium* infections in children have been found to contribute to malnutrition

status by causing growth faltering and inhibiting weight gain (56, 57). However, there is conflicting data on the effects of micronutrient deficiencies. As mentioned previously, iron supplementation has been found to increase disease susceptibility to malaria (36).

Innate immunity to malaria

Natural killer (NK) cells are innate lymphocytes. These cells are essential for the production of cytokines, such as IFN-γ and TNF-α, which are important for inflammatory responses and cell signaling. NK cells also have granules that contain perforin and granzyme B, which are cytotoxic proteins that facilitate NK cell-mediated killing of target cells and microorganisms (58). These cells are effective at fighting tumors and viruses and have recently been found to be anti-parasitic (59, 60). In addition to target cell killing and cytokine production, NK cells also contribute to immune cell recruitment and activation.

NK cells initially develop within the bone marrow, acquiring the surface receptor NK1.1 before migration in mice (61, 62). The maturity of splenic NK cells in C57 mice can be determined using the expression of CD27 and CD11b (63). CD27 is acquired near the end of the bone marrow stage of development and maintained through the initial stages of development outside of the bone marrow (64, 65). A linear model of maturation based upon induction of activation by NK cells has been characterized by the progression from $CD27^{-}CD11b^{-}$ → $CD27^{+}CD11b^{-}$ → $CD27^{+}CD11b^{+}$ → $CD27^{-}CD11b^{+}$ (61, 63-65). This increase in maturity is correlated with a decrease in proliferative ability and an increase in effector function determined by gene expression (63). Since micronutrient deficiencies can impact population dynamics, determining the effects on maturation was deemed important.

Success against malaria hinges on a functional and robust IFN-γ response (66). NK cells were found to be the dominant producers of IFN-γ during the early stage of infection (0-48h), with T cells predominating after 48h (67). Infected mice lacking NK cells were unable to survive the infection, while a robust NK cell response can lead to spontaneous resolution of *Plasmodium* infection (68). Activation of the IFN-γ NK cell response is induced by IL-2, which is secreted by the $CD4^+$ T cells, indicating an inflammatory network crosstalk to combat blood-stage malaria (67). NK cells directly bind and kill parasitized erythrocytes via cytotoxic granules, while a reduction in NK cells leads to a significant increase in parasitemia (69). Blood-stage *Plasmodium* parasites evade the immune response in order to survive. One such mechanism is the stimulation of regulatory T (Treg) cells to produce soluble fibrinogen-like protein 2 (sFGL2) (70). This molecule inhibits macrophage recruitment ability and in turn decreases the amount of NK cells. This decreased recruitment of NK cells leads to a decrease in IFN-γ production, thus benefitting the parasite (70).

PEM reduces the activity of NK cells (71), but has no impact on the number of NK cells. NK cells in both young and old mice are decreased in number and have impaired functionality in a calorie-restricted diet when infected with influenza, compared to ad libitum fed mice (72). Protein deficiency alone has been shown to decrease splenic NK cells (73). In a similar model without infection, NK cell populations were skewed, with a significant decrease in mature NK cells and an increase in $CD127^+$ NK cells in the spleen and lymph nodes (74). These $CD127^+$ NK cells primarily produce cytokines as opposed to cytotoxic granules, causing a skew in NK cell population functionality (74). Calorie restriction was also found to increase the cytokine potential of the $CD127^+$ NK cells, but decreased the functionality of the $CD127^-$ NK cells (74).

Adaptive immunity to malaria

Following the innate arm of the immune system, is the adaptive arm. Made up of the B and T lymphocytes, this response is specific to the pathogen. A proper functioning adaptive immune response is vital to pathogen clearance due to its ability to specifically target the infectious agent. The adaptive arm is also responsible for forming a memory to the pathogen; however, immune memory to *Plasmodium* infection is unable to fully develop in humans (75). Vaccinations are dependent upon the functionality of the adaptive immune system to create this memory response. Malnutrition has profound effects on the adaptive response; however, the mechanistic understanding of how these cells are specifically affected is lacking.

Both B and T cells function in the defense against malaria; however, T cells, primarily $CD4^+$ T cells, are the primary lymphocyte against malaria (76). $CD4^+$ T cells function in immune cell activation, engaging in crosstalk with other immune cells to drive the response (67). These cells lead to an inflammatory response, with T cells being the primary IFN-γ producing cells after 48 hours post-infection (p.i.). Loss of T cells is fatal in murine malaria using an athymic model, while deprivation of B cells can be compensated for by the T cells that are present (77, 78). B cells function in antibody synthesis and have been shown to create an incomplete memory response to malaria after repeated *Plasmodium* infection, with antibodies from adults in endemic regions imparting an increased, but incomplete, defense in both children and non-endemic individuals (76, 79).

As for the effects of malnourishment on adaptive immune cells, children who were malnourished and hospitalized with bacterial infections showed no difference in the number of $CD4^+$ or $CD8^+$ T cells, compared to well-nourished bacterial-infected hospitalized children (80). While the total numbers of $CD4^+$ and $CD8^+$ T cells were analogous to controls, specific subsets

of T cells were found to be reduced, such as $CD4^+CD62L^-$ and $CD8^+CD28^-$ effector T cells and $CD4^+CD45RO^+$ memory T cells (81, 82). Some of the effects on T cells may be a second-hand effect of malnutrition as previously stated. Decreased antigen presentation due to malnutrition affects T cell differentiation and specific effector function. Evidence of this can be seen by a reduction in key cytokines in the peripheral blood mononuclear cells (PBMCs) for T_H1 differentiation and function in malnourished children with bacterial infections (83). This reduction in T_H1 cytokines was accompanied by an overexpression of T_H2 cytokines, impacting the immune response (84).

In this study, I aim to determine the effects of moderate malnutrition on NK cell population, maturity, and functionality during *P. chabaudi* infection. I hypothesize that NK cell percentage, maturity, and functionality would be diminished in malnourished mice during *P. chabaudi* infection. I believe this will occur due to the previously observed effects of immune dysfunction attributed to the singular effects of low protein, iron deficiency, and zinc deficiency, which are all combined in my model.

CHAPTER 2

Materials and Methods

Mice and parasite

Adult C57BL/6 mice were obtained from Harlan labs and a breeding colony maintained in my 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 20-10). Male mice aged 8-14 weeks were used for experiments with consistency in both the malnourished and control groups as male mice have increased pathology to *P. chabaudi* compared to females attributed to testosterone.

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 a sublethal dose of $1x10^5$ *P. chabaudi* intraperitoneally and other groups of control and malnourished mice were left uninfected to serve as negative controls. For splenocyte analysis, all mice were sacrificed 3-, 5-, or 9-days post infection (p.i.) via CO_2 desiccation with adherence to my approved IACUC protocol 20-10. The spleens were harvested and placed in ice-

cold PBS supplemented with 2% FBS and 3mM HEPES (Atlanta Biologicals S11150H, Flowery Branch, GA).

Preparation of blood for flow cytometry

For whole blood analysis, mice were tail snipped for blood collection at days 0, 2, 5, 7, and 9 p.i. 4 μL of blood was collected into Eppendorf tubes with 50 μL FACS buffer and 2 μL EDTA. Fluorescent antibodies were directly added to the samples and incubated for 30 minutes in the dark at room temperature. The incubation included: FITC-conjugated anti-CD11b (Biolegend 101206), PE-conjugated anti-NK1.1 (Biolegend 108708), PE-Cy7-conjugated anti-CD27 (Biolegend 124215), and APC-conjugated anti-CD4 (Biolegend 116014). The VL1 channel of the Attune NxT flow cytometer functioned as a fifth target by allowing for differentiation of leukocytes from RBCs based on violet absorbance and light-scatter. After incubation, samples were filtered into 5 mL polystyrene tubes with 3.5 mL of FACS buffer then collected on an Attune NxT flow cytometer (ThermoFisher, Pleasanton, CA).

Preparation of spleen tissues for flow cytometry

The spleens were cleaned by manually removing all residual adipose residues with scissors and forceps. The spleens were then mashed through a 70μm cell strainer with 3 mL of ice-cold PBS supplemented with 2% FBS and 3mM HEPES. The single cell solution was centrifuged at 1,200 rpm at 4°C for 5 minutes and treated with RBC lysis for 2 minutes to eliminate red blood cells followed by addition of 5mL of PBS to stop the lysis. The samples were centrifuged again under the same conditions and the pelleted cells were resuspended in 1mL FACS buffer for cell counting. The cells were counted on a microscope using a hemocytometer

at a 1:200 dilution with PBS and trypan blue. $3x10^6$ cells were aliquoted out from each sample into a 24-well plate for surface staining & incubation for intracellular cytokine determination.

Flow cytometry (surface & intracellular cytokine staining of spleens)

For surface & intracellular 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 1µL of cell stimulation cocktail containing the golgi blocker Brefeldin A. The 24-well plate was then placed into a HERAcell 150i incubator set at 37°C and 5% CO_2 for 5 hours. After incubation, the cells were collected into 5-mL round-bottom polypropylene tubes and centrifuged at 1200 rpm at 4°C for 5 minutes, then washed with FACS buffer. The cells were incubated with Fc block in the dark at 4°C for 20 minutes to ensure blockade of Fc receptors and enhance specific binding. Fluorescent antibodies were used against surface markers to label targets of interest: PerCP-conjugated anti-CD11b (Biolegend 101230), PE-conjugated anti-NK1.1 (Biolegend 108708), and BV605-conjugated anti-CD27 (Biolegend 124249) for the innate panel & FITC-conjugated anti-MHC II (Tonbo Biosciences 35-5321-U100), PerCP/Cy5.5-conjugated anti-CD62L (Biolegend 104431), PE-conjugated anti-CD86 (Tonbo Biosciences 50-0861-U100), PE-Cy7-conjugated anti-CD4 (Tonbo Biosciences 60-0042-U025), APC-conjugated anti-CD19 (Biolegend 115512), and Alexa 700-conjugated anti-CD44 (Biolegend 103026) for the adaptive immune cell panel at 4°C 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 incubated with Fc block in the dark at 4°C for 20 minutes. The samples were stained for intracellular

targets: FITC-conjugated anti-GranzymeB (Biolegend 372206), PE-Cy7-conjugated anti-TNF-α (Biolegend 506324), APC-conjugated anti-Perforin (Biolegend 154304), and BV421-conjugated IFN-γ (Biolegend 505829) for the innate immune cell panel & BV421-conjugated IFN-γ (Biolegend 505829) and BV605-conjugated anti-TNF-α (Biolegend 506329) for the adaptive immune cell panel. Finally, the cells were resuspended in 500 μL of FACS buffer and filtered into 5 mL polystyrene tubes for analysis on the Attune NxT flow cytometer.

Data analysis

Raw data collected from the Attune NxT flow cytometer were analyzed using FlowJo software (Ashland, OR). Data were formatted in Microsoft Excel. Graphs were made in Prism GraphPad (San Diego, CA). Data were analyzed in IBM SSPS Statistics for significance using a two-way ANOVA. Normality test, combined effects of malnutrition and infection, and main effects of malnutrition and infection were determined. Significance was defined by a *p*-value less than 0.05 determined by two-way ANOVA with Tukey's test for post-hoc.

CHAPTER 3

Results

P. chabaudi *infection increases circulating leukocytes including NK cells by day 9 post-infection*

To determine the effects of moderate malnutrition with deficiencies in iron, zinc, and low protein content on the immune system using a chronic infection model, I subjected mice to a 4-week diet regimen followed by *P. chabaudi* infection that lasted for nine-days, correlating with the peak of malaria disease. Because lymphocytes are essential contributors to protection against malaria (85), I sought to investigate the kinetics of the leukocyte distribution in the blood over the first nine days of the infection. Due to differences in violet light scatter between red and white blood cells, I was able to use a gating strategy in which I isolated leukocytes from whole blood without having to stain (**Figure 1A**). I observed that circulating leukocytes were the same early during infection, and slightly increased by day 7. By day 9 p.i, there was a significant increase of total leukocytes in both the infected well-nourished (WND) and malnourished (PDD) groups (**Figure 1B**).

I next measured percent NK cells and observed that they were high at day 2 in all groups including the infected and uninfected groups. This population decreased by day 5 then started increasing again at day 7 p.i, and by day 9 p.i, the proportions of this cell population were significantly increased in the blood of the infected groups alone and not the uninfected groups. Importantly, there was no difference between the proportions of these cells between the infected well-nourished or the moderately malnourished groups (**Figure 1C**). I observed no differences in circulating $CD3^+$ or $CD4^+$ cells (data not shown). Upon further investigation of the leukocytes (**Figure 1A**), I observed 3 different populations L1, L2 and L3, which were differentiated based

on their granularity (**Figure 1D**). L1 was the first population based on SSC and had the most cells at D0 followed by L2 then L3. As the infection progressed the L1 population decreased especially in the infected group while the L2 population increased in the infected groups (**Figures 1E & 1F**). The L3 population was consistent across all treatments at all days and was by far the smallest population with no real changes during the course of the 9 days of infection (data not shown). Taken together, this data suggests that there is a leukocyte population that is increased early in the infection and that may be essential early in the infection before the adaptive immunity is activated to enhance protection. Therefore, I focused on two time points for the rest of the study (day 3 and day 9 post-infection).

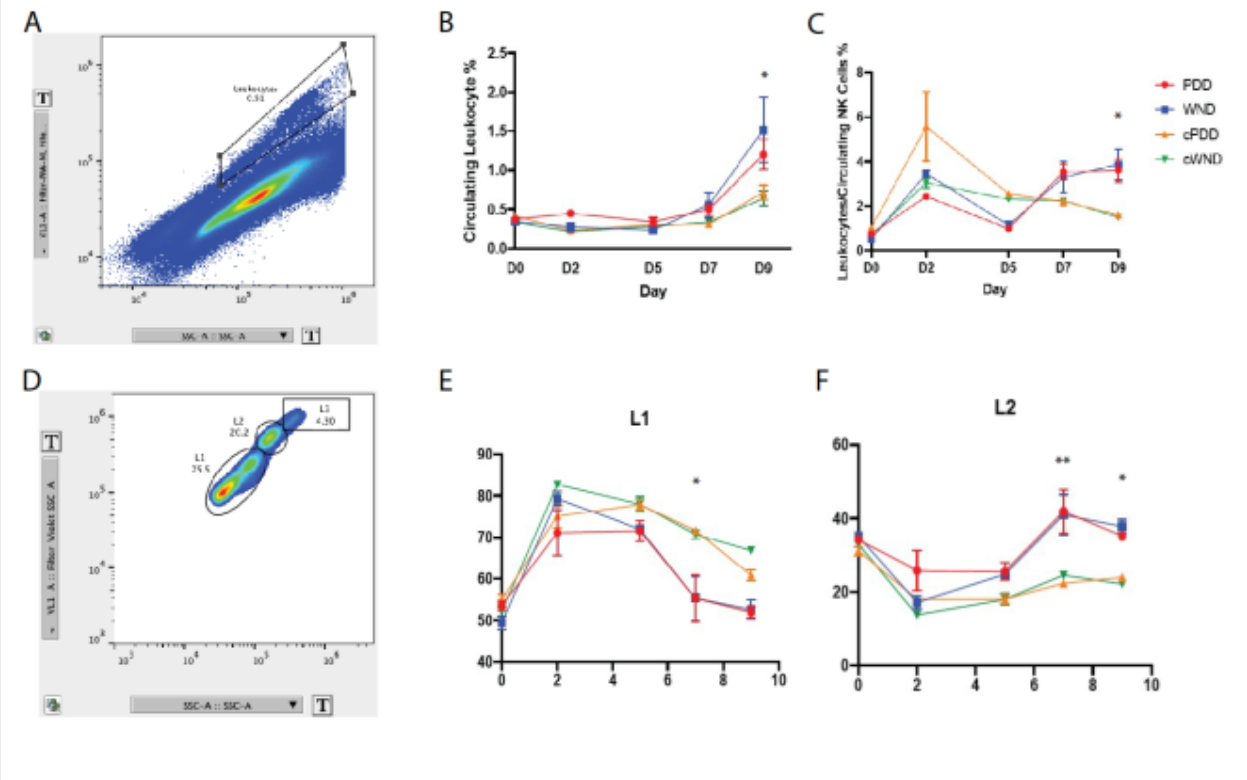

Figure 1: *P. chabaudi* infection increases circulating NK cells by day 7 and circulating leukocytes by day 9. Mice were infected with 1x10^5 iRBCs after 4 weeks of feeding on the experimental diet. The infected and uninfected controls had blood drawn via tail snip over the course of infection for analysis using flow cytometry. Graphs showing A) Leukocyte Gate, B) Circulating Leukocytes, C.) Circulating NK Cells, D.) Leukocyte Population Gates, E.) L1 Leukocytes Population, F.) L2 Leukocytes Population. All data are shown as means +/- SEM, n=3-5 male mice per group, representative of 2 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. PDD=Infected moderately malnourished, WND=infected well-nourished, cPDD=control moderately malnourished, cWND=control well-nourished.

Moderate malnutrition increases the proportions of lymphocytes and mature NK cells in the spleen during* P. chabaudi *infection.

Since NK cells are known to be an important early lymphocyte contributor to protection against malaria (86), I sought to investigate splenic NK cells, since malaria is a blood disease, and the spleen is the essential lymphoid organ for blood borne pathogens. At day 3 of infection, moderately malnourished mice had an increased percentage of splenocytes, but there was no difference in the uninfected groups (**Figure 2A**). As for the NK cells, although there was a trend, there was no significant differences for interaction (p=0.065), but main effects were significant for both infection (p=0.04) and diet (p=0.008) (**Figure 2B**). This indicated that there is a elevation in the NK cell percentage in the malnourished mice compared to the well-nourished, as well as in the controls elevated compared to the uninfected. However, this could just be a result of the elevated uninfected malnourished, as the other three groups are similar in value.

The maturation of NK cells can be determined by the expression of surface markers CD27 and CD11b, with the progression being double negative→CD27 single positive→double positive→CD11b single positive (63, 74). Well-nourished mice had a significantly higher percentages of immature NK cells ($NK1.1^{+}CD27^{-}CD11b^{-}$) determined by the main effect (p=0.028) with no impact for infection. At this early stage, the infected moderate malnourished mice showed higher early mature ($NK1.1^{+}CD27^{+}CD11b^{-}$) cells than the other groups, with significant main effect for infection (p=0.021) and trending for diet (p=0.067) (**Figure 2D**). The most mature NK cell populations ($NK1.1^{+}CD27^{+}CD11b^{+}$ and $NK1.1^{+}CD27^{-}CD11b^{+}$) were the same in all the groups with no significant interaction or main effects (**Figures 2E&F**).

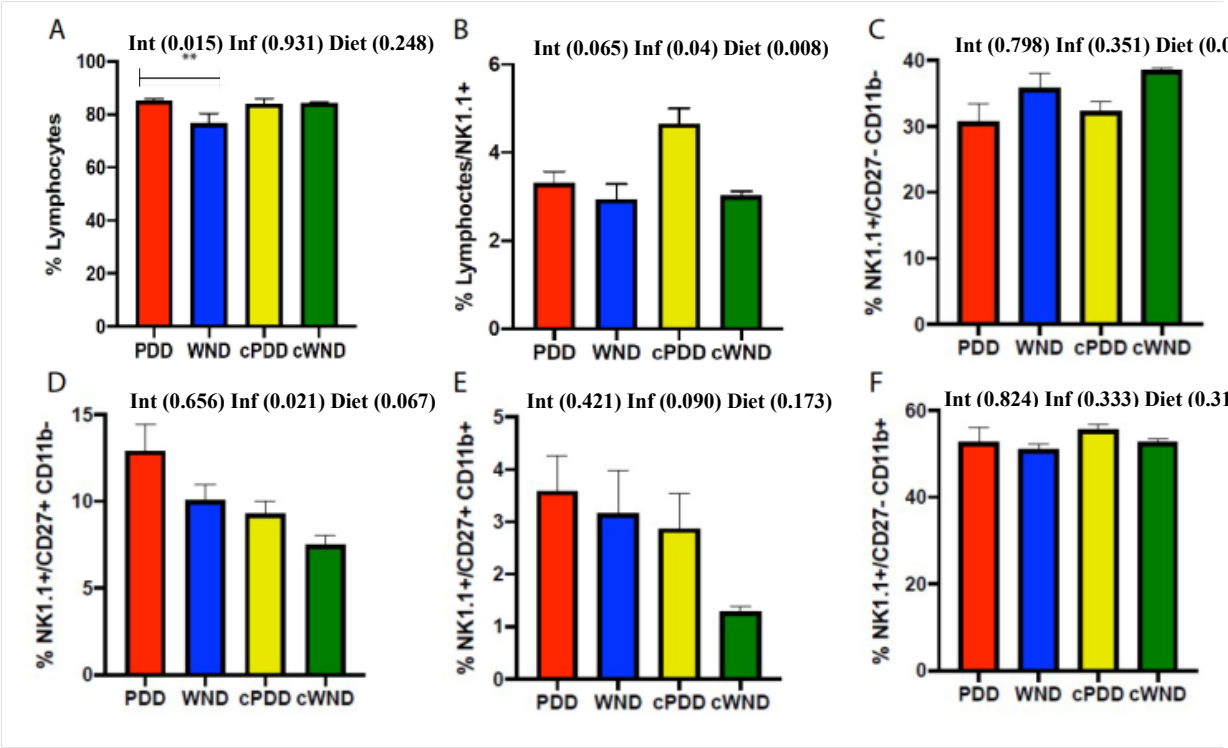

Figure 2: **Moderate malnutrition increases the proportion of splenic lymphocytes during infection and increases the maturity NK cells in the Spleen early in *P. chabaudi* infection.** Mice were infected with 1x10^5 iRBCs after 4 weeks of feeding on the experimental diet. The infected and uninfected controls were then sacrificed on day 3 post-infection for analysis using flow cytometry. Graphs showing A) Lymphocytes, B) NK cells, C-F) NK cell maturity. All data are shown as means +/- SEM, n=3-5 male mice per group, representative of 2 independent experiments. Statistical analysis was performed using a two-way ANOVA with Tukey's test. Interaction (Int) and each independent variable main effect, infection (inf) and diet (diet), p-values are above each graph. PDD=Infected moderately malnourished, WND=infected well-nourished, cPDD=control moderately malnourished, cWND=control well-nourished.

Moderate malnutrition decreases NK cell percentage and maturity during the peak of* P. chabaudi *infection

While NK cells are most important during early infection, they have been shown to be highly increased in both adults and children suffering from malaria (87). Based on my observation of higher proportions of these cells in circulation at day 9 p.i. in **Figure 1**, I wondered if a similar phenotype would be observed in the spleen. I observed significantly higher proportions of lymphocytes in the spleens of both infected and uninfected moderately malnourished mice compared to well-nourished mice at day 9 p.i, showing a significant effect of the diet (p=0.003) (**Figure 3A**). NK cell percentages were lower in the infected groups by day 9 p.i, with the malnourished mice still being elevated (p=0.002) (**Figure 3B**). Immature NK cells ($NK1.1^{+}CD27^{-}CD11b^{-}$), were significantly decreased in the uninfected malnourished mice, with significant main effects for both infection (p=0.024) and diet (p=0.015) (**Figure 3C**). CD27 expression alone was not impacted by either infection or diet and co-expression of CD27 and CD11b was very low (**Figure 3D&F)** The most mature NK cell population ($CD27^{-}CD11b^{+}$) were significantly lower in the infected mice (p=0.026) and the well-nourished mice (p=0.023) (**Figure 3E&F**).

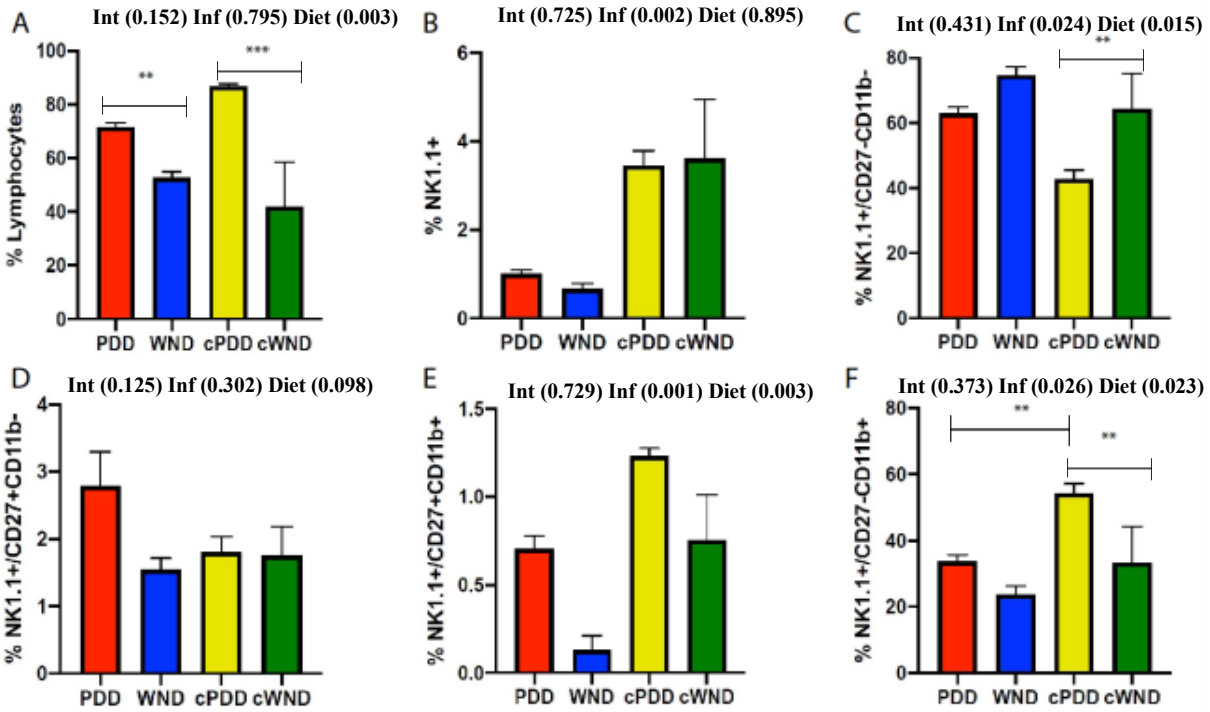

Figure 3: Moderate malnutrition decreases NK cell number and maturity during the peak of *P. chabaudi* infection. Mice were infected with 1x10^5 iRBCs after 4 weeks of feeding on the experimental diet. The infected and uninfected controls were then sacrificed on day 9 post-infection for analysis using flow cytometry. Graphs showing A) Lymphocytes, B) NK cells, C-F) NK cell maturity. All data are shown as means +/- SEM, n=3-5 male mice per group, representative of 2 independent experiments. Statistical analysis was performed using a two-way ANOVA with Tukey's test. Interaction (Int) and each independent variable main effect, infection (inf) and diet (diet), p-values are above each graph. PDD=Infected moderately malnourished, WND=infected well-nourished, cPDD=control moderately malnourished, cWND=control well-nourished.

Moderate malnutrition increases inflammatory cytokine production early in **P. chabaudi** *infection*

In addition to enumerating the splenic NK cells and determining their maturity, I also wanted to measure the functionality. Previous studies have indicated impacted functionality of NK cells based on nutrition status, so I wanted to examine the inflammatory capability at day 3 of infection (74). To determine inflammatory functionality, I measured the IFN-γ and TNF-α production by NK cells in my diet groups using flow cytometry and intracellular cytokine staining. At day 3 p.i, there was a larger percentage of IFN-γ-producing NK cells in the moderate malnourished mice compared to the well-nourished (p=0.003) (**Figure 4A)**. Co-production of inflammatory cytokines was also found to be elevated in the moderate malnourished mice (p=0.045) **(Figure 4B).** Unlike IFN-γ, neither malnutrition nor infection impacted TNF-α production (**Figure 4C**).

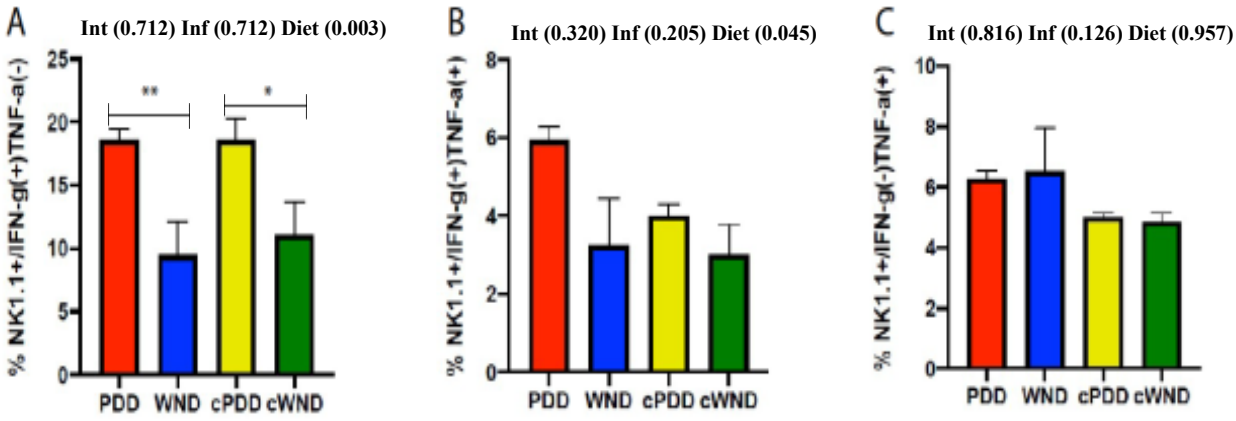

Figure 4: Moderate malnutrition decreases inflammatory cytokine production early in *P. chabaudi* infection. Mice were infected with 1x10^5 iRBCs after 4 weeks of feeding on the experimental diet. The infected and uninfected controls were then sacrificed on day 3 post-infection for analysis using flow cytometry. Graphs showing A) IFN-γ Single-Positive NK cells, B) IFN-γ, TNF-α Double-Positive NK cells, C) TNF-α Single-Positive NK cells. All data are shown as means +/- SEM, n=3-5 male mice per group, representative of 2 independent experiments. Statistical analysis was performed using a two-way ANOVA with Tukey's test. Interaction (Int) and each independent variable main effect, infection (inf) and diet (diet), p-values are above each graph. PDD=Infected moderately malnourished, WND=infected well-nourished, cPDD=control moderately malnourished, cWND=control well-nourished.

Moderate malnutrition increases perforin and decreases granzyme B production by NK cells at the peak of* P. chabaudi *infection

Following the results in **Figure 1** showing higher NK cells later in infection, I sought to determine the functionality of NK cells at 9 days p.i. in addition to 3 days p.i. To determine inflammatory functionality, I again measured the IFN-γ and TNF-α production by NK cells in my diet groups. At day 9 p.i, IFN-γ production was elevated in the infected mice ($p<0.001$), with the moderate malnutrition infected mice being slightly higher than the uninfected (**Figure 5A)**. Co-production of the cytokines was also found to be elevated in the infected mice ($p<0.001$) **(Figure 5B).** TNF-α production displayed a similar trend with the infected groups being elevated ($p<0.001$) and the moderate malnourished mice slightly higher than the well-nourished in the infected (**Figure 5C**).

In addition to inflammatory cytokine production, NK cell are cytotoxic. NK cells can synthesize and release cytotoxic granules, perforin and granzyme B, to directly kill cells (58). Moderate malnutrition was found to elevate perforin expression by day 9 p.i. ($p=0.034$) (**Figure 5D**). Granzyme B expression was nearly absent in the uninfected groups (**Figures 5E & Figure F**) while present in both infected groups ($p<0.001$, $p=0.002$) (**Figures 5E & Figure F**).

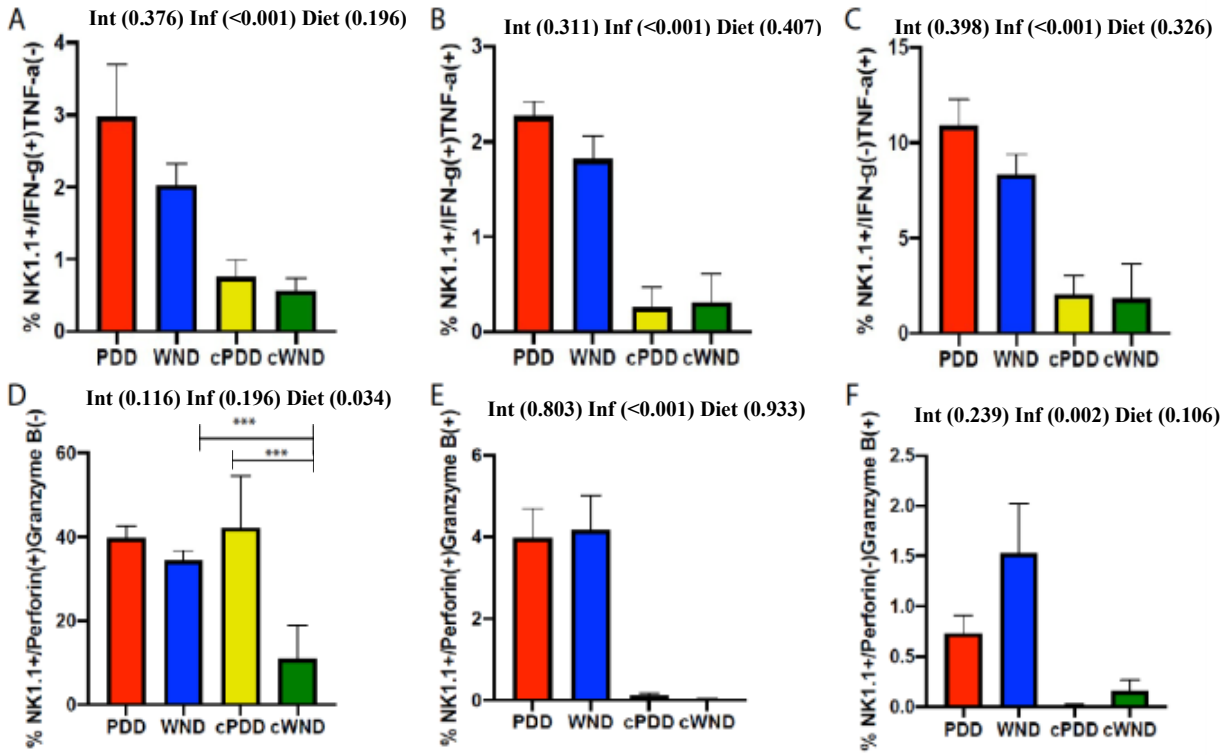

Figure 5: Moderate malnutrition increase perforin and decreases Granzyme B production by NK cells at the peak of *P. chabaudi* infection. Mice were infected with 1x10^5 iRBCs after 4 weeks of feeding on the experimental diet. The infected and uninfected controls were then sacrificed on day 9 post-infection for analysis using flow cytometry. Graphs showing A) IFN-γ Single-Positive NK cells, B) IFN-γ, TNF-α Double-Positive NK cells, C) TNF-α Single-Positive NK cells D.) Perforin Single-Positive NK Cells E.) Perforin, Granzyme B Double-Positive NK cells F.) Granzyme B Single-Positive NK cells. All data are shown as means +/- SEM, n=3-5 male mice per group, representative of 2 independent experiments. Statistical analysis was performed using a two-way ANOVA with Tukey's test. Interaction (Int) and each independent variable main effect, infection (inf) and diet (diet), p-values are above each graph. PDD=Infected moderately malnourished, WND=infected well-nourished, cPDD=control moderately malnourished, cWND=control

Moderate malnutrition increases splenic T_H cell activation and functionality early in **P. chabaudi** *infection*

Based on observations from **Figure** 1 showing elevated leukocytes by day 9 p.i, I wanted to see if adaptive lymphocytes could be impacted by moderate malnutrition during chronic infection. Since NK cells were investigated at days 3 and 9, I decided to do the same with the adaptive lymphocytes. As malaria is a blood-borne infection, and $CD4^+$ cells function in immune activation and are vital to the malaria response (78), splenic $CD4^+$ T cells (T_H cells**)** were measured. Neither infection nor moderate malnutrition appeared to have an impact on $CD4^+$ cell percentages in the spleen by day 3 p.i. (**Figure 6A**). The activation and inflammatory capability was determined by CD44 expression and IFN-γ production by $CD4^+$ cells (88). Moderate malnutrition increases activation in both infected and uninfected malnourished groups compared to well-nourished mice (p=0.045) (**Figure 6B**).

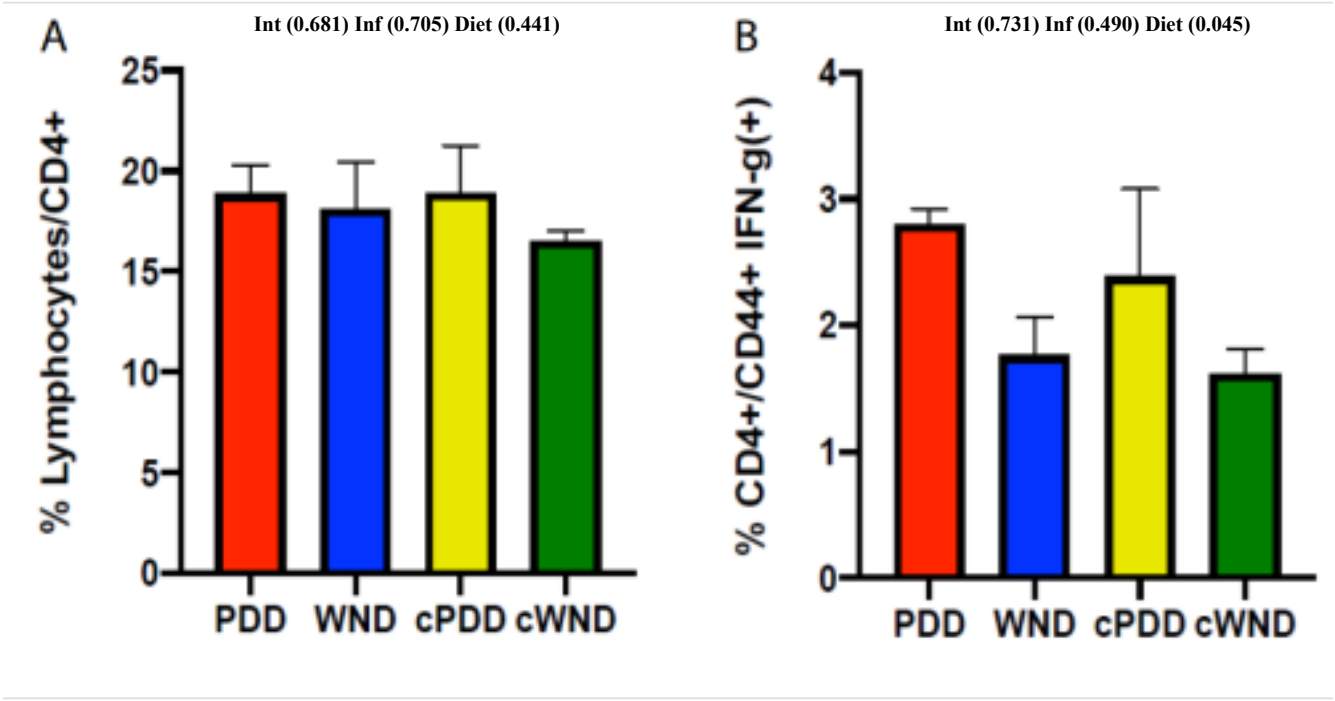

Figure 6: Moderate malnutrition increases splenic T_H cell functionality early in *P. chabaudi* infection. Mice were infected with 1x10^5 iRBCs after 4 weeks of feeding on the experimental diet. The infected and uninfected controls were then sacrificed on day 3 post-infection for analysis using flow cytometry. Graphs showing A) $CD4^+$ T_H cells, B) CD44, IFN-γ Double-Positive T_H cells. All data are shown as means +/- SEM, n=3-5 male mice per group, representative of 2 independent experiments. Statistical analysis was performed using a two-way ANOVA with Tukey's test. Interaction (Int) and each independent variable main effect, infection (inf) and diet (diet), p-values are above each graph. PDD=Infected moderately malnourished, WND=infected well-nourished, cPDD=control moderately malnourished, cWND=control well-nourished.

Moderate malnutrition decreases splenic T_H cells and increases activation during peak of P. chabaudi *infection*

Following the innate immune system, the adaptive lymphocytes predominate the immune response to malaria, including IFN-γ production (67). For this reason, T_H cell population percentage and functionality were determined for day 9 p.i. At the peak of infection, $CD4^+$ cells were now decreased in the infected mice ($p<0.001$), with both well-nourished groups being trending toward elevated compared to their respective moderate malnourished groups ($p=0.059$) (**Figure 7A**). As with day 3 p.i, activation and inflammatory cytokine production were measured. Moderate malnourished mice had more activation than well-nourished mice ($p=0.005$) (**Figure 7B**). Infection also appeared to elevate the activation of T_H cells ($p=0.013$) (**Figure 7B**).

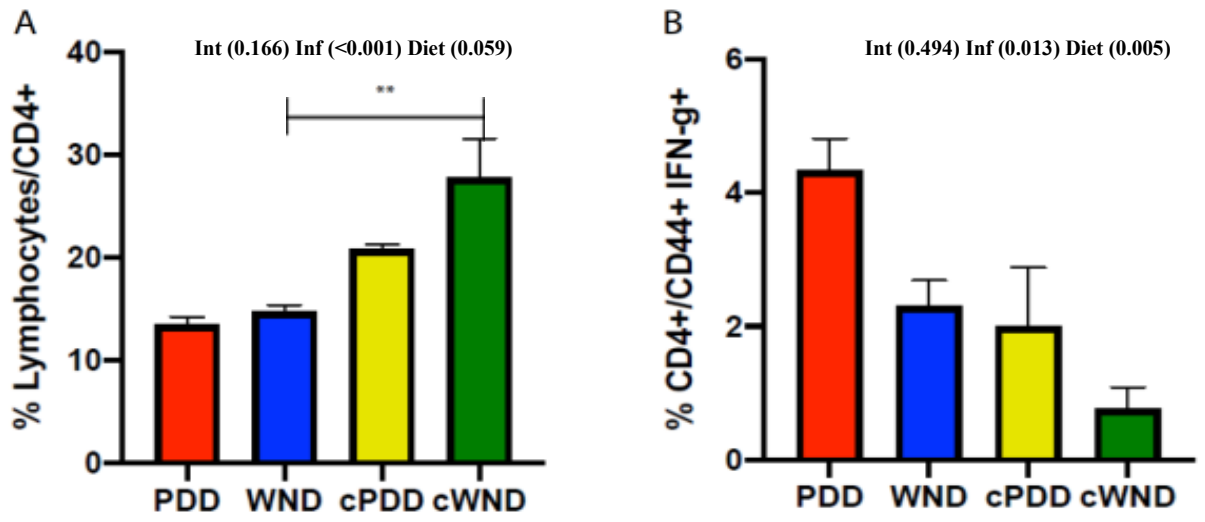

Figure 7: Moderate malnutrition decreases splenic T_H cells and increases activation during peak of *P. chabaudi* infection. Mice were infected with 1x10^5 iRBCs after 4 weeks of feeding on the experimental diet. The infected and uninfected controls were then sacrificed on day 9 post-infection for analysis using flow cytometry. Graphs showing A) $CD4^+$ T_H cells, B) CD44, IFN-γ Double-Positive T_H cells. All data are shown as means +/- SEM, n=3-5 male mice per group, representative of 2 independent experiments. Statistical analysis was performed using a two-way ANOVA with Tukey's test. Interaction (Int) and each independent variable main effect, infection (inf) and diet (diet), p-values are above each graph. PDD=Infected moderately malnourished, WND=infected well-nourished, cPDD=control moderately malnourished, cWND=control well-nourished.

Moderate malnutrition lowers B cell percentage but increases B cell activation early in **P. chabaudi** *infection as well as increases both by the peak of* **P. chabaudi** *infection*

B cells are the other major lymphocyte in the spleen that combats *P. chabaudi* infection and function in a T-cell dependent manner (75). At day 3 p.i. without infection, moderate malnutrition was found to slightly decrease splenic B cells, with no significant differences across all groups (**Figure 8A**). B cell activation was determined by looking at the expression of surface markers MHCII and CD86 (89). Moderate malnutrition was found to increase the activation of B cells (p=0.005), (**Figure 8B).**

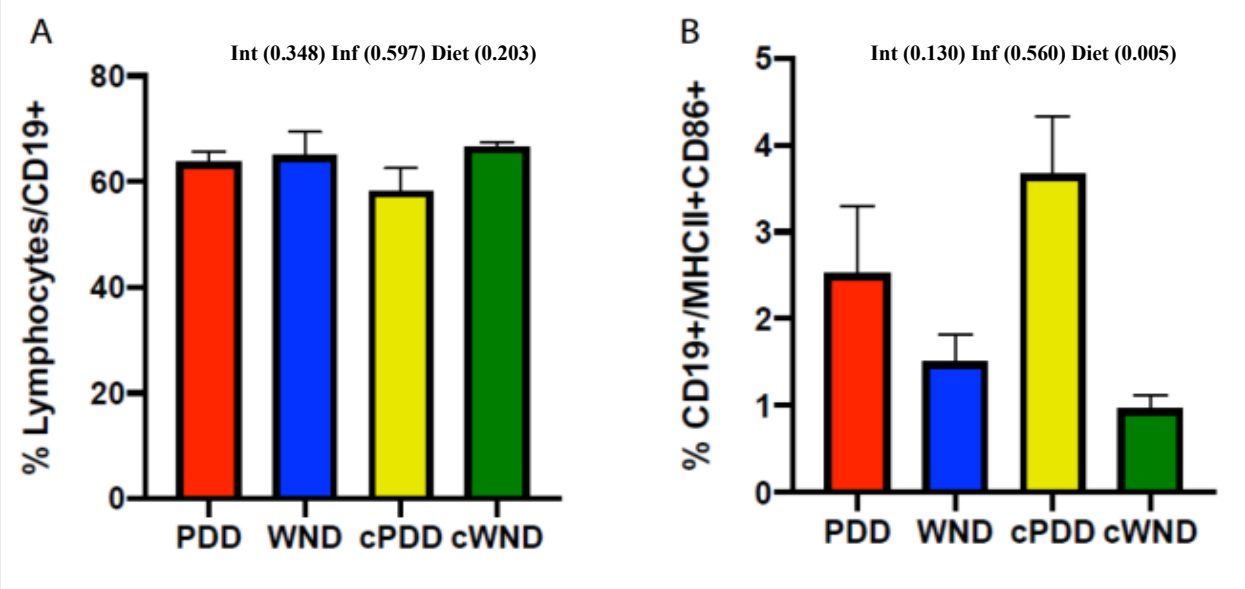

Figure 8: Moderate malnutrition lowers B cell percentage but increases B cell activation early in *P. chabaudi* infection. Mice were infected with 1x10^5 iRBCs after 4 weeks of feeding on the experimental diet. The infected and uninfected controls were then sacrificed on day 3 post-infection for analysis using flow cytometry. Graphs showing A) B cells, B) MHCII, CD86 Double-Positive B cells. All data are shown as means +/- SEM, n=3-5 male mice per group, representative of 2 independent experiments. Statistical analysis was performed using a two-way ANOVA with Tukey's test Interaction (Int) and each independent variable main effect, infection (inf) and diet (diet), p-values are above each graph. PDD=Infected moderately malnourished, WND=infected well-nourished, cPDD=control moderately malnourished, cWND=control well-nourished.

Moderate malnutrition and infection increase B cell percentage at peak of **P. chabaudi** *infection*

In line with the rationale for observing T_H cells at day 9 p.i, B cell population percentages were also determined at day 9 p.i. By day 9, infected mice had significantly increased B cell percentages (p=0.004) (**Figure 9A**). Malnourished controls were also found to have significantly higher splenic B cells than well-nourished controls (p=0.017) (**Figure 9A**). As with day 3 p.i, B cell activation was investigated. Activation of B cells between the infected groups by day 9 p.i. was showing similar trends to the activation at day 3 but not significantly so (p=0.094), with moderate malnourished mice having slightly elevated B cell activation (**Figure 9B**). However, the elevation in the uninfected malnourished compared to the uninfected well-nourished mice observed at day 3 p.i. was reversed at day 9 p.i. (**Figure 9B**).

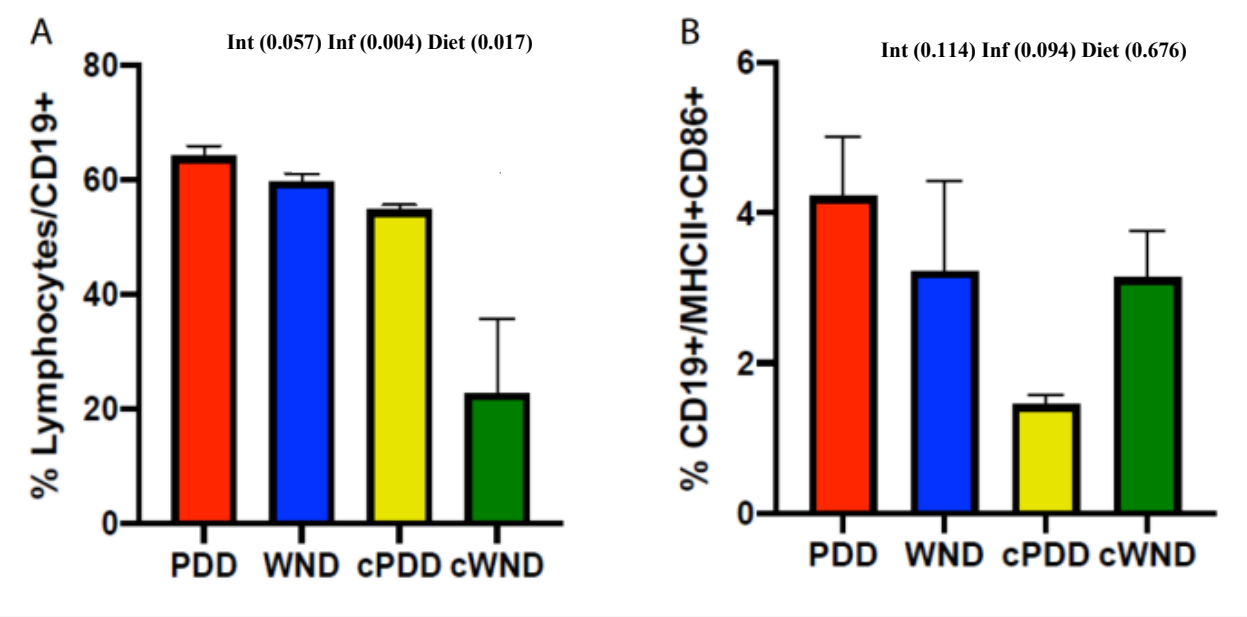

Figure 9: Moderate malnourished mice have increase splenic B cells by peak of *P. chabaudi* infection and increased activation. Mice were infected with 1x10^5 iRBCs after 4 weeks of feeding on the experimental diet. The infected and uninfected controls were then sacrificed on day 9 post-infection for analysis using flow cytometry. Graphs showing A) B cells, B) MHCII, CD86 Double-Positive B cells. All data are shown as means +/- SEM, n=3-5 male mice per group, representative of 2 independent experiments. Statistical analysis was performed using a two-way ANOVA with Tukey's test. Interaction (Int) and each independent variable main effect, infection (inf) and diet (diet), p-values are above each graph. PDD=Infected moderately malnourished, WND=infected well-nourished, cPDD=control moderately malnourished, cWND=control well-nourished.

CHAPTER 4

Discussion

Malaria and malnutrition have consistently been found in sub-Saharan Africa and are known to have significant impacts on one another, yet the mechanisms of how malnutrition affects immunity to malaria have yet to be fully unraveled (25, 90). In recent studies, the roles of innate lymphocytes such as NK cells, γδ T cells, and ILCs against *Plasmodium* infection have increased, but with limited knowledge on factors that could influence their function in controlling the parasite (44, 60, 85, 91-93). In this study, I have demonstrated that moderate malnutrition affects the proportion and activation of splenic lymphocytes during the first weeks of *P. chabaudi* infection. As for circulating leukocytes, infection was found to be the only contributor to an increase in leukocytes, specifically the NK cells. The increase in these innate immune lymphocytes was similar in both the malnourished and well-nourished mice, suggesting that micronutrient deficiency may not alter the circulating innate lymphocytes.

During the early stage of infection defined as day 3 p.i., splenic lymphocytes including NK cells were increased in the infected moderate malnourished mice, compared to the well-nourished mice. This was inconsistent with literature where it has been reported that protein malnutrition alone decreased splenic NK cells (73). In these previous studies, the diet was completely deficient in protein as opposed to my diet where I have 3% protein with similar caloric content between my malnourished and well-nourished diets. Indeed, my uninfected controls showed no difference in lymphocyte percentages, but the moderately malnourished group had increased splenic NK cell percentage. This indicates that my malnourished model may lead to increased proportions of NK cells in the spleen.

To further understand the impact of malnutrition on NK cells, I investigated the maturation status as documented by Chiossone et al. via the expression of the CD27 and CD11b surface markers. As expected, malnutrition altered splenic NK cell population maturity which translated to an increase in the effector functions observed even in the uninfected mice (63). A similar study by Gardner et al. using calorie restricted and ad-libitum-fed influenza infected mice reported reductions in influenza-induced NK cells, decreased cytotoxicity, and increased susceptibility in calorie-restricted aged mice (94). Both the calorie restricted, and ad libitum diets consisted of 18% protein, similar to the well-nourished diet used in my model, while the energy restriction was a 40% reduction, compared to my diet which is calorie matched. Using Gardner et al.'s diet model without infection, the maturation status of NK cells was determined by Clinthorne et al. who demonstrated that calorie-restriction resulted in decreased CD11b expression, increased granzyme B and TNF-α production, along with reduced IFN-γ production compared to ad libitum fed mice. Although we found quite different results defined by an increase in maturity and IFN-γ-producing cells, this could be attributed to the equal protein in Clinthorne's model, as the limited protein has been found to lead to gut epithelium damage increasing bacterial translocation and antigen presentation.

Protein and energy malnutrition in humans has been shown to depress peripheral blood NK cell activity determined by cytotoxicity assay (95). My data show that a calorie matched-low protein diet with iron and zinc deficiency increases CD11b expression along with increased IFN-γ production and no difference in TNF-α production between the two diet groups at day 3 of *P. chabaudi* infection. The increase in inflammatory cytokine production correlates with increased CD11b expression as the mature NK cells are more active. This goes against data on zinc deficiency alone as it has been found to reduce NK cell activity, however this study used aged

mice (96). While total production was not determined, the increase in cytokine- and cytotoxic-producing cells correlated with data indicating increased effector function with increased CD11b expression.

Iron deficiency, on its own also, impacts NK cells by reducing the cytotoxicity in rats as NK cells upregulate metabolism upon activation and require iron (97, 98). Littwitz-Salomon et al. showed that iron deficiency exacerbates viral infections by limiting the functionality of NK cells by day 7 of infection, as they were unable to adequately access metabolic necessities such as iron (98). IFN-γ production by NK cells in mice is also decreased by iron deficiency, though the decrease in IFN-γ may be due to deficiencies in cells such as dendritic cells and macrophages that activate NK cells by producing IL-2 as opposed to intrinsic changes to the NK cells based on diet (99).

At the peak of infection defined as day 9 p.i, NK cells were lower in the infected groups compared to the uninfected mice. These data correlate with circulating leukocyte data, indicating that the lack of splenic NK cells in the infected mice (**Figure 3**) can be attributed to the increase in circulating NK cells by day 9 p.i. (**Figure 1**). This decrease is sensical as by day 9, the adaptive immune response is active so innate lymphocytes like NK cells would be in lower percentages in the spleen. Infected mice had increased proportions of $CD27^{-}CD11b^{-}$ immature NK cells compared to the uninfected mice. Also, the well-nourished mice continued to have increased double-negative immature NK cells, compared to their malnourished groups. I can speculate that the increased immature NK cells in the infected mice is a result of the more mature splenic NK cells migrating to the bloodstream to combat the infection. With the more mature NK cells migrating out, the immature cells remain in the spleen leading to population changes. As with day 3, NK cells expressed more CD11b in the malnourished mice. Infection elevated the

production of inflammatory cytokines, with the malnourished slightly higher in production of both IFN-γ and TNF-α.

Cytotoxic function was determined by measuring the production of perforin and granzyme B. Uninfected malnourished mice produced similar amounts of perforin as the infected groups and significantly more than the well-nourished uninfected mice. This high presence of perforin in the uninfected malnourished might may be attributed to zinc deficiency as it drives increased inflammatory responses by decreasing T_H2 and M2 cells in the spleen, disrupting the immune cell population dynamics. Perforin and granzyme B co-production was similar between the infection groups, while granzyme B production alone was slightly higher in the well-nourished infected mice. However, neither of the uninfected groups produced noticeable amounts of granzyme B, indicating that while the malnourished mice have higher levels of perforin, infection is required for the production of granzyme B. A similar differential production of perforin and granulysin, another cytotoxic granule produced in conjunction with perforin, is not seen in NK cells of healthy controls but has been documented across multiple types of cancer where NK cells populations are decreased in addition to having a significant decrease in granzyme B-producing cells compared to perforin (100). The elevated perforin in the uninfected malnourished mice, may lead to a faster and more robust cytotoxic response upon activation. Since iron deficiency has been shown to reduce cytotoxicity in rats, the elevated perforin in the uninfected moderate malnourished mice compared to the uninfected well-nourished using my multi-deficient model is interesting. However, the model discussed for iron deficiency did not limit protein or zinc, but only iron. A possible explanation for the elevated perforin despite iron and zinc deficiencies could be related to damaged gut epithelium and gut leakage associated with the moderate malnourished mice (101). Moderate malnutrition has been shown to cause bacterial

translocation, which may partially activate the NK cells in the moderate malnourished mice leading to the elevated perforin in both infected and uninfected mice (101).

Adaptive immune cells are required for malaria protection (102). $CD4^+$ T cells are necessary to properly combat malaria (103) with specific subsets of these cells correlating with increased protection in malaria-endemic regions (104). B cells have been found to be protective determinants in regard to the decreased rates of cerebral malaria observed in adults compared to children following repeated infections (105). Due to their importance in malaria defense and the elevation in NK cells in malnourished mice, I decided to investigate the effects on the adaptive lymphocytes from the onset to peak of infection. Early in infection, the $CD4^+$ T cell numbers were similar across all groups. This was unexpected as iron deficiency alone has been shown to decrease $CD4^+$ T cells (106), but those data were reported in a human model of children with long-term iron deficiency, while my model is iron deficient over a shorter period. Activation and inflammatory cytokine production were higher in $CD4^+$ T cells for both malnourished groups vs. the well-nourished, with no differences between the infected and uninfected on the same diet. Protein deficiency alone has been shown to decrease $CD4^+$ T cell function to vaccines measured by IL-2 production (107). Although IL-2 was not measured in my experiment, the activation of these cells was determined by the upregulation of CD44 and increased IFN-γ production in the moderate malnourished mice. This difference could be attributed to the lack of zinc. Zinc deficiency has been shown to drive increased inflammatory responses by decreasing T_H2 and M2 cells in the spleen disrupting the immune cell population dynamics (108). Iron deficiency decreases T-cell mediated immunity; however, the impact of zinc deficiency appears to outweigh the effects of iron deficiency in regard to T cell activation (109).

By the peak of infection, $CD4^+$ T cell percentages were decreased in the infected groups vs the uninfected, with the uninfected well-nourished being elevated compared to the uninfected malnourished. This was interesting as there was no difference in circulating blood $CD4^+$ T cells between the infected and uninfected groups throughout the infection. However, a longer time point might be necessary to observe this. Also, by day 9, the adaptive lymphocytes should be elevated in the spleen, not diminished so this could be an avenue for future study. Moderate malnourished mice had increased activation and inflammatory cytokine production and infected groups were higher than their uninfected groups. These results are in line with data from both protein deficiency and zinc deficiency. There is a smaller $CD4^+$ cell population from protein deficiency in addition to increased activation from zinc deficiency (107, 108).

Similar to the $CD4^+$ T cells, there was no difference in the proportions of the B cell population between the diets in the infected groups at day 3 p.i. Unlike the $CD4^+$ T cells, the uninfected groups did show a difference with the malnourished mice having a significantly smaller proportion of B cells in the lymphocyte population. This decrease in B cells for the moderate malnourished mice is in line with research showing that zinc deficiency depletes B cell lineage cells in the bone marrow (40). Protein deficiency alone has been reported to have no impact on the B cell responses to vaccine in mice. Therefore, differences observed here point to zinc as the primary cause (107). As with the other lymphocytes, B cell activation was elevated in the malnourished mice compared to the well-nourished in both infected and uninfected groups.

By the peak of infection, the infected groups had an elevated percentage of B cells compared to the uninfected. In addition, the infected moderate malnourished were now slightly higher than the well-nourished. In the uninfected controls, the well-nourished population was significantly decreased from day 3, when it was significantly higher than the moderately

malnourished. This difference in the controls can be attributed to an unexpected diverse distribution in the uninfected well-nourished group that affected cell percentages. At this later timepoint, the infected groups had similar B cell activation, while the uninfected well-nourished had increased activated B cells compared to the malnourished mice, which contrasted day 3. This could also be attributed to the unexpected diverse distribution impacting cell percentages. The data on the lymphocyte immune cells indicate that a diet with low-protein content and deficient in zinc and iron leads to increased splenic lymphocyte activation and inflammatory potential in addition to differential splenic NK cell maturity during *P. chabaudi* infection.

Recent research has demonstrated that NK cells can impart increased protection from malaria disease based on specific phenotypes. Hart et al. showed that an endemic population had developed adaptive NK cells defined by the loss of the transcription factor promyelocytic leukemia zinc finger (PLZF) and Fc receptor γ-chain that increased resistance and lowered parasitemia (91). Taking my diet and designing a re-infection model could allow for the investigation into the effects of moderate malnutrition on the memory defense. It is well documented that the immune memory to malaria takes multiple infections to develop if at all and is ineffectively maintained by the immune system, but the effects on NK cell memory have yet to be investigated.

Another possible expansion of this project could include a longer time point for the circulating leukocytes, up to 15 days. NK cells are innate cells yet were found to increase in the blood at day 7 and 9 of infection, not earlier. This could indicate that circulating adaptive lymphocyte population differences may not be apparent until after day 9 p.i. Also, I could determine the importance of each individual nutrient in my model, to ascertain which one is

responsible for the increased NK cells in the moderate malnourished mice not seen in caloric restriction diets.

Further investigation into why splenic $CD4^+$ T cells were decreased in the infected mice at the peak of infection could be done using this model. This experiment should include examining IL-2, IL-4, and IL-10 production to see any differences in cytokine production and immune environment. IL-10 and IFN-γ production by $CD4^+$ T cells indicates a proper functioning Th1 malaria response as the IFN-γ activates macrophages leading to an active inflammatory response while IL-10 is immunomodulatory and helps to prevent immunopathology (110). The T_H2 response by $CD4^+$ T cells against malaria leads to the production of IL-4 following stimulation by dendritic cells. In addition, an examination into potential differences between cell percentages and cell numbers could be conducted in all lymphocytes.

CHAPTER 5

Conclusion

Malnutrition is termed the largest acquired immunodeficiency in the world and is found to exacerbate many infections yet the mechanisms behind these effects are still unclear. My data indicates that a low-protein diet that is also deficient in iron and zinc leads to increased splenic lymphocyte activation and inflammatory potential in addition to differential splenic NK cell population maturity during *P. chabaudi* infection. I have shown that a combination of these deficiencies is responsible for the observed effects, as research into these nutrient deficiencies individually has produced both confirming and conflicting results dependent upon the model. The full effects of this differential immune environment are not fully understood, but it is apparent that moderate malnutrition is impacting the host immune system. As such, in a real-world environment I would recommend supplementing both protein and zinc to prevent an increased inflammatory environment as that increases the risk of severe malaria. As for iron, both deprivation and supplementation have been shown to increase malaria severity, therefore caution should be taken when supplementing.

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Vita

James Jackson Erny was born in Thibodaux, Louisiana in 1996. Diagnosed with osteogenesis imperfecta, he was always interested in medicine and science. This fascination was encouraged by his parents and grandparents' backgrounds in academia. At the start of his undergraduate degree at LSU, James intended to pursue medical school. After taking immunology and parasitology classes in undergrad, he realized that research was the field in which he wanted to work. The passion for furthering his understanding of these topics along with his love for the Appalachian Mountains led to him pursuing a master's degree in cellular and molecular biology at Appalachian State University as a part of the Opata lab (Team Malaria).

During his second year in the master's program, James gave an oral presentation at Appalachian State's Celebration of Student Research in 2022 after writing and receiving several competitive university sponsored research grants for funding of his projects. Additionally, James has presented his research at the American Society of Microbiology Branch Conference 2022. James hopes to work as a research scientist in cancer immunology with a focus on immunotherapies. James has taught Introductory biology lab for non-biology majors, Introductory biology for science majors and the senior/graduate level Immunology lab. He has also trained several students in lab techniques. As a leader, he was the social chair of the Biology Graduate Students Association (BGSA) in his first year in graduate school and is currently the vice-president.

In his free time, James likes to enjoy the Appalachian Mountains primarily by hiking in addition to other outdoor activities. He also enjoys spending time with his friends and family whether it be sharing a meal or going to events like live music or sporting events.