MODERATE MALNUTRITION DECREASE MALARIA-SPECIFIC EFFECTOR $CD4^+$ T CELLS

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

August 2021
Department of Biology

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Abstract

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Malnutrition is known to predispose people to infections by affecting immune cell populations, but it is not known how moderate malnutrition affects the survival of effector $CD4^+$ T cells that could be protective against chronic infections such as malaria. In our current study, we hypothesized that moderate malnutrition leads to a reduction of malaria-specific $CD4^+$ T cells resulting in lower numbers of activated effector $CD4^+$ T cells that have poor survival potential due to decreased *Bcl-2/Bcl-xL* expression. Using flow cytometry, we determined effector and malaria-specific $CD4^+$ T cells in the spleens of mice, after *Plasmodium chabaudi* infection. We observed that moderate malnutrition does not decrease the total number of lymphocytes and polyclonal $CD4^+$ T cells, but the moderate malnourished mice had lower spleen weights compared to well-nourished mice. Using adoptive transfer technique, we found that moderate malnutrition decreases malaria-specific $CD4^+$ T cells that express Thy1.2 molecule, along with reduced numbers of activated malaria-specific effector $CD4^+$ T cells. The decrease in activated malaria-specific effector cells was accompanied by reduced cytokine production. We also found that *Bcl-2* expression is downregulated, but *Bcl-xL* may play a compensatory role in the infected malnourished group, hence promote some kind of survival during the effector phase in the malaria-specific

$CD4^+$ T cells. These findings suggest that moderate malnutrition does impair pathogen specific $CD4^+$ T cell populations during chronic infection, which may have a significant effect on other immune cells.

Acknowledgments

Throughout the writing of this dissertation, I have received supports and assistance. I would first like to thank and express my sincerest gratitude to my faculty mentor Dr. Michael Opata for his patience and guidance throughout this program and process. His insightful feedback pushed me to sharpen my thinking and writing skills and brought my work to a higher level. I would also like to acknowledge my committee members Drs. Maryam Ahmed and Andrew Bellemer for their guidance, advice, and encouragement through my thesis research. Also, my sincerest gratitude to my colleagues in the Opata Lab, for helping and guiding me through my research. I would also like to give thanks to Monique Eckerd for her patience and kindness for taking care of our laboratory mice. In addition, I would like to thank my parents for their support and encouragement through this challenging journey. My sisters and brother, who have supported and have always listened to what I have to say when I am at my lowest. Lastly, my two greatest friends, Yang and Michelle, for supporting me emotionally and mentally through this difficult journey. I would have not accomplished this and be who I am today without you all.

Dedication

I would like to dedicate this thesis to my parents, siblings, and friends. Thank you for the unwavering support, love, patience, words of wisdom, and encouragement through this challenging journey.

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List of Abbreviations

iRBCs: infected red blood cells

RBCs: Red blood cells

IFN-γ: Interferon gamma

TNF-α: Tumor necrosis factor alpha

MSP-1: Merozoite or Major Surface Protein-1

B5 TCR Tg: Merozoite Surface Protein-1 transgenic mice or B5 T Cell Receptor Transgenic

Thy1.1: BALB/c mice

Thy1.2: B5 TCR Tg mice

Cntrl: Control

Mal: Malnourished

P.i.: Post infection

FACS: Fluorescence-activated cell sorting

Unif Ctrl: Uninfected Control

Unif Mal: Uninfected Malnourished

IL-2: Interleukin 2

IL-2R: Interleukin 2 Receptor

IL-7: Interleukin 7

NC: No Cell

NC-Ctrl: No Cell Controls

NC-Mal: No Cell Malnourished

SEM: Standard error of the mean

Foreword

This thesis has been formatted according to the style guide from *The Journal of Immunology*, a peer-reviewed medical journal owned and published by the American Association of Immunologists.

Chapter 1

INTRODUCTION

Malaria

While an average of 1,700 cases of malaria are reported yearly in the United States, the global world cases stand at 229 million with majority of the cases being reported in sub-Saharan Africa (1). Climate affects both the mosquito and parasite (2). Mosquitoes are unable to survive in low humidity, therefore rainy seasons and a tropical climate are more beneficial for female *Anopheles* mosquitoes to breed and lay eggs (2). The optimum temperature range in the environment that is suitable for the development of these parasites are between 25°C and 30°C (3), allowing regions near the equator like Sub-Saharan Africa to have higher cases of malaria (2, 3).

There are five known species that infect humans namely; *Plasmodium falciparum (P. falciparum)*, *P. vivax*, *P. ovale*, *P. knowlesi*, and *P. malariae* (4, 5). The most prevalent and lethal species that is responsible for high mortality rates in sub-Saharan Africa and globally is *P. falciparum* (5, 6), which causes chronic malaria infection. *P. falciparum* is known to be more virulent than the other parasite species because it multiplies rapidly in the blood and can cause severe forms of diseases including anemia, (7), cerebral malaria and mental retardation (8). The *P. falciparum* parasites can stay in the body for a year, and the infected red blood cells (iRBCs) burst every 48 hours. It also leads to splenomegaly which can affect immune cells and their ability to secrete cytokines (9). To better understand malaria pathogenesis, there are four rodent parasite strains that infect mice; these include *P. chabaudi, P. vinckei, P. berghei*, and *P. yoelii* (10). These rodent strains display different parasite biology and pathogenicity, thus being able to study different aspects of the

pathological and immunological features of human malaria infections (10). Our lab used the *P. chabaudi* rodent malaria strain to understand how moderate malnutrition affect immune response to *Plasmodium* infection, since the blood stage life cycle and pathology induced by this model is similar to that induced by *P. falciparum*.

Malaria Life Cycle

During a *P. falciparum* infection, the parasite has a complex life cycle (Fig. 1). The sporozoites mature in the gut of the female *Anopheles* mosquitoes and travel to the salivary glands (11). When an infected female *Anopheles* mosquito bites a human, these sporozoites are released into the skin. The sporozoites travel to the liver via the circulatory system and infect the hepatocytes, where they undergo an asexual pre-erythrocytic liver stage life cycle (6, 11, 12). Over a period of 7-12 days, the sporozoites grow into schizonts and develop into merozoites, which rupture the infected hepatocytes (6). The merozoites that are released from the ruptured hepatocytes then infect red blood cells (RBCs) (11, 12). During the red blood cell stage, the symptoms for the disease occur. Some common symptoms are fever, vomiting, jaundice, headache, and nausea (5, 13). In more severe cases, malaria frequently results in anemia or cerebral malaria, respiratory distress, and death (13). As RBCs are infected, they burst and release more merozoites to infect new RBCs, leading to the continuous life cycle of parasite invasion and replication (11, 12). Some blood stage parasites switch to sexual development, producing male and female gametocytes, which is an important stage for transmission to the mosquitoes (11, 12). When another female *Anopheles* mosquito ingest erythrocytes containing male and female gametocytes, sexual reproduction takes place in the mosquito's midgut, where the gametocytes develop into ookinetes (11). These ookinetes

travel to the mosquito's stomach wall and develop into oocysts, which rupture and release sporozoites (11). The sporozoites crawl to the female *Anopheles* mosquito's salivary glands and when the mosquito takes a blood meal from another human, the cycle starts all over again (11, 12).

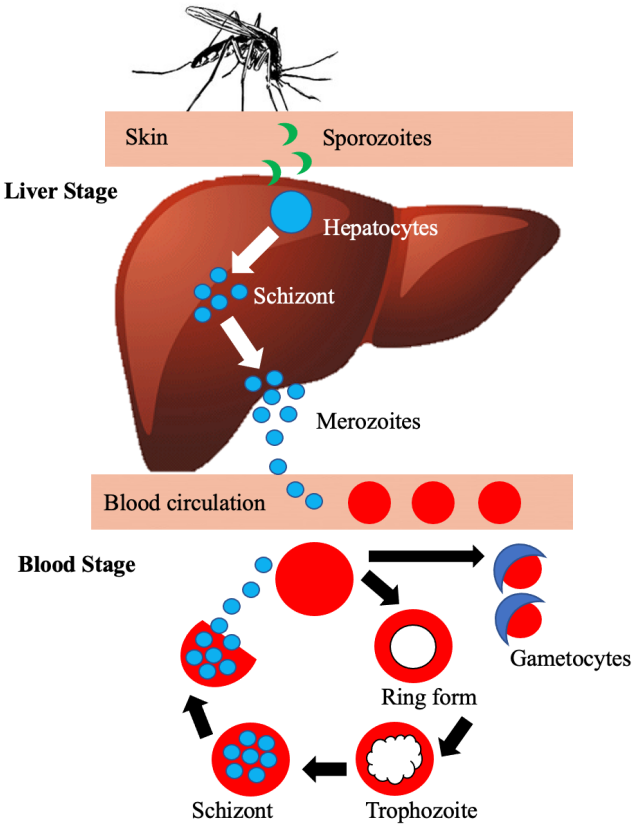

FIGURE 1. Schematic of a malaria life cycle.

Preventions

All individuals are at risk for malaria infections, but children between the age of six months to five years are the most vulnerable to malaria infections due to losing maternal immunity and having an immune system that is not fully developed (14). They are more likely to develop anemia, have frequent seizures, and sepsis (12, 15). Apart from children, pregnant mothers are at risk for malaria infection as well. This can cause preterm delivery,

miscarriages, and low birth weight to the fetus (16, 17). The low birth weight has detrimental effects on overall growth of children brought up in a malnourished environment.

Despite poor understanding of immune response to malaria, other public health strategies have been developed to control the rate of malaria infections. The two main vector controls strategies are insecticide-treated mosquito bed nets and indoor residual spraying (IRS) (1, 18). Sleeping under an insecticide-treated net can reduce contact between mosquitoes and humans because there is a physical barrier (1). In 2019 alone, about 46% of people who were at risk for malaria were protected by an insecticide-treated net (1). Indoor residual spraying is another powerful way to reduce malaria transmission, which involves spraying the inside of a house with insecticide once or twice per year (1). Studies have shown that IRS can reduced re-infection with malaria parasites (18, 19). However, over the years, IRS has become very expensive and in high transmission areas this would require multiple rounds of spraying to be protected (18). Another problem is that IRS can cause health risks for humans and to the environment, because some insecticide such as dichlorodiphenyltrichloroethane (DDT) are environmental hazards (18). According to the World Health Organization (WHO), IRS should only be used under specific conditions (1).

Malnutrition

In malaria-endemic areas, malnutrition is also an issue that can lead to a poorly developed immune system that cannot fight off pathogens (20). According to WHO, 462 million people are underweight (21), due to nutritional deficiencies in iron, zinc, and protein (22). Lack of nutrients can be a significant health issue, as lack of proper amount of nutrients can alter the immune system's functions. In this case, zinc, iron, and protein are significantly

vital nutrients for the immune system. These macro- and micronutrients are essential for cell growth, proliferation, and effective functionality. With deficiencies in these nutrients, many immune cells do not proliferate nor survive well. These nutrients are also crucial for the normal development of an individual's organs (23–25). While malnutrition affects all groups in a community, infants and young children are the most vulnerable because they need higher nutritional requirements for growth and development (26).

Iron is necessary for all living organisms and plays an essential role in surveillance of immune cells (27). Studies suggest that iron deficiency stops cell proliferation (28), can impair a child's growth, cognition, and neurological development as well as their immune functions (23, 24). However, malaria parasites need iron for survival, because they feed on healthy RBCs (23). This has been supported by studies showing that iron deficiency can protect against malaria, because of a decrease in RBCs (23, 29, 30). Despite these outcomes, iron deficiency can result to severe anemia and malnutrition.

Similar to iron, zinc is also an important micronutrient for cell growth, differentiation, and survival (31). It is crucial for the normal development and functions of the innate immune system, that includes neutrophils, natural killer cells, and macrophages (25). Some studies have shown that zinc deficiency predominately affects cell mediated immune cells (32–34), causing defects in their growth and functions (25, 35). However, *Plasmodium* parasites require zinc for replication and basic cellular processes (36). Some studies suggest that zinc may reduce clinical disease caused by malaria infections (36–38), while other studies found no evidence (39, 40). Despite these findings, zinc deficiency can result to malnutrition and cause other detrimental effects to an individual's health.

A diet sufficient in protein is important for the activation of T and B cells, innate immune cells, cell proliferation, and the production of antibodies and cytokines (41). Thus, insufficient protein intake leads to impaired cell-mediated immunity, phagocytic functions, and cytokine productions in humans (42, 43). Studies suggests that protein deficiency can protect the host against malaria morbidity and mortality (38, 44). But some research indicates that protein deficiency can predispose an individual to excess malaria infections and other diseases (38, 43). Despite this debate, protein deficiency can lead to malnutrition and affect normal development of an individual.

The Immune System and Immune Cells

The immune system comprises of the innate and adaptive immune responses, which play a significant role in fighting off acute and chronic infections. Adaptive immune cells take time to be activated, so they come into play later after the innate immunity, which starts immediately if there is an infection. One of the body's main type of immune cells are lymphocytes. These comprise the T cells, B cells, and natural killer (NK) cells (45). They have different abilities or functions to destroy cells that cause damage and are important during malaria infections.

T cells recognize and respond to particular epitopes to specific antigens (46). Also, T cells become activated when an infection lasts more than four days. They either have a helper function to stimulate different immune cells or cytotoxic properties to kill infected cells directly. CD4 T cells, also called helper T cells play a critical role in stimulating other immune cells as infections progress. They provide a helper function by secreting cytokines that activate other immune cells to come to the site of infection. Helper T cells also activate

B cells, and cytotoxic T cells by licensing the dendritic cells to present antigens effectively (47–49). The CD4 helper T cells have been shown to play a central role in immune control of infections with *Plasmodium* parasites (50). CD4 T cells can directly control the growth and development of pre-erythrocytic *Plasmodium* stages, direct the immune response elicited by the erythrocytic stage of malaria, and control the pathogenesis of infection (50, 51).

Early in the infection, effector CD4 T cells are important in promoting protection. Effector T cells develop from naïve T cells upon exposure to antigens, which trigger their activation and expansion (Fig. 2). Activated effector T cells can protect better than memory T cells in general, because they express high levels of effector molecules, making them to be the most suited T cell subset for protection against infectious diseases (52), but they are short-lived (53, 54). As the infection progresses into the chronic phase, memory T cells become critical in fighting the infection. Repeat exposure to the same infection will subsequently induce a faster response from memory T cells (55). Thus, malaria being a chronic infection requires good survival of memory T cells (56). Memory T cells have a longer survival rate than their naïve counterparts (57). Therefore, these antigen-specific memory cell populations can persist for years to a lifetime in humans (57). Also, memory T cells have the advantage of circulating through both the secondary lymphoid tissues and peripheral non-lymphoid tissues where they directly encounter foreign antigens that might be there (57).

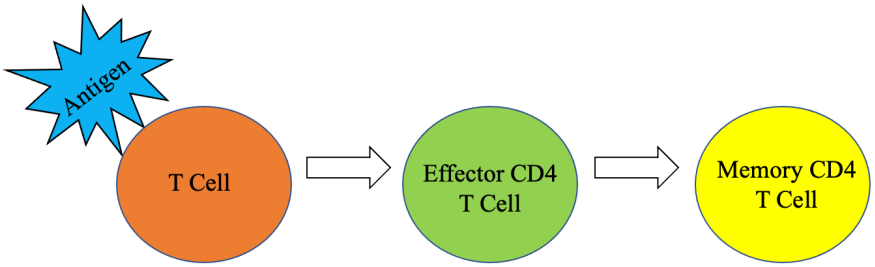

FIGURE 2. Schematic of T cell differentiation.

As malaria infection lasts for several months, survival of T cells is important during this infection. There is a balance between survival and death of the cells during the chronic phase and this survival depends on the anti-apoptotic proteins of the B cell lymphoma 2 (Bcl-2) family (58). Both naïve and memory T cells express the anti-apoptotic Bcl-2 molecules, which inhibit the induction of apoptosis (59). Bcl-2 family proteins control the contraction phase of effector cells to support the development of memory cells (59). Studies have shown greater portions of effector cell population surviving the contraction phase in mice overexpressing Bcl-2. This led to more effector cells differentiating into memory cells (59). In the presence of cytokines such as IL-7 that promote survival of immune cells, memory populations are seen to survive longer as well.

T cells produce different types of cytokines as they support other immune cells in the control of malaria infection (46, 60–62). Important cytokines that are secreted by T cells during most infections are Interferon gamma (IFN-γ) and Tumor Necrosis Factor alpha (TNF-α) (63). There has been a significant body of research on IFN-γ and TNF-α for controlling and protection of the *Plasmodium* infection in rodent models (50, 60, 64) and humans (65–67).

IFN-γ is an inflammatory cytokine that induces and modulate many types of immune responses (68). These responses include activation of macrophages, enhanced antigen presentation, and directing growth and maturation in many cell types (68, 69). Importantly, IFN-γ play a protective role against *Plasmodium* parasites (70), by controlling the infection during both the liver and blood stage life cycles (71). Studies have shown that mice lacking IFN-γ have higher and more prolonged blood stage parasitemia compared to mice that have IFN-γ when infected with rodent strains of malaria (70, 71). This demonstrates that IFN-γ is a key pro-inflammatory cytokine that can control *Plasmodium* parasites during the blood stage (70).

TNF-α on the other hand is a pleiotropic cell-signaling cytokine, but also involved in inflammation (72, 73). In malaria infections, TNF-α can have both beneficial and detrimental effects (74, 75). Studies have shown that TNF-α can prevent parasite development during the pre-erythrocytic stage in hepatocytes as well as the erythrocytic phase in both humans and mice (74, 75). However, if there is an excess secretion of TNF-α during a malaria infection, this can lead to cerebral malaria or more fatal outcomes (75, 76), since it plays an essential role in the development of chronic inflammatory diseases (77).

While it is known that malnutrition decreases immune response in general, the direct effect of moderate malnutrition on the development of effector malaria-specific CD4 T cells has not been well studied. Therefore, we employed an adoptive transfer technique using major surface protein-1 (MSP-1) T cell receptor transgenic mice, that contain CD4 T cells specific to the *Plasmodium* major surface protein-1 (MSP-1), to understand how moderate malnutrition affects the activation of effector malaria-specific $CD4^+$ T cells in Thy1.1 BALB/c mice. We hypothesized that moderate malnutrition leads to a reduction of malaria-

specific $CD4^+$ T cells resulting in lower numbers of activated effector $CD4^+$ T cells that have poor survival potential due to decreased *Bcl-2/Bcl-xL* expression.

Chapter 2

MATERIALS AND METHODS

Mice and Parasites

Adult congenic Thy1.1 BALB/c and Merozoite Surface Protein-1 transgenic mice (B5 TCR Tg) were used. Merozoite Surface Protein-1 transgenic mice contain the Merozoite Surface Protein-1 (MSP-1) on their T cell receptors, making them able to respond specifically to the *Plasmodium* MSP-1 antigen. All mice were maintained at the Appalachian State University vivarium and kept on a 12:12 light/dark cycle. All mice were cared for under the guidelines set by the Appalachian State approved IACUC (protocol 20-10). To understand human chronic malarial disease caused by *P. falciparum*, we used the rodent strain called *P. chabaudi*. This rodent strain causes chronic blood-stage malaria and similar pathology, as seen with the human malaria disease caused by the *P. falciparum*. All Thy1.1 mice were infected with $1x10^5$ of *P. chabaudi* infected red blood cells (iRBCs).

Experimental Diets

To test the effects of moderate malnutrition, two diets were used:

A well-nourished diet (Control) comprised of 17% protein and sufficient in all micronutrients, including zinc and iron. A moderate malnutrition diet (Mal) consisted of 3% protein and was deficient in zinc and iron. Both diets contained the same amount of calories to induce moderate malnutrition. The experimental mice were fed their specific diets for four weeks to induce moderate malnutrition before the adoptive transfer of B5 TCR transgenic $CD4^+$ T cells is performed.

Weights and Percent Weight Change of Thy1.1 BALB/c Mice

a) Weights Before Infection

During the four weeks that the Thy1.1 BALB/c mice were fed their specific diets, each mouse was weighed using a scale (Intelligent Weighing Technology, Camarillo, CA) in grams. Each weight was then input into a Google Excel sheet to determine the average and standard error of the mean (SEM) for the well-nourished (Control) and moderate malnourished (Mal) group.

b) Percent Weight Change

After the four weeks period, the Thy1.1 BALB/c mice were infected with *P. chabaudi*, then weighed continuously until day 9 post-infection (day of sacrifice). Each weight was input into a Google Excel sheet to determine percent weight change. Percent weight change was calculated by subtracting the weights on each day post-infection from the day of infection divided by day zero and multiplied by 100 as shown below.

$$\%\ Weight\ Change = \frac{weight\ on\ day\ post\ infection - weight\ on\ day\ of\ infection}{initial\ day\ zero}\ x\ 100$$

Spleen Weights and Lengths of Thy1.1 BALB/c Mice

After Thy1.1 BALB/c mice were sacrificed, their spleens were harvested and weighed using a scale (Intelligent Weighing Technology, Camarillo, CA) in grams. Each spleen was put on a white plate and measured with a ruler in centimeters. All spleen weights and lengths measurements were input into a Google Excel to determine average and SEM.

Adoptive Transfers

$CD4^+$ T cells were purified from the spleens of the B5 TCR transgenic (Thy1.2) using the Miltenyi Biotec CD4 T cell isolation kit (Miltenyi Biotech, San Diego, CA). This kit selected for $CD4^+$ T cells by magnetic microbeads. Once the $CD4^+$ T cells were purified, $1x10^6$ T cells were transferred to each experimental Thy1.1 mouse. All mice were then infected with $1x10^5$ of *P. chabaudi* iRBCs one day after cell transfer. The recipient mice were continuously fed on the experimental diets for the duration of the experiment until day nine post-infection when mice are sacrificed.

Flow Cytometry

a) Surface staining

Spleens were collected from the Thy1.1 mice in ISCOVEs media, mashed through mesh screens, and lysed with 1x RBC lysis buffer. The cells were then resuspended in complete ISCOVEs media and counted using a hemocytometer. An aliquot representing $3x10^6$ cells for each sample were transferred into new 5-mL round-bottom polystyrene tubes. The cells were washed using FACS buffer supplemented with 2% FBS and 0.01% sodium azide, then incubated with Fc receptor blocking antibody for 20 minutes. After 20 minutes, a master mix of antibodies was added for surface staining to determine: CD4 T cells activation (CD44, CD62L, Thy1.2, CD4) and CD25/CD127 panel (CD25, CD127, Thy1.2, CD4) and incubated for 40 minutes. The stained cells were then washed, resuspended in FACS buffer, and filtered with nanomesh screens for data collection by flow cytometry using FC500 Beckman Coulter (Indianapolis, IN).

b) Intracellular staining

For intracellular cytokine staining, an aliquot of $3x10^6$ cells of each sample were stimulated with a cell stimulation cocktail (Tonbo Bioscience, San Diego, CA), except for an unstimulated control for four hours. Cells were then harvested after four hours and stained with CD4 and Thy1.2 antibodies for surface staining followed by a 40 minute incubation in the fridge. The cells were then fixed with 2% Paraformaldehyde for 20 minutes. After washing, the cells were permeabilized using perm/wash buffer, and stained with IFN-γ and TNF-α antibodies for 30 minutes. The stained cells were then washed three times with perm wash buffer to remove excess unbound antibodies and filtered for data collection using FC500 Beckman Coulter flow cytometer (Indianapolis, IN).

Real-Time PCR

RNA was isolated from spleen purified $CD4^+Thy1.2^+$ cells from using the Qiagen RNeasy Plus Mini Kit (Qiagen Sciences Inc., Germantown, MD) at day 9 post-infection. To make cDNA from the RNA, the High-Capacity cDNA Reverse Transcription Kit was used (Applied Biosystems). The samples were then loaded into a thermal cycler (Eppendorf 5341 Mastercycler epGradient Thermal Cycler) and stored at 4°C until qRT-PCR was done. To amplify the survival genes, the cDNA was mixed with the SYBR Select Master Mix (Applied Biosystems) and *Bcl2, Bcl-xL* and *Gapdh* primers shown in **Table 1**. All primers were bought from Invitrogen Inc. All PCRs were analyzed in real time using the 7500 Real-Time PCR System and software from Applied Biosystems.

Table 1. Primers used for Real-Time PCR analysis.

Gene	Forward Primer	Reverse Primer
Bcl-2	GAGTCCCAGCCTCCGTTAT	GCATCCCAGCCTCCGTTAT
Bcl-xL	ACCACCTAGAGCCTTGGATCC	TCTCGGCTGCTGCATTGTT
GAPDH	GATGGGTGTGAACCACGAGA	AGATCCACGACGGACACAT

Data Analysis

Raw data collected from the flow cytometer were analyzed using FlowJo software (Ashland, OR). Data calculations were done in Microsoft Excel. Graphs and statistics on analysis were performed using the Prism GraphPad version 8 software (San Diego, CA). Statistical evaluations were done using student's two-tailed T test to compare pairs of groups and some experiments that were five group comparisons were done using non-parametric One-way ANOVA in Prism GraphPad version 8 software (San Diego, CA). A p value of less than 0.05 was considered significant.

Chapter 3

RESULTS

Infected Thy1.1 malnourished mice lost more weight after Plasmodium chabaudi infection

Malnutrition in the form of lower nutrient content leads to loss of weight over time. To induce moderate malnutrition, but not bias our study, we fed different groups of mice on a well-nourished control or moderate malnourished diets and monitored their weights over a four-week period. The malnourished mice slightly lost some weight in the first week of feeding, then remained constant for the remainder of the three weeks, while the mice that were fed the well-nourished control diet did not lose any weight (Fig. 3A)**.** The mice were then infected with $1x10^5$ malaria parasites and weights were monitored from day zero (day of infection) to day nine which represents the peak of infection. We continued to weigh the mice after infection to determine if there would be a change in weight due to the infection. To do this, we calculated the percent weight change. The malnourished mice lost more weight overall starting on day three post-infection and continued to lose up to day nine, while those fed on the control diet only lost slightly at the peak of infection (days eight and nine) (Fig. 3B)**.** This suggests that moderate malnutrition contributes to severity of malaria disease as the malnourished mice lost more weight after infection.

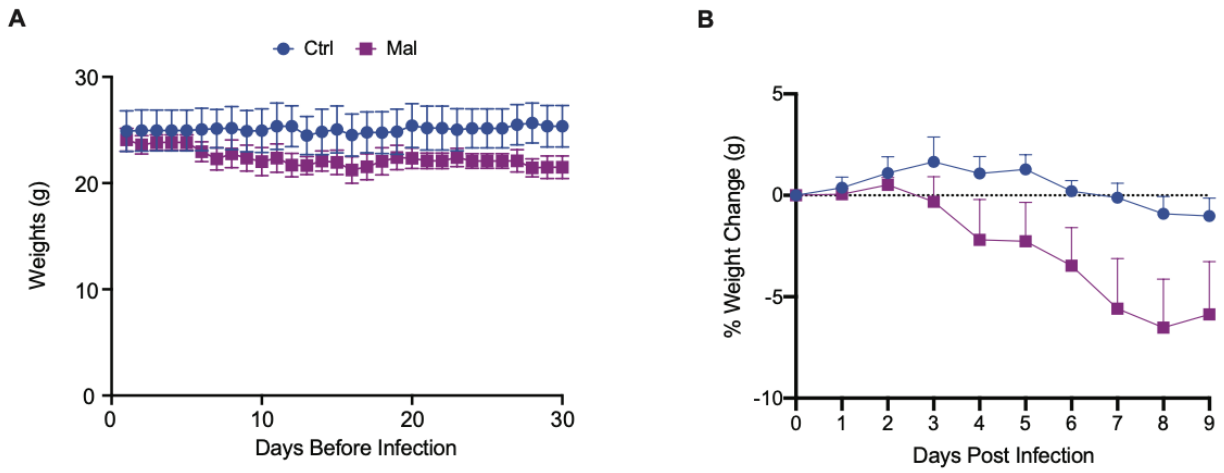

FIGURE 3. Infected malnourished mice lost more weight than control fed mice after infection. **(A)** Thy1.1 mice were weighed in grams for four weeks. **(B)** Percent weight change was calculated by subtracting the weights on each day post-infection from the day of infection divided by the initial day zero and multiplied by 100 to get percent change in weight. Data represents ten Thy1.1 mice per group. Error bars represent SEM. Ctrl = Control diet, Mal = Malnourished diet.

Infected malnourished Thy1.1 mice have less heavier spleens after malaria infection

Malaria infection in both mice and humans is characterized by splenomegaly as a result of increased confinement of the parasite, damaged red blood cells and immune cells in the spleen. To determine if moderate malnutrition had an impact on splenomegaly, we sacrificed the mice on day 9 post-infection and harvested the spleens from both well-nourished and malnourished groups, measured the spleen lengths, and weighed them. Looking physically at the spleens of the infected mice, both control and malnourished fed mice had enlarged spleens as demonstrated by their lengths (Fig. 4A). Even though the spleen lengths were not significantly different between the control and malnourished groups when measured, the spleens from the control fed mice weighed significantly more than the spleens from the malnourished group (Fig. 4B).

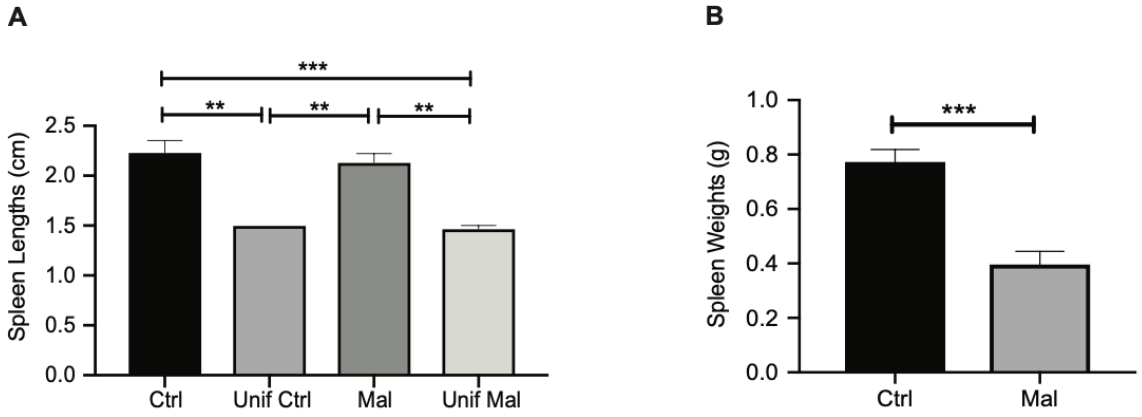

FIGURE 4. Infected control mice have heavier spleens than malnourished mice after infection. Thy1.1 mice were sacrificed day 9 p.i. and spleens harvested. **(A)** Spleen lengths from infected (Ctrl, Mal) and uninfected (Unif Ctrl, Unif Mal) Thy1.1 mice. **(B)** Spleen weights from infected Ctrl and Mal Thy1.1 mice. Data represents 3-5 mice per group from 2 independent experiments. Error bars represent SEM and significance was determined by a One-way ANOVA (** $p < 0.01$, *** $p < 0.001$) and two-tailed t-test (*** $p < 0.001$). Ctrl = Control diet, Unif Ctrl = Uninfected Control diet, Mal = Malnourished, Unif Mal = Uninfected Malnourished diet.

Moderate malnutrition diet does not affect the number of lymphocytes and polyclonal CD4$^+$ T cells

Since infected malnourished mice lost more weight after infection accompanied by spleens weighing less than the control fed mice, we sought to investigate if this had an impact on the number of lymphocytes between the groups. Lymphocytes including both B and T cells are important during chronic infections such as malaria. When looking at the number of lymphocytes using flow cytometry, there was no significant difference between the groups after infection, even though the uninfected naïve mice had significantly lower numbers. This result indicates that the malnourished diet did not affect the number of lymphocytes in response to infection (Fig. 5A). We next looked at the CD4 T cell population, as they provide a helper function for other immune cells during an infection. Infected mice on both diets did not have a difference in the number of CD4 T cells, suggesting that the

malnourished diet does not affect the number of polyclonal CD4 T cells despite having spleens that are lighter. Interestingly, the background representing naive CD4 T cells were significantly higher in the uninfected control mice (Fig. 5B).

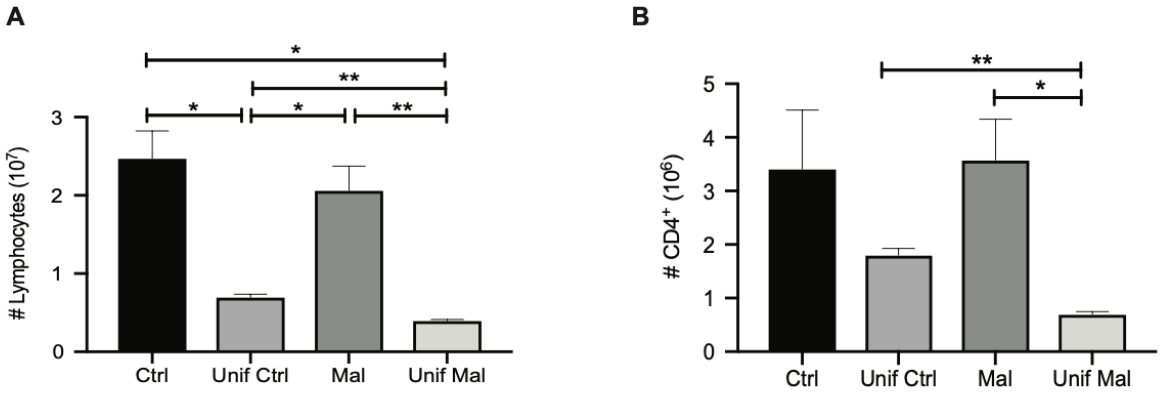

FIGURE 5. Malnourished diet does not affect the number of lymphocytes and polyclonal CD4$^+$ T cells in infected Thy1.1 mice. Thy1.1 mice were sacrificed on day 9 p.i. **(A)** The number of lymphocytes from Thy1.1 mice that received adoptive transfer of B5 Tg CD4$^+$ T cells (Ctrl, Unif Ctrl, Mal, Unif Mal) and Thy1.1 mice that did not receive any cells (NC). **(B)** Number of CD4 T cells determined from the lymphocytes. Data represents 3 mice per group. Error bars represent SEM and significance was determined by a One-way ANOVA (* $p < 0.05$) and two-tailed t-test (* $p < 0.05$, ** $p < 0.01$). Ctrl = Control diet, Unif Ctrl = Uninfected Control diet, Mal = Malnourished, Unif Mal = Uninfected Malnourished diet.

Moderate malnutrition diet does not affect the number of CD25$^+$ CD127$^-$ CD4 T cells

Because we observed no significant effect on the polyclonal CD4 T cell response in the malnourished mice in figure 3, we wondered if their activation was affected. When CD4 T cells are activated, they respond to IL-2 via IL-2 receptor, which is also known as CD25. In addition, activated effector cells down regulate CD127 or the IL-7 receptor, which help promote survival of naive T cells. We therefore wanted to determine if the diet affects the number of activated effector cells measured by higher expression of CD25 and lower expression of CD127 in the polyclonal CD4 T cells population. Again, malnutrition had no observable impact on the number of activated CD4 T cells defined as CD25$^+$ CD127$^-$, and the

uninfected control had significantly higher background representing activated phenotype (Fig. 6).

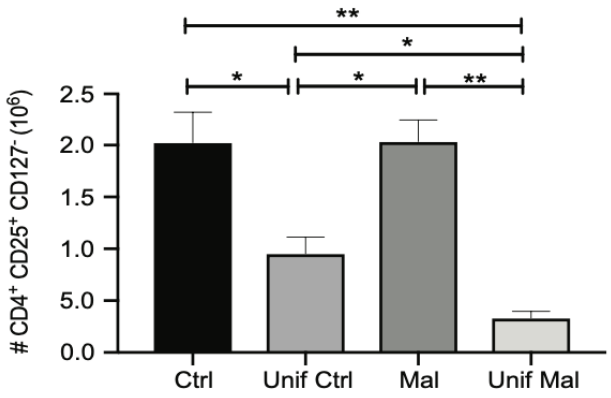

FIGURE 6. Malnutrition does not affect the number of $CD25^{+}CD127^{-}$ in infected Thy1.1 mice. Thy1.1 mice were sacrificed at day 9 p.i. The activation of the cells was determined by looking for $CD25^{+}$ $CD127^{-}$ CD4 T cells. Data represents 3 mice per group. Error bars represent SEM and significance was determined by a One-way ANOVA (* $p < 0.05$, ** $p < 0.01$) and a two-tailed t-test (* $p < 0.05$). Ctrl = Control diet, Unif Ctrl = Uninfected Control diet, Mal = Malnourished, Unif Mal = Uninfected Malnourished diet.

Moderate malnutrition decreases the number of malaria-specific $CD4^{+}$ T cells

While polyclonal $CD4^{+}$ T cells provide some cross-protection against disease, this protection is limited. Since we did not observe any differences in the effect of moderate malnutrition on polyclonal CD4 T cells, we employed an adoptive transfer model to determine the effect of this diet on malaria-specific CD4 T cells. We adoptively transferred $1x10^{6}$ purified MSP-1 TCR transgenic CD4 T cells that express Thy1.2 into Thy1.1 BALB/c mice that are fed the control or moderately malnourished diets (Fig. 7A). The recipients were then infected with $1x10^{5}$ *P. chabaudi* the same day. MSP-1 TCR transgenic $Thy1.2^{+}$ cells within the Thy1.1 mice splenocytes were determined at day 9 post-transfer (Fig. 7A). We observed slightly lower numbers of $Thy1.2^{+}$ malaria-specific $CD4^{+}$ T cells from the moderate

malnourished (Mal) recipients compared to the well-nourished (control) group (Fig. 7B). Just like the polyclonal CD4 T cells, slightly fewer Thy1.2 cells were recovered in the uninfected malnourished mice, compared to their uninfected control counterparts. Even though there were no significant differences, these results suggest that the moderate malnourished diet slightly decrease the malaria specific $CD4^+$ T cells.

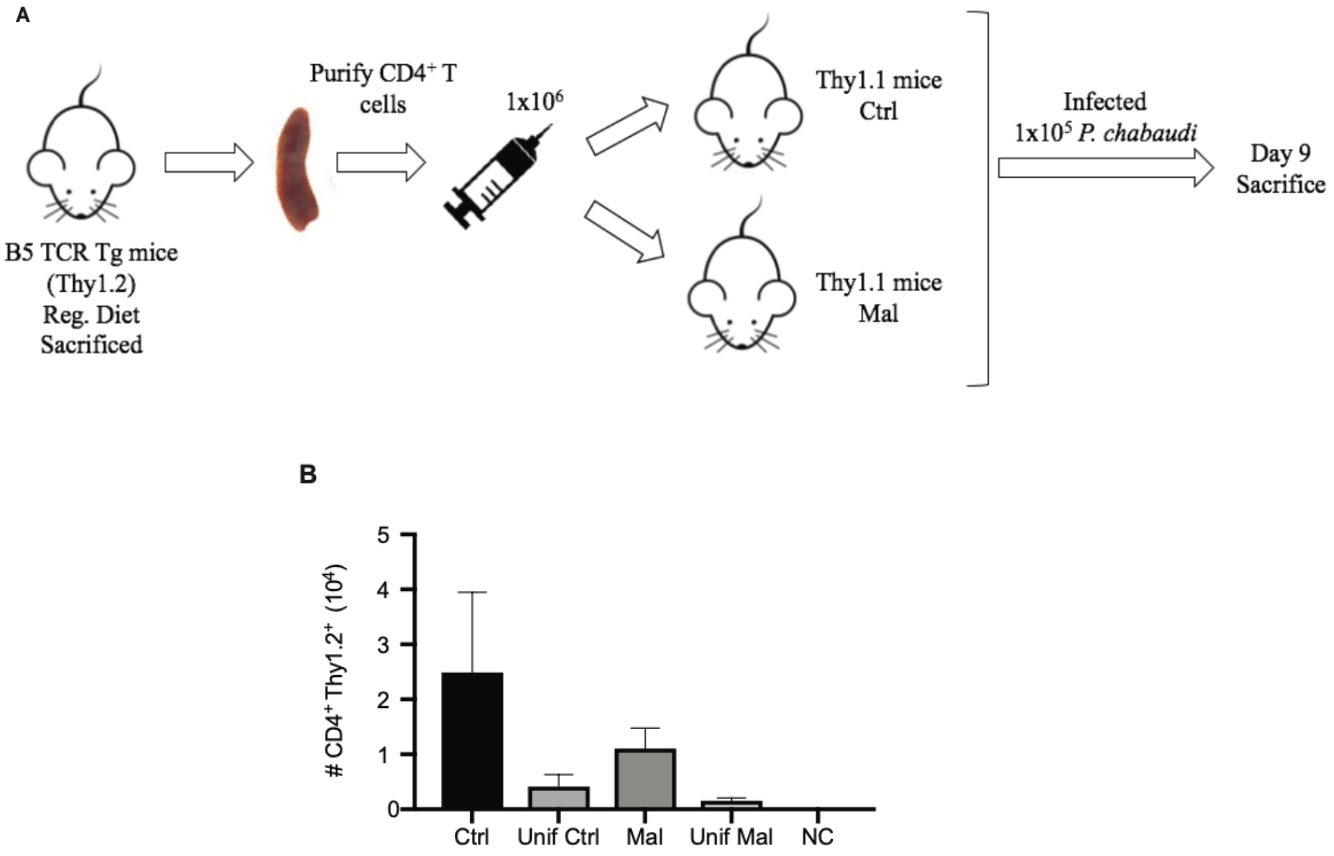

FIGURE 7. Malnourished diet decreases the survival of malaria specific $CD4^+$ T cells. **(A)** Ctrl and Mal Thy1.1 mice received $1x10^6$ of purified $CD4^+$ T cells from B5 TCR Tg mice. All Thy1.1 mice were then infected with $1x10^5$ *P. chabaudi* the following day and sacrificed on day 9 p.i. **(B)** Spleen cells were surfaced stained using anti-CD4 and Thy1.2 to determined MSP-1 transgenic T cells that are malaria-specific by flow cytometry. Data represents 3 mice per group. Error bars represent SEM. Ctrl = Control diet, Unif Ctrl = Uninfected Control diet, Mal = Malnourished, Unif Mal = Uninfected Malnourished diet, NC = No-Cell controls.

Moderate malnutrition reduces activated malaria-specific effector CD4$^+$ T cells

Since we observed lower number of Thy1.2 malaria-specific CD4$^+$ T cells in the mice fed on the malnourished diet, we wondered if this decrease was associated with lower activation status of malaria-specific effector CD4$^+$ T cells. During the early stages of malaria infection, effector T cells are generated to induce cell-mediated immunity in response to the infection. Upon activation, effector T cells can produce inflammatory cytokines and cytotoxic molecules to help eliminate the infection (78). Therefore, higher activation will lead to more functional effector T cells. Using CD44, and CD62L, we observed lower numbers of activated effector malaria-specific CD4$^+$ T cells in the moderate malnourished group than the control group after infection (Fig. 8). Taken together with figure 5, these data suggest that the activation of effector malaria-specific CD4$^+$ T cells is decreased during moderate malnutrition.

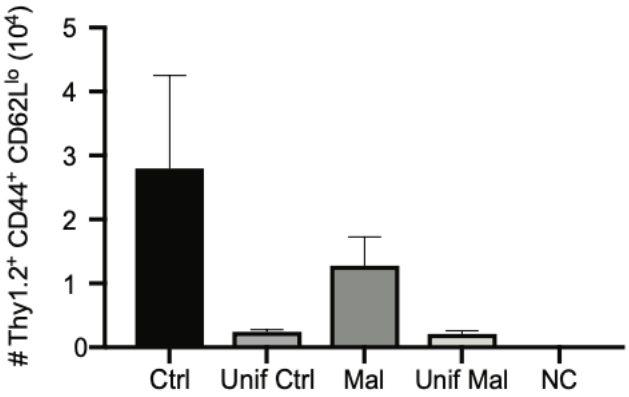

FIGURE 8. Moderate malnutrition reduces the number of activated malaria-specific effector CD4$^+$ T cells. The Ctrl and Mal Thy1.1 mice that received Thy1.2 donor cells were sacrificed on a day 9 p.i., and spleens were harvested. Spleen cells were surfaced stained with anti-Thy1.2, CD44, and CD62L to determine activation by flow cytometry. Thy1.2 is a surface marker for MSP-1 transgenic T cells that are malaria-specific. CD44 is a surface marker for effector CD4$^+$ T cells, and CD62L is a surface marker for naïve CD4$^+$ T cells. Data represent 3 mice in each group. Error bars represent SEM. Ctrl = Control diet, Unif Ctrl = Uninfected Control diet, Mal = Malnourished, Unif Mal = Uninfected Malnourished diet, NC = No-Cell controls.

Moderate malnutrition decreases cytokine secretions from malaria-specific effector CD4$^+$ T cells

With lower numbers of activated malaria-specific CD4 T cells in the malnourished donor cells, we next investigated the effect of malnutrition on the functionality of the malaria-specific CD4 T cells. Thus, we determined IFN-γ and TNF-α production by the recovered Thy1.2 donor cells upon stimulation *ex vivo* with a cell stimulation cocktail, which helps activated cells to produce cytokines. We observed lower production of IFN-γ (Fig. 9A) and TNF-α (Fig. 9B) by Thy1.2 cells from the malnourished group even though it was not significantly different from the control group. This suggests that moderate malnutrition affects the functionality of malaria-specific effector CD4$^+$ T cells to secrete important cytokines for protection against the infection.

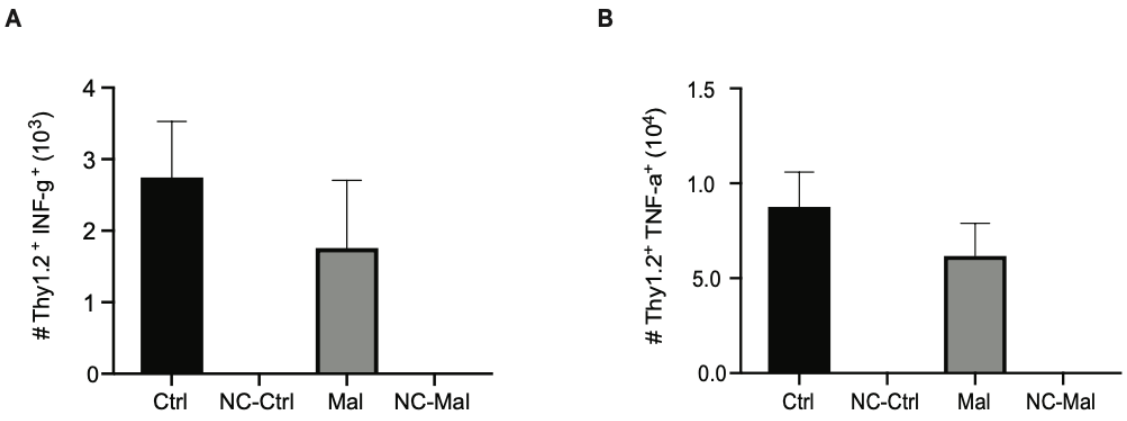

FIGURE 9. Moderate malnutrition slightly decreases cytokine secretion by the malaria-specific effector CD4 T cell**.** Thy1.1 mice spleen cells were intracellularly stained with IFN-γ and TNF-α antibodies on day 9 p.i. **(A)** Number of IFN-γ and **(B)** TNF-α from infected Thy1.1 mice. Data represent three mice in Ctrl and Mal group and two mice in NC-Ctrl and NC-Mal group. Error bars represent SEM. Ctrl = Control diet, NC-Ctrl = No-Cell Control diet, Mal = Malnourished, NC-Mal = No-Cell Malnourished diet.

Moderate malnutrition decreases malaria-specific effector CD4$^+$ T cell survival

An important aspect of immune response to chronic infections such as malaria is the survival of responding antigen-specific cells. We therefore hypothesized that the slight reduction observed in Thy1.2 malaria-specific cells (Fig. 7) may be due to a decrease in survival of these cells. Two anti-apoptotic proteins from the Bcl-2 family that are commonly known to induce T cells survival, are Bcl-2 and Bcl-xL (59, 79). Therefore, we determined the expression of *Bcl-2* and *Bcl-xL* genes within purified malaria-specific effector CD4$^+$ T cell population using Real-time PCR. Consistent with our hypothesis, we observed lower *Bcl-2* and *Bcl-xL* expression in the uninfected malnourished group compared to the uninfected control (Fig. 10A, 10B). Upon infection, *Bcl-2* was down regulated in the malnourished mice, but surprisingly, *Bcl-xL* was upregulated in the malnourished mice compared to the uninfected malnourished group. These data suggest that Bcl-2 may be downregulated in moderate malnutrition after infection, but Bcl-xL may play a compensatory role to promote survival of the malaria-specific effector CD4$^+$ T cells at least by day 9 after infection.

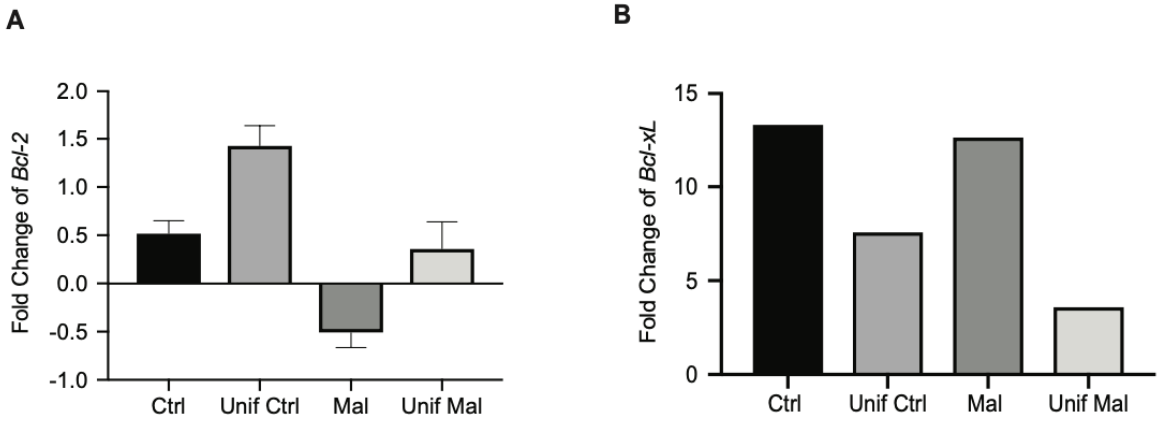

FIGURE 10. Moderate malnutrition decreases *Bcl-2* but not *Bcl-xL* in malaria-specific effector CD4$^+$ T cells. RNA was isolated from purified CD4$^+$Th1.2$^+$ cells at day 9 p.i. Fold change of *Bcl-2* **(A)** and *Bcl-xL* **(B)** within the Thy1.2 population. Data represents three mice per group. Error bars represent

SEM. Ctrl = Control diet, Unif Ctrl = Uninfected Control diet, Mal = Malnourished, Unif Mal = Uninfected Malnourished diet.

Chapter 4

DISCUSSION

Although there are strategies in improving malnutrition and preventing malaria transmission, deaths caused by the disease are still high, and vaccine studies are limited by the lack of knowledge in the direct effect of moderate malnutrition on the development of effector malaria-specific $CD4^+$ T cells. To enable progress in this area, our laboratory used a transgenic mouse model and the rodent malaria strain, *P. chabaudi*, which has been used mostly in laboratories to study chronic malaria (9).

Mouse models are useful to understand infections because they have physiological similarities to humans (80). These similarities enable scientists to investigate the progression of diseases, which is more difficult to study in humans (10, 81). It is more feasible to manipulate different things in a mouse model to mimic human diseases and, therefore, understand how different parameters can alter immune cell populations (80). In this study, we employed the adoptive cell transfer technique to investigate the effects of moderate malnutrition on effector malaria-specific $CD4^+$ T cells that are known to be protective during malaria infections (52).

Inadequate micronutrient intake causes malnutrition and can lead to weight loss. Most studies found that high rates of malnutrition in children are due to a deficiency or inadequate intake of micronutrients leading to weight loss (26, 82, 83). In animal models, it has also been demonstrated that mice fed a malnourished diet lost more weight overall compare to a control group (84, 85). In our study, we did not see a loss of weight in the Thy1.1 mice before infection. Instead, the mice adjusted to the diet within a week and their weights remained constant. This could possibly be due to the fact that both diets contained the same

amount of calorie content. Furthermore, this confirms that we induced moderate malnutrition as opposed to previous studies which investigated general malnutrition.

There has been a significant body of research over the years on the relationship between malnutrition and infections (86–88). Researchers suggest that people who are malnourished are at risk for diseases and suffer long-term infections (86–88). Infections are mediated by the effect of nutritional deficiencies on the immune defense of an individual (86, 87). Various deficiencies can hamper immune cell functions, thereby enhancing the susceptibility of an individual to a number of infections (87). Previous studies have suggested that malaria may increase the severity of malnutrition, while malnutrition may increase the risk of malaria infections (38, 89, 90). Despite these suggestions and ongoing research in this area, the relationship between malaria and malnutrition remain complex as some studies suggest that malnutrition may be protective against malaria, while others indicate that malnutrition increase malaria associated morbidities and death (44, 91).

During a malaria infection, individuals tend to lose more weight (90, 92). Support for this observation has also been seen in animal models showing that malnourished mice had increased weight loss during a malaria infection (86, 93). Similar to these previous reports, the malnourished Thy1.1 mice in our study lost more weight overall compare to the control group after a *P. chabaudi* infection. This decrease in body weight change is possibly due to the malaria infection causing appetite loss leading to rapid weight loss, and a deficiency in important micronutrients like zinc, iron, and protein that are critical for normal organ functions in the body (92).

In addition to weight loss, splenomegaly is another symptom associated with malaria infection. As shown in previous studies, infected protein energy malnourished mice had

enlarged spleens, but the spleens were less heavy (84, 94, 95). Similar observations were seen in our current study, despite us using a moderately malnourished diet which was deficient in zinc, iron, and low protein content, but similar calorie concentration between the diet. Thus, this may suggest that these micronutrients are critically important, and their absence could cause deformities in the spleen structures and architecture, making the spleens weigh less (84, 96).

The spleen consists of many critical immune cells that are part of the innate and adaptive immunity. Cellular and humoral immune responses are important during a malaria infection, where they can elicit an effective immunity together (97). It has been demonstrated in experimental murine models that moderately malnourished mice had reduced lymphocytes in their spleens (98, 99), however, this was not the case in our study. This suggests that, with adequate calorie intake as is the case with our moderate malnutrition diet, there might not be defects in the white pulp of the spleen which is mostly comprised of lymphocytes (96, 100), despite having lighter spleens.

Cell mediated immunity is largely $CD4^+$ T cell-driven and is essential for most *Plasmodium* infections and protection against malaria in humans (97). Importantly, there is evidence in experimental murine models that T cells are necessary for the development of protective immunity to blood stage malaria infections (101–103). However, malnutrition has been found to alter T cell numbers by affecting both T cell survival and proliferation (104). In our current study, the number of polyclonal $CD4^+$ T cells were not affected by malnutrition. Two possible explanations for this would be 1) the fact that we used a moderate malnourished diet with similar caloric content to the control diet and 2) only malaria-specific T cells respond to the *Plasmodium* parasite.

When $CD4^+$ T cells are activated by an antigen, they produce interleukin-2 (IL-2), which is a cytokine that mediates proliferation and clonal expansion of T cells (105, 106). IL-2 binds to the IL-2 receptor (CD25) on activated T cells which regulates tolerance and immunity (107). Studies in both malnourished infected children and mice have shown that there are fewer activated $CD4^+$ T cells because of an impairment of IL-2 and its receptor, CD25 (84, 86, 98, 108). However, this was not observed in our current study with the malnourished Thy1.1 mice that were infected with *P. chabaudi*. This observation may support the idea that polyclonal T cells are not significantly affected by moderate malnutrition during a *P. chabaudi* infections (98, 99). While we did not measure the concentrations of IL-2, it would be interesting to determine how this proliferative cytokine is affected by the malnourished diet.

Adoptive transfer experiments allowed us to test the response and functionality of malaria-specific $CD4^+$ T cells using a transgenic T cell receptor (TCR) that recognizes the MSP-1 molecule of the parasite. Upon adoptive transfer of MSP-1 TCR Tg Thy1.2 splenocytes into Thy1.1 BALB/c recipients, we observed lower numbers of malaria-specific $CD4^+$ T cells in the malnourished mice, similar to observations from investigators using other infectious agents (103, 109). This suggests that the expansion of malaria antigen specific $CD4^+$ T cells require proper micronutrients. Indeed, the lower numbers were accompanied by lower activation shown by CD44/CD62L markers for responsive and expanding CD4 T cells.

$CD4^+$ T cells are a major source of IFN-γ in response to both liver and blood stage malaria (71). Studies have demonstrated that IFN-γ is responsible for protective immunity against *Plasmodium* parasites (70, 71, 110). An early production of IFN-γ after a malaria infection has been correlated with protection against the development of clinical symptoms

of malaria in some studies (71, 111). Mice that are IFN-γ deficient and infected with *Plasmodium* are unable to control malaria infection and showed delayed elimination of the parasites (70). These experimental murine models show that IFN-γ is an essential pro-inflammatory cytokine for controlling blood-stage *Plasmodium* parasites (70, 71).

TNF-α also play an important role in the development of immunity and pathology in malaria infections (112). However, TNF-α has been shown to have an effective anti-parasitic activity (74). TNF-α is found circulating during the erythrocytic phase of a malaria infection in humans and mouse models (46, 74). Some studies have shown that TNF-α inhibits the hepatic development of different rodent malaria strains along with *P. falciparum* (74). In experimental murine models, mice that were injected with recombinant TNF-α were protected against blood stage infection with *P. chabaudi*, while mice deficient for TNF-α controlled the *P. chabaudi* blood infections less efficiently (74). This suggests that TNF-α is essential for malaria infection control (76).

However, malnutrition combined with malaria infection can lead to lower productions of both IFN-γ and TNF-α (84, 113). It has been shown in human models that infected malnourished children had a significant decrease in IFN-γ and TNF-α production (84, 113). This has been confirmed in experimental murine models that malnutrition impaired the ability of T cells to proliferate and produce cytokines as well (104, 114). According to our study, moderate malnutrition slightly affected the functionality of effector $CD4^+$ T cells to secrete important cytokines. This suggests that T cells are highly influenced by nutrients uptake, and changes in nutritional status can alter their functionalities to elicit proper immune response against infectious agents (104).

As the malaria infection progresses to the chronic phase, some effector T cells survive to become memory T cells. This transition is controlled by the Bcl-2 family proteins to either inhibit or induce apoptosis of the effector T cells (58, 59). This survival also depends on the IL-7R. Two important anti-apoptotic members of the Bcl-2 family proteins that help with T cells survival, are Bcl-2 and Bcl-xL. Bcl-2 is known to bind to pro-apoptotic proteins, like Bax, to inhibit apoptosis, through blocking cytochrome c release thereby preventing apoptotic protease activating factor 1 (APAF-1) and caspase-9 activation (115). Even though Bcl-xL binds to Bax and Bak, just like Bcl-2, it's mechanism of action is not well known. Some investigators have reported that Bcl-xL and Bcl-2 differ in their mechanism of inhibiting apoptosis (116, 117).

Malnourished murine models show low level expression of the anti-apoptotic Bcl-2 family proteins after an infection (84, 118). However, other studies have found that the anti-apoptotic Bcl-2 family proteins were upregulated in malnourished mice (119, 120). According to our study, moderate malnutrition decreased *Bcl-2* gene after malaria infection, but *Bcl-xL* gene was upregulated compared to the uninfected malnourished controls within the malaria-specific $CD4^+$ T cell population. This could be due to these genes acting differently in a control and malnourished environment or the different mechanism of inhibiting apoptosis as reported by other groups (116, 117).

The survival of these malaria-specific effector $CD4^+$ T cells were determined at the effector timepoint, during this time, a majority of effector cells are undergoing cell death, and may not be the best timepoint for survival proteins. Because the parasite is not completely eliminated, the effector cells could upregulate Bcl-xL to promote their survival as they fight the present parasite at this time point. Therefore, it would be interesting to investigate these

survival molecules after 60 days of infection, when true memory cells are present in this infection model. In addition, investigating the expression of other anti-apoptotic and pro-apoptotic proteins of the Bcl-2 family at both the effector and memory timepoints can help make better conclusions on the survivability of these cells and how these genes change based on the duration of infection or change from the effector to memory phase during moderate malnutrition.

After elimination of an infection by the effector cells, a small population remain that transition into memory to protect from the chronic nature of the infection (121). These antigen-specific memory T cells survive for years following initial exposure to an antigen, and play an essential role for long-term maintenance of protective immunity against reinfection (122). However, malnutrition can cause memory T cells to be dysfunctional by affecting their development, maintenance, and function (123). Therefore, it is highly possible that the proportion of memory T cells that develop later are affected by the moderate malnourished diet as well. The memory T cell population at day 60 post-infection would need to be investigated in the future.

In conclusion, our current report shows that moderate malnutrition slightly reduced the malaria-specific effector $CD4^+$ T cell population, probably due to lack of expansion, as there were less activated cells in the malnourished mice that are infected with malaria. Malnutrition combined with malaria infection can reduce antigen-specific CD4 T cells, which play a significant helper role in the immune system. Also, there needs to be a balance of nutrients to produce a robust immune response to the infection. A deficiency in certain nutrients like zinc, iron, and protein, can alter how immune cells function. A good nutrient diet can help regulate the immune response to function better. More research focused on the

impact of how moderate malnutrition affect survival of these antigen-specific cells during the memory phase can help immunologists and vaccinologists develop ideas on how to make these immune cells survive longer and maintain a stronger activation status.

Future mechanistic investigations to determine how moderate malnutrition influence the survival of the malaria-specific $CD4^+$ T cells can determine the signaling pathways downstream of important survival cytokines such as IL-7R (CD127), including the JAK/STAT family of proteins. Upon IL-7R engagement, JAK1 phosphorylation leads to a signaling cascade through PI3K or STAT5 that promote an increase in Bcl-2 family proteins hence survival and viability of immune cells (124). While we did not investigate the IL-7R in the malaria-specific cells, down regulation of Bcl-2 protein in the malnourished infected mice suggest that the molecules downstream of key survival receptors such as IL-7R on the cell surface may be affected by the diet. In addition, we would look at differences in the expression of key caspases that induce programed cell death.

The decrease in spleen weights, but similar numbers of lymphocytes and polyclonal CD4 T cells was unexpected. We speculate that the overall spleen architecture may be deformed due to 4 weeks of exposure to moderate malnutrition. Indeed, other investigations in the lab has shown that our malnutrition model may lead to gut leakage and potentially gut microbial dysbiosis (125). Thus, leakage of gut microbiota could impact peripheral organs including the spleen. In addition, immune response to this microbiota could result in deformities in the lymphoid organ architecture. This can be investigated by determining the white pulp and germinal centers in the control and malnourished groups.

Chapter 5

CONCLUSIONS

While it is known that malnutrition decreases immune response in general, our data suggest that moderate malnutrition can decrease the activated effector $CD4^+$ T cells that could be protective against chronic infections like malaria. We observed that infected Thy1.1 mice on the malnourished diet lost more weight overall compared to the infected control mice and had less heavier spleens even though the spleen lengths were similar in sizes. Despite having less heavier spleens, we observed that moderate malnutrition does not affect the number of lymphocytes, polyclonal $CD4^+$ T cells, along with $CD25^+$ $CD127^-$ $CD4^+$ T cell. Upon adoptive transfer of malaria-specific cells, we observed that moderate malnutrition decrease effector malaria-specific $CD4^+$ T cells that express Thy1.2 molecule, along with reduced numbers of activated population and their cytokine production. We also found that without proper nutrients intake, *Bcl-2* expression is downregulated, but *Bcl-xL* may play a compensatory role, hence promote some kind of survival during the effector phase in the malaria-specific $CD4^+$ T cells. Overall, we propose that moderate malnutrition does impair pathogen specific $CD4^+$ T cell populations, which may have a significant effect on other immune cells. Future research will focus on investigating the effect of moderate malnutrition to memory $CD4^+$ T cells in malaria infection.

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Vita

Emily Xiong was born in Valdese, North Carolina, in 1996. At an early age, she knew she wanted to pursue a career in the health and science field. Therefore, during her high school years, she took certified nursing aid (CNA) courses and did clinicals at Shaire Center located in Lenoir, North Carolina. With bigger dreams and goals, and to expand her knowledge and interests in science, she attended Western Carolina University where she received her bachelor's degree in biology with a pre-med concentration. During her times at Western Carolina, she had a passion and interest of learning disease pathology, medicine, and immunology. Therefore, these interests led her to further her education by pursuing a master's degree in cell and molecular biology at Appalachian State University, where she joined the Opata Lab.

She attended the North Carolina American Association of Microbiology (NC-ASM) conference in 2019-2020 and was able to present her work virtually in 2020. She also attended the Office of Student Research Annual Celebration of Student Research and Creative Endeavors in 2021 and was able to present her work virtually. She has also received awards and funding from the Student Faculty and Excellence (SAFE) Fund and the Office of Student Research.

Outside of academics, she enjoys fishing in the spring and summer time as well as hiking along the blue ridge mountains. She also enjoys walking downtown in big cities and enjoying her coffee and matcha from Starbucks. Apart from this, she loves traveling and trying new foods.