IMMUNOLOGICAL EFFECTS OF *MORINGA OLEIFERA* ON MALARIA AND MALNUTRITION DURING *PLASMODIUM CHABAUDI* INFECTION

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Abstract

IMMUNOLOGICAL EFFECTS OF *MORINGA OLEIFERA* ON MALARIA AND MALNUTRITION DURING *PLASMODIUM CHABAUDI* INFECTION

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Malaria is a worldwide problem that affects millions of people yearly, especially in rural areas where life-saving anti-malarial drugs are not easily accessible. Residents in such rural areas also suffer from malnutrition due to limited supply of varieties of nutritious foods, thus they use herbal plants for both the treatment of various diseases and as nutritional supplements. One such herbal treatment, that also serves as a nutritional supplement, is *Moringa oleifera*. Both malaria and malnutrition are believed to be the associative cause of about half of the deaths in the world and children are most affected, suffering the greatest morbidity and death. As Moringa is used as a treatment regimen in malaria endemic areas, studies to determine its immunological effects on the infection are limited. Therefore, we hypothesized that in addition to suppression of parasite as reported by other investigators, Moringa treatment increases T helper 1 immunity in response to malaria infection.

Using a rodent parasite strain, *Plasmodium chabaudi* that mimics disease manifestations of the most virulent strain of human malaria, *Plasmodium falciparum,* we

observed that Moringa treatment reduced parasite burden compared to the untreated controls. Interestingly, mice treated with high dose Moringa (60 mg/mouse) for a short time (7 days) or low dose Moringa (30 mg/mouse) for a longer time (3 weeks), exhibited increased numbers of effector $CD4^+$ T cells accompanied by an increase in Tbet expression. To further investigate this, we treated mice with Moringa after infection (curatively) or before infection (prophylactically) and observed that mice that were treated with Moringa after infection exhibited increased IFNγ and TNFα secretion. Surprisingly, the mice that were treated before infection had significantly higher Tbet expression. These data suggest that Moringa treatment leads to $CD4^+$ T cell activation and production of pro-inflammatory cytokines after malaria infection.

To determine the contribution of Moringa on malnourished malaria infected mice, we induced malnutrition by limiting access to food to only 4 hours a day for 4 weeks, while control mice had unlimited access to mouse lab chow. We observed reduced numbers of $CD4^+$ T cells, TNFα proportions, and significantly greater Tbet expression in the food limited group compared to controls. Supplementation with Moringa in the limited group slightly restored $CD4^+$ T cell activation, IL-2, and IL-10 production. Taken together, our results suggest that Moringa may be immunologically useful in the treatment of malaria and malnutrition.

Acknowledgements

I would like to express my sincerest gratitude to my faculty mentor Dr. Michael Opata for this unwavering support, patience, guidance, and expertise throughout this life changing process. I would also like to acknowledge Dr. Nathan Mowa for serving as my co-advisor and encouraging me as well as providing me with Moringa extract and cassava flour. Special appreciation and gratitude to Dr. Maryam Ahmed for her encouragement, time, and dedication as my committee member. Also, my sincerest gratitude to my colleagues in the Opata lab: Kadra Ibrahim, Andrea Deras, Isaac Ogden, and Karla Monroig for their support, encouragement, and hard work throughout this thesis. I would like to give special thanks to Lyndsay Richard for being the ideal lab mate- for all the days and nights we spent in lab in the pursuit of knowledge. I would also like to give thanks to Monique Eckard for her expertise, patience, and kindness towards our laboratory mice. Lastly, I would like to thank my family and in-laws for their unwavering support, love, and encouragement through my ups and downs in this challenging and exciting time in life. I could not have accomplished this without all of you, thank you.

Dedication

I would like to dedicate this thesis to my wonderful husband and best friend. You have seen me through this entire process and have always supported me, even when I was at my worst. Thank you for growing with me and providing love, patience, and encouragement through this difficult and exciting chapter in my life. I hope to experience many more exciting chapters together in the years to come.

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List of Abbreviations

AKI: Acute kidney injury

CHMI: Controlled human malaria infection

Cnt: Control

CSP: Circumsporozoite protein

DI: Deionized

IFNγ: Interferon γ

IL-2: Interleukin 2

IL-10: Interleukin 10

iRBC: Infected red blood cells

Mal: Malnourished

PfSPZ: *Plasmodium falciparum* sporozoite vaccine

P.i.: Post infection

Pre: Moringa Treatment before infection

Post: Moringa Treatment after infection

RBC: Red blood cells

RTS,S/AS0: *Plasmodium falciparum* vaccine

SEM: Standard error of the mean

Tbet: Master transcriptional regulator for T helper 1 cells

Tcm: Central memory T cell

Teff: Effector T cell

Tem: Effector memory T cell

Th1: T helper 1 subset of $CD4^+$ T cells

Th2: T helper 2 subset of $CD4^+$ T cells

TNFα: Tumor necrosis factor

Chapter 1

INTRODUCTION

Malaria is a life-threatening parasitic infection that is caused by four different strains of the *Plasmodium* parasite and impacts millions of people yearly. In 2017 there were 219 million cases of malaria which resulted in 435,000 deaths [1]. Of the four *Plasmodium* parasite strains, *Plasmodium falciparum* (*P. falciparum*) is the most deadly, accounting for almost all of the malaria-related deaths in Sub-Saharan Africa [2].

In *P. falciparum* infection, the parasite goes through 3 phases and involves 2 hosts. The life cycle begins when the female *Anopheles* mosquito takes a blood meal and inoculates *Plasmodium* sporozoites into the mammalian host [3]. These sporozoites will migrate to the liver where they undergo asexual reproduction infecting hepatocytes to form schizonts; a process known as exo-erythrocytic schizogony (liver phase) [4]. This phase is largely asymptomatic and lasts approximately 7-10 days after which the schizont ruptures releasing merozoites into the bloodstream. This is known as the erythrocytic schizogony (blood phase) of malaria infection. The merozoites infect red blood cells and mature into trophozoites, eventually lysing the red blood cells and releasing up to 32 progeny [5]. During this blood phase, clinical symptoms of malaria infection which include fever, headache, chills, vomiting, anemia, and eventually enlargement of the spleen (splenomegaly) are observed [1]. Also, during this phase gametocytes are produced, which can be ingested by other mosquito vectors when they take a blood meal. Once in the mosquito's stomach, the parasite's sexual life cycle (sporogonic cycle) begins [3] and parasite zygotes are generated. These zygotes become motile and elongated, now called

ookinetes. The ookinetes migrate from the stomach to the midgut of the mosquito where they develop into oocysts which later mature into sporozoites [6]. These sporozoites migrate to the mosquito's salivary glands and can be inoculated into another host when the mosquito takes a blood meal [7].

Unlike other *Plasmodium* strains, *P. falciparum*, causes chronic infection where parasite is often present in the blood 50 to 500 days post infection [8]. The host immune response is characterized by a robust T helper 1 (Th1) induced inflammatory response [9]. During the blood stage, merozoites lyse a large number of red blood cells which releases antigens into the bloodstream. These antigens activate resident macrophages and circulating monocytes which phagocytize the free merozoites and present them to $CD4^+$ T cells in the spleen. The naïve T cells are then polarized and express transcriptional regulator Tbet leading to their differentiation into *Plasmodium* specific Th1 cells [10]. The proliferation and accumulation of these immune cells within the spleen causes the splenomegaly observed in malaria infection. These *Plasmodium* specific Th1 cells differentiate into effector and memory cells. Effector $CD4^+$ T cells exit the spleen and enter the bloodstream where they produce large amounts of pro-inflammatory cytokines: TNFα, IFNγ, IL-12, and IL-18 [4]. These pro-inflammatory cytokines induce systemic inflammation and aid in the activation and recruitment of macrophages, B cells, and cytotoxic $CD8^+$ T cells [11,12]. IFNγ and TNFα, most notably, help to boost cell mediated immunity as well as humoral immunity by inducing more monocyte/ macrophage activation and isotype switching in B cells [13]. The B cells produce parasite specific antibodies which assist in neutralization and opsonization of the parasite as well as tagging it for destruction by the complement system [14]. This immune response is unique to

malaria as most parasitic infections such as helminth infections are heavily reliant on early eosinophilic action and are largely T helper 2 (Th2) mediated [15]. *P. falciparum* is capable of suppressing eosinophilia [16] and Th1 mediated systemic inflammation is necessary in early blood phase to generate protective immunity and promote parasite clearance. Th2 responses are necessary to protect against tissue damage due to inflammation, but these occur later in infection [17]. Therefore, the outcome of the infection is heavily reliant on proper timing of these responses by the adaptive branch of the immune system to not only control the infection but to generate protective immunity [9,18].

Naturally acquired protective immunity to malaria has been the topic of research for many years, as it is not generated quickly and requires chronic, repeat infections [9]. An individual will usually be infected before the age of 5 and develop severe malaria leading to complications such as severe anemia, respiratory distress in relation to metabolic acidosis [1], cerebral malaria [19], and acute kidney injury (AKI) [20]. These symptoms are life-threatening, but can easily be alleviated using anti-malarial drugs which clear the parasite by targeting the various life-cycle stages of the *Plasmodium* parasite as well as activating the immune response to control and eliminate the parasite [21,22]. Upon repeat exposure to the parasite, an individual will only develop mild and eventually asymptomatic malaria [23], but without chronic exposure these protective memory $CD4^+$ T cell populations wane over time [24]. This slow development of immunity makes it difficult to develop efficient vaccines against malaria.

To enhance immunity to malaria and reduce mortality rates, there are 2 current vaccine candidates in advanced clinical trials, the RTS,S/AS01 and the PfSPZ (*Plasmodium falciparum* sporozoite vaccine). Both are pre-erythrocytic vaccines aimed at

inducing cell mediated and humoral responses to malaria antigens [25,26]. The RTS,S/AS01 is a recombinant protein vaccine that co-expresses the *P. falciparum* circumsporozoite protein (CSP) sequence on the hepatitis B surface antigen. CSP mediates the transition between mosquito midgut and mammalian host hepatocyte. Therefore, the vaccine induces anti-CSP $CD4^+$ T cells and antibodies which provides protection, with or without vaccine boosters [27]. The RTS,S/AS01 has been shown to possess a 35.9% efficacy for the first year post vaccination but this decreased by 2.5% in the fourth year and 4.4% in the seventh year post-vaccination [27]. The PfSPZ vaccine candidate is an attenuated sporozoite vaccine that is administered three times over 8-week intervals at a dose of $9.0x10^5$ *P. falciparum* sporozoites. Clinical trials have shown that 64% of vaccinated volunteers exposed to controlled human malaria infection (CHMI) remained without parasitemia compared to the unvaccinated controls who all exhibited parasitemia post exposure. Of this vaccinated cohort six underwent repeat CHMI at 33 weeks after final immunization; 83% remained free of parasitemia [28]. This indicates that booster vaccination may enhance protection against the parasite.

The PfSPZ has demonstrated long-term protection in controlled human malaria against a variety of strains of *Plasmodium* by inducing *Plasmodium* specific $CD4^+$ and $CD8^+$ T cells. Despite these results these vaccine candidates are not yet on the market so the current treatment for malaria is by anti-malarial drugs. But drug resistance has been frequently noted in most of the commonly used anti-malarial drugs including chloroquine, sulphadoxine-pyrimethamine, quinine, piperaquine and mefloquine [29]. To combat development of drug resistance, combination therapies of anti-malarial drugs with artemisinin are being used [30]. Although these combination therapies have allowed for

treatment of these resistant strains, recent epidemiological studies have shown the emergence of artemisinin-resistant *P. falciparum* in Thailand, Laos, and Cambodia [31]. This growing resistance calls for the development of effective anti-malarial therapies and *Moringa oleifera* is a promising candidate [32].

Moringa oleifera, known as drumstick tree, is an edible plant from the *Moringaceae* species cultivated in many parts of the world, including sub-Himalayan tracts of India, Pakistan, Bangladesh, Afghanistan [33] and many parts of Africa [34]. It's a perennial, pan-tropical tree characterized by fast growth, drought resistance, and a deciduous nature. It can be eaten raw, cooked, or dried [35] and has been used for centuries to prevent and/or treat a variety of disease in many rural malaria-endemic areas where access to conventional treatment is limited [36,37]. Several researchers have shown that Moringa possess many medicinal properties including: antimicrobial [37-43], anticancer [44], antioxidant [45-47], and anti-plasmodial [30,32,40,48,49]. Traditionally, this herbal tree is used to treat scurvy, purgation, headaches, fevers, otitis, sore throat, bronchitis, eye infections [40], STDs/STIs [41], and malaria [34,50].

Studies performed to determine the anti-plasmodial effect of Moringa showed that it possesses potent suppressive and curative properties in *Plasmodium* infections. A dose dependent suppression of parasite growth up to 90% and an 80% reduction in parasite burden was observed [32]. Using both crude ethanolic and n-hexane Moringa leaf extracts, other investigators showed that the crude ethanolic extract inhibits parasitemia by 74.7-95.6% and n-hexane extract inhibited by 59.3-87.9% [48]. Moringa has also been found to be effective in combination therapies with artesunate. In one study, a dose dependent suppression of parasitemia up to 91% was observed when Moringa is combined with

artesunate, compared to 50% with artesunate alone [30]. On the immune response, a study by Sijabat, and colleagues showed that a combination therapy of methanolic Moringa extract and artemisinin not only reduced parasite burden but increased the percentage of $CD4^+$ T cells in a dose dependent manner [49]. These studies demonstrate that Moringa could be beneficial by inhibiting the parasite directly or activating immune $CD4^+$ T lymphocytes.

In other studies to determine the biochemical compounds with medicinal properties in Moringa, it was reported that Moringa is a rich source of vitamins, essential amino acids, carotenoids, polyphenols, phenolic acids, and flavonoids [37,42,43]. Many of these components are responsible for the plant's medicinal and nutritional attributes. Anti-parasitic effects of Moringa and other plants have been shown to be a result of a variety of chemical compounds concentrated within the leaves, gum, and flowers [51-53]. These compounds include saponins, isothiocyanates, tannins, phenolic compounds, and isoflavones [54,55]. Saponins have been shown to cause permeation of the cell membrane of parasites and induce vacuolization and disintegration of teguments. Isothiocyantes, such as 4-[(α-*L*-rhamnosyloxy) benzyl] isothiocyanate [56] found in Moringa, inhibit energy metabolism which interferes with the motor activity of parasites [54]. Tannins and phenolic compounds uncouple oxidative phosphorylation which inhibits energy generation as well as binds the glycoproteins on cuticles of worms and cause death. Lastly, isoflavaones inhibit enzymes of glycolysis and glycogenolysis, interrupt calcium balance, and negatively impact parasitic nitric oxide activity [54].

In addition to its medicinal activity, Moringa is also been consumed widely as a nutritional supplement in many malaria endemic areas that are effected by malnutrition

[57]. Malnutrition is the implicated with approximately half of the deaths reported in children under the age of five in many countries [58]. The effect of malnutrition on malaria has been the topic of much controversy over the years as some studies suggest that malnutrition exacerbates the already life-threating symptoms of malaria infection, leading to greater malaria morbidity and mortality [59], while others suggest that malnutrition reduces parasitemia, leading to less disease severity [60,61]. Studies have shown that undernourished and stunted children [62] were more susceptible to malaria infection because of a notable reduction in T lymphocytes, impaired antibody production, decreased complement production, and atrophy of the thymus and other lymphoid tissues [63]. This was backed up by another study comparing malnourished, stunted, and wasted children [61]. In their report, the investigators showed that malnourished and stunted children had reduced anti-*P. falciparum* IgG antibodies [64]. Another study to determine the effect of protein energy malnutrition found that controlled trials of vitamin A and zinc supplementation led to significant reduction in clinical malaria attacks [59]. Thus, malnutrition has a direct link with malaria immunity and/or severity.

To combat malnutrition, individuals consume Moringa for its vitamins, minerals, and protein content [65]. The leaves and seeds can be eaten green, roasted, seeped for teas, as well as dried and ground into a powder- commonly added to curries. Moringa is thought to be a high source of fiber, protein, calcium, iron, vitamin C, and carotenoids [33]. Specifically, Moringa provides more than 7 times the amount of Vitamin C in oranges, 10 times the amount of Vitamin A in milk, 9 times the amount of protein in yogurt, 15 times the amount of potassium found in bananas, and 25 times the amount of iron in spinach [66]. Studies have also shown that 8 grams of Moringa leaf powder can provide a toddler with

14% of the protein, 23% of the iron, and 40% of the calcium recommended daily [35]. Studies in a cohort of malnourished children in rural India found that Moringa improved protein energy malnutrition in 70% of grade II malnourished children and 60% of grade I malnourished children [67]. The growing resistance to anti-malarial drugs as well as the nutritional and medicinal properties observed by different researchers make Moringa a promising candidate in both malnutrition and malaria infection.

Therefore, the first aim of our study was to assess the effect of Moringa on *P. chabaudi* parasite burden and malaria immunity. We hypothesized that Moringa would decrease parasite burden and increase the number of activated $CD4^+$ T cells and cytokine secretion. Given that malnutrition is common in many rural malaria endemic areas our second aim was to assess the effect of food limitation induced malnutrition on malaria immunity and determine if Moringa can be used as a supplement to remediate malnutrition and improve immune response. We hypothesized that food limitation would decrease the number of activated $CD4^+$ T cells and impair cytokine secretion, but Moringa would remediate these detrimental effects by increasing the number of activated $CD4^+$ T cells and cytokine secretion.

Chapter 2

MATERIALS AND METHODS

2.1 Mice and parasite

C57BL/6 mice were obtained from Harlan labs and breeding colonies were maintained at the Appalachian State University animal facility under a 12:12 light/dark cycle. All mice were cared for under the guidelines set by the IACUC (protocol 17-04).

The rodent strain of malaria *Plasmodium chabaudi* AS (*P. chabaudi*), an established mouse model for human malaria, was used in this study to mimic the chronic nature of the human parasite strain *P. falciparum*. The parasite was a kind gift from Dr. Robin Stephens at the University of Texas Medical Branch, with permission from Dr. Jean Langhorn (Francis and Crick Institute, UK).

2.2 *Preparation of Moringa pellets and Moringa extract*

Moringa leaves and extract were a kind gift from Dr. Chishimba Nathan Mowa in the Department of Biology at Appalachian State University, or obtained from Natural Market (Boone, NC). Otto's cassava flour was obtained from Earth Fare natural market (Boone, NC). Cassava flour was used in this study to make Moringa pellets as it makes a good binding agent and contains empty calories. Three doses of Moringa pellets were utilized in this study: low, high, and food limitation nutritional dose (see dosages below).

a) Extract

The extract was prepared by extracting Moringa leaves into 100% ethanol and ethanol was evaporated off utilizing a rotary evaporator and resuspended in DI water at a concentration of 1.00g/mL.

b) Low dose Moringa pellets

Moringa pellets were made every 3 days using 10.10g of cassava flour and 30mg of Moringa leaf powder per mouse under a sterile hood. These were mixed with sterile DI water to form pellets and ~2g peanut butter mixed in for flavor enhancement. The control pellets were made using 10.40mg of cassava flour and ~2 g of peanut butter. Six pellets were made formed for both groups and allowed to air dry. Mice were given two pellets daily along with standard mouse chow for 3 days and another batch was made fresh.

c) High dose Moringa pellets

Moringa pellets were made every 3 days using 10.10g of cassava flour and 60mg of Moringa leaf powder per mouse under a sterile hood. These were mixed with sterile DI water to form pellets and ~2g of peanut butter mixed in for flavor enhancement. The control pellets were made using 10.70g of cassava flour and ~2 g of peanut butter. Six pellets were formed for both groups and allowed to air dry. Mice were given two pellets daily along with standard mouse chow for 3 days and another batch was made fresh.

d) *Food limitation nutritional dose Moringa pellets*

Moringa pellets were made every 3 days using 20.10g of cassava flour and 500mg of Moringa leaf powder per mouse under a sterile hood. These were mixed with sterile DI

water to form pellets and ~4g of peanut butter mixed in for flavor enhancement. Twelve pellets were formed and allowed to air dry. Mice were given four pellets daily along with standard mouse chow for 3 days and another batch was made fresh.

2.3 Treatment of mice

a) Moringa experiments

Adult female C57BL/6 mice were utilized in these experiments to determine the effect of Moringa on generation of malaria immunity. Mice were fed Moringa pellets daily for 7 or 23 days before infection (pre-infection) or after infection (post-infection). Control mice were fed control pellets for 7 days. In prophylactic/curative studies mice were fed for 3 weeks before and throughout infection (prophylactic) or 9 days after infection (curative). Mice were infected with a $1x10^5$ dosage of *P.chabaudi* AS and sacrificed at day 9 post-infection (p.i) to harvest spleen for effector time points or day 60 (p.i.) for memory time points.

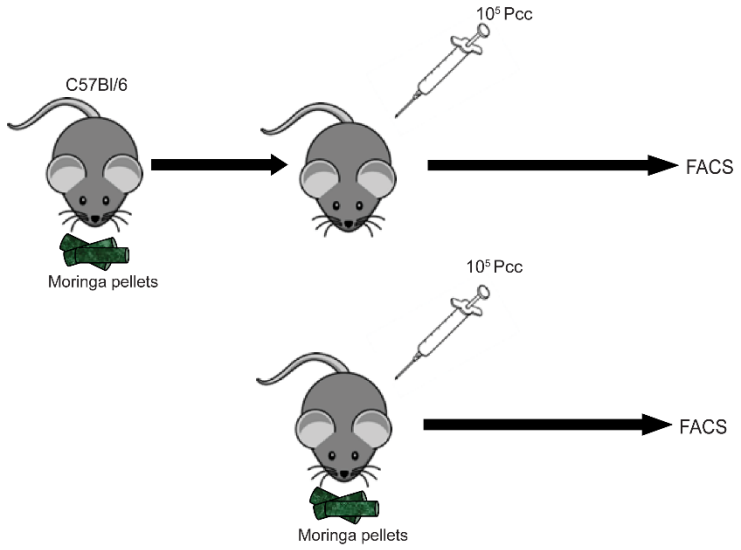

Figure 1: **Schematic for Moringa treatment timeframes.** Mice were treated for either short term (7 days), long term (3 weeks), prophylactically (3 weeks and continuous feeding until sacrifice), or curatively (9 days). Mice were sacrificed at either 9 days post infection (p.i), 23 days p.i., or 60 days p.i. depending on the populations that were studied.

b) Food limitation experiments

Adult and young C57BL/6 mice were utilized in these experiments to determine the effect of food limitation induced malnutrition on malaria immunity. Young mice were weaned at 3 weeks and food limitation began when mice reached a body weight of 10-12g. Food limitation was performed by only allowing the "limited group" of mice to access to standard lab mouse chow for 4 hours daily while controls were given unlimited access 24/7. Mice were weighed every other day or weekly and infected with a $1x10^5$ dosage of *P.chabaudi* AS. Experimental mice were sacrificed at day 9 or day 60 (p.i) to determine immune response in spleen cells.

c) *Food limitation and Moringa experiments*

Adult C57BL/6 mice were utilized in these experiments to determine if Moringa could remediate the effects of food limitation induced malnutrition on malaria immunity. Food limitation was performed by only allowing limited groups access to standard mouse chow for 4 hours daily while controls were given unlimited access 24/7. A third group of the food limited mice were supplemented with Moringa pellets as a nutritional supplement after food was removed. All mice were weighed every other day or weekly and infected with a $1x10^5$ dosage of *P.chabaudi* AS and sacrificed at day 9 p.i. to determine immune response in spleen cells.

2.4 Determining Parasitemia

Parasite burden was determined using thin blood smears obtained by bleeding the tail of the mice at days 3, 5, 7, 8, and 9 post infection with *P.chabaudi* AS. The slides were stained with Diff-Quik and parasites were counted by microscopy in 10 to 50 different fields depending on the parasite load and day of infection. To determine percent parasitemia, the number of infected red blood cells was divided by the total number of red blood cells and multiplied by the counted fields. The outcome was multiplied by 100 as shown in the formula below.

$$\%Parasitemia = \frac{iRBC}{(Total\ RBC)} * cells\ counted\ *100$$

Cells counted= (*total RBC* fields*)

2.5 Flow cytometry analysis

Mouse spleens were collected in ISCOVEs media and mashed through mesh screens to obtain a single cell suspension. Cells were incubated with red blood cell (RBC) lysis buffer to lyse red blood cells which was stopped by adding media. The cells were then resuspended in complete ISCOVEs media. The cells were counted using a hemocytometer and an aliquot was taken for staining for extracellular molecules using fluorochromes FITC, PE, and PE-Cy5, and PE-Cy7 to determine T cell activation. For intracellular cytokine staining, an aliquot from the counted cells were stimulated *in vitro* with a cell stimulation cocktail (Tonbo Biosciences, San Diego, CA) for 5 hours. After 5 hours of stimulation, cells were stained with CD4 FITC and incubated for 40 minutes in the fridge before fixation with 2% Paraformaldehyde. Cells were then permeabilized using

perm/wash buffer (Tonbo Biosciences, San Diego, CA), and stained with IFNγ PE, TNFα PE-Cy7, IL-2 PE, and IL-10 FITC (all from Biolegend, San Diego, CA). Data was collected on an FC500 (Beckman Coulter, Indianapolis, IN) and analyzed by FlowJo (Ashland, OR).

2.6 Data Analysis

All flow cytometry data were analyzed using the FlowJo software (Ashland, OR). The number of cells was determined by the counts taken using the hemocytometer. We determined the average and standard error of the mean for all groups using Microsoft excel number of cells. We ran Student's two-tailed t tests and generated graphs using the Prism GraphPad 5 software (La Jolla, CA).

Chapter 3

RESULTS

3.1 Moringa Experiments

Moringa Treatment Reduces Parasitemia

Given that Moringa possesses anti-plasmodial properties, we sought to determine the effect of Moringa extract and Moringa pellets on parasite burden in infected mice. To do this, we fed Moringa pellets **(Figure 2a)** or extract (via oral gavage) **(Figure 2b)** along with their standard mouse chow. We gave Moringa to the mice for 7 days before (pre) or 7 days after (post) infection with *P. chabaudi* AS. We took blood smears by tail bleeds at days 3, 5, 7, and 8 to determine parasitemia by microscopy. Consistent with literature we observed lower parasite burden in Moringa treated groups, but this was not significantly different from the control mice (**Figure 2**). This data indicates that Moringa treatment with solid pellets or ethanolic extract have an inhibitory effect on parasitemia. As there was no difference in the 2 methods of moringa administration, the rest of the experiments were done with moringa pellets.

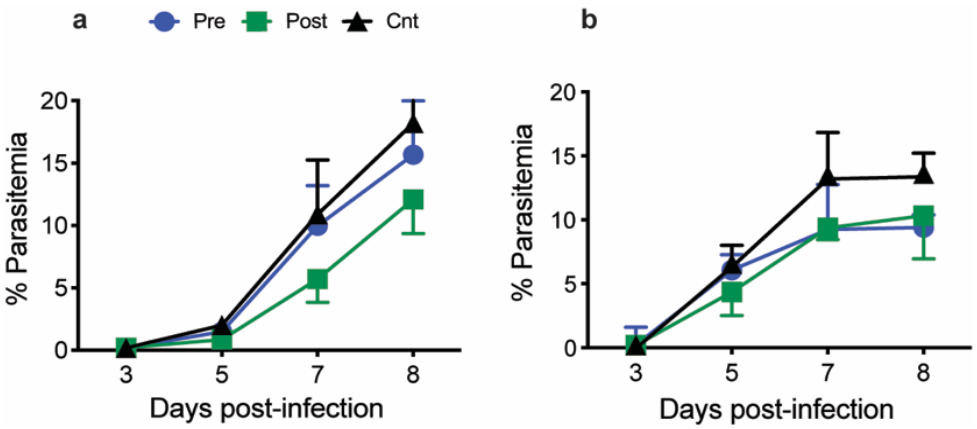

Figure 2: **Moringa treatment decrease parasitemia**. C57BL/6 mice were fed **(a)** Moringa leaf pellets or **(b)** Moringa leaf ethanolic extract for 7 days pre-infection or post infection; control mice were fed peanut butter control pellets. All groups were infected with a $1x10^5$ infected red blood cells (iRBCs) of *P.chabaudi* AS. Graphs show average parasitemia of 3-4 mice per group from two similar experiments. Error bars represent SEM and no significant difference was observed between groups. Significance was determined by two-tailed t test with 95% confidence and p values of >0.05. Pre = Moringa treatment before infection, Post = Moringa treatment after infection, Cnt = Control with no Moringa treatment.

Short term treatment with Moringa pellets post-infection leads to decreased effector CD4$^+$ T cell numbers.

Since Moringa is used for many ailments and our results showed reduced parasitemia in Moringa treated mice, we wondered if Moringa treatment had an effect on immune response. We fed Moringa pellets to the mice for 7 days pre or post infection with *P. chabaudi*. We then sacrificed all mice at day 9 post infection to determine the percentages and numbers of effector CD4$^+$ T cells, B cells, and cytokine secretion. We observed no differences in B cell activation between the groups, (data not shown), but there was a significant reduction in the number of effector CD4$^+$ T cells in mice treated post-infection with Moringa pellets (**Figure 3a**). We did not observe significant differences in the percentage or number of pro-inflammatory cytokines IFNγ and TNFα between the groups (**Figure 3b&c**).

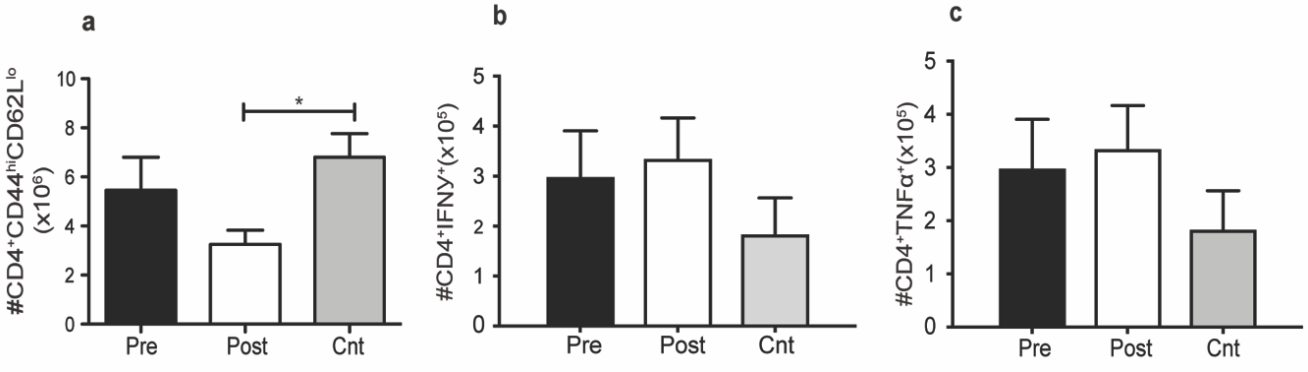

Figure 3: Number of effector T cells are reduced in mice fed on Moringa pellets after infection. C57BL/6 mice fed low dose Moringa pellets for 7 days pre or post-infection with $1x10^5$ dose of *P. chabaudi AS* and control mice were given control pellets prior to infection. Graphs show **(a)** effector T cells identified

using $CD4^+CD44^{hi}CD62L^{lo}$, **(b)** IFNγ and **(c)** TNFα producing $CD4^+$ T cells determined using intracellular cytokine stimulation assay and analyzed using flow cytometry.Data represent 4 mice per groups from 2 independent experiments. Error bars represent SEM and significance was determined by two-tailed t test with 95% confidence, *$p<0.05$. Pre = Moringa treatment before infection, Post = Moringa treatment after infection, Cnt = Control with no Moringa treatment.

High dose Moringa treatment increased IFNγ and Tbet expression in pre-infection treated Mice

Given that individuals in developing nations consistently take Moringa, it is likely that they consume it at a higher dose than the recommended 100g as a nutritive supplement for humans [57]. Therefore, since we observed a reduction in activated effector $CD4^+$ T cells in post-treated mice with the low dose Moringa treatment, we were interested in determining the effect of a higher dose of this treatment on the activation of $CD4^+$ T cells. Mice were given high dose Moringa pellets-60mg per mouse (2-fold increase) per day for 7 days pre or post infection with appropriate controls. The mice were sacrificed on day 9 post infection and percentage and numbers of effector $CD4^+$ T cells were evaluated, as well as pro-inflammatory cytokines (IFNγ & TNFα) and Tbet. We did not observe a significant difference in parasitemia and T cell numbers (**Figure 4a&b**). Interestingly, there was a significant decrease in the proportion of IFNγ production in the post-treated mice **(Fig 4c&d)**. Production of IFNγ and TNFα is associated with increased expression of Tbet, a master regulator of $CD4^+$ T helper 1 (Th1) subset [68]. Therefore, we determined the effect of high dose Moringa treatment on Tbet. We observed an increase in both the percentage (**Figure 4g**) and number (**Figure 4h**) of $CD4^+$ T cells expressing Tbet in the mice that were given Moringa before infection.

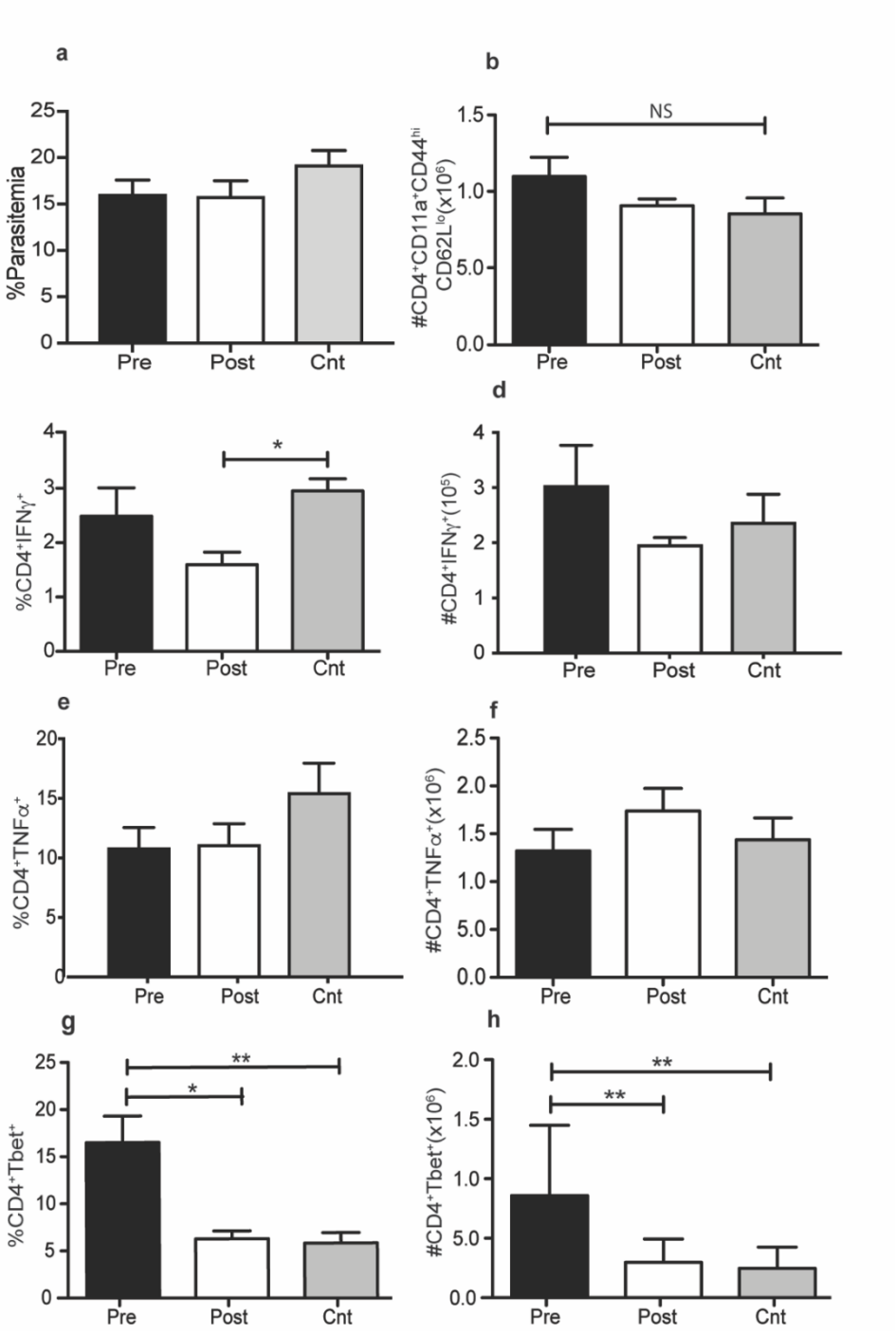

Figure 4. Tbet expression is increased in mice fed high dose Moringa before infection. Mice were fed high dose Moringa pellets (60mg/mouse) for 7 days pre-infection or post infection, including respective controls without Moringa treatment. Mice were infected with a $1x10^5$ dose of *P. chabaudi* AS, and sacrificed at day 9 post-infection. Graphs show **(a)** Percent and (**b)** number of activated effector $CD4^+$ T cells are identified as $CD4^+CD44^{hi}CD62L^{lo}$, (**c,e)** Percent and **(d,f)** number of IFNγ and TNFα producing $CD4^+$ T cells determined using intracellular cytokine staining and analyzed using flow cytometry. **(g)** Percent and **(h)** number of Tbet expression on $CD4^+$ T cells determined using flow cytometry.

Long-term treatment with Moringa pellets post-infection leads to increased numbers of effector and TNFα producing CD4$^+$ T cells

As most people in malaria endemic areas consistently consume Moringa for a longer time and given the reduction in cytokine production that we observed in short term post-infection treated mice **(Figure 3)**, we wondered if long term Moringa treatment would affect the immune response more. To accomplish this, we fed Moringa to the mice for 3 weeks pre or post infection with *P. chabaudi* with appropriate controls that were only fed normal lab chow. We then sacrificed the mice after a total of 6 weeks and determined the percentages and number of effector CD4$^+$ T cells and pro-inflammatory cytokine secretion. Interestingly, when Moringa was given for a long duration (3 weeks), we observed an increase in the number of effector CD4$^+$ T cells in mice treated post infection compared to the pre-infection treated mice and the control groups, but no significant difference in the percentages of these cells (**Figure 5a&b)**. We also observed an increase in TNFα secretion, but no statistical difference in IFNγ secretion (**Figure 5c&d**) between groups.

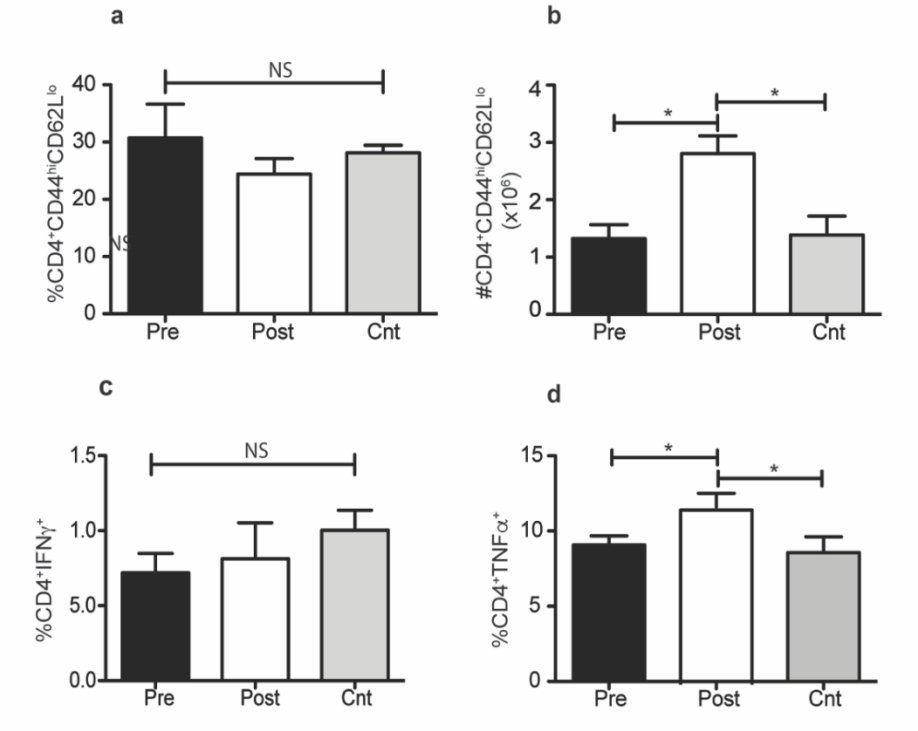

Figure 5: ***Long-term treatment with Moringa pellets increases the number of activated effector and TNF α secretion in post infection treated mice.*** C57BL/6 mice were fed for 3 weeks pre-infection or post infection; control mice were fed control pellets. Mice were infected with a $1x10^5$ *P. chabaudi AS*. Mice were sacrificed 6 weeks later at day 23 post infection. **(a)** Percent and **(b)** number of activated effector $CD4^+$ T cells identified using $CD4^+CD44^{hi}CD62L^{lo}$ and analyzed by flow cytometry. Percentage of $CD4^+$ T cells producing **(c)** IFNγ and **(d)** TNFα respectively. Data represent 3 mice/group. Error bars represent SEM and significance was determined by two-tailed t test with 95% confidence, *$p<0.05$. Pre = Moringa treatment before infection, Post = Moringa treatment after infection, Cnt = Control with no Moringa treatment.

Low dose prophylactic or curative consumption of Moringa leads to increased $CD4^+$ T cell activation and pro-inflammatory cytokine secretion.

Traditionally individuals who consume Moringa for its anti-plasmodial properties consume it either before being sick as a prophylactic or after getting sick for cure (curatively) [50]. To better mimic these conditions, we fed mice a lower dose of Moringa pellets prophylactically for 3 weeks prior to and throughout infection or curatively after infection with *P. chabaudi*. Blood smears were taken on day 9 before sacrifice to determine

parasite and we observed a significant decrease in parasite load in the Moringa treated mice compared to the untreated controls **(Figure 6).** Upon sacrifice, we determined the proportions and number of activated effector $CD4^+$ T cells, cytokine production and Tbet expression. Consistent with lower parasitemia, we observed significantly higher numbers of activated $CD4^+$ T cells in curative (post) treated group compared to controls. The prophylactically treated mice had a trend towards increased cell numbers but did not reach statistical difference (**Figure 6b**). We also observed higher proportions and number of $CD4^+$ T cells secreting pro-inflammatory cytokines (IFNγ and TNFα) in Moringa treated mice compared to control mice **(Figure 6c&d)**. Strikingly, the prophylactic treated group had significantly higher proportions of Tbet expression **(Figure 6e)**. Both groups of Moringa treated mice exhibited increased numbers of $CD4^+$ T cells expressing Tbet compared to control mice (**Figure 6f**).

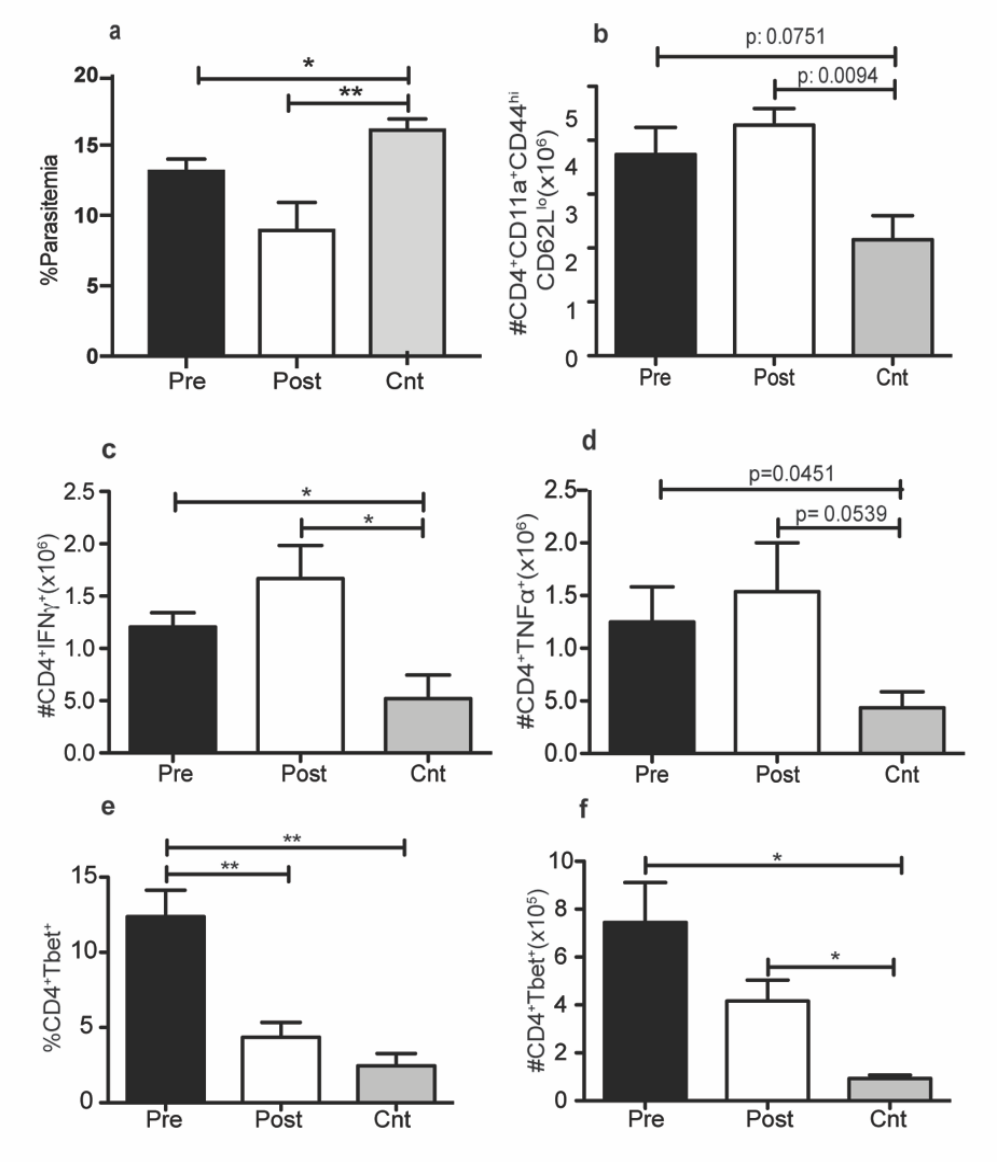

Figure 6: Prophylactic and curative treatment with Moringa increase the number of activated CD4$^+$ T cells, pro-inflammatory cytokines, and Tbet expression after malaria infection. Mice were fed low dose Moringa pellets prophylactically for 3 weeks prior to and throughout infection or curatively after infection with appropriate controls. All mice were infected with a 1x10^5 dose of *P. chabaudi AS* and sacrificed at day 9 post-infection. Summary graphs show **(a)** parasitemia at day 9 p.i, **(b)** effector CD4$^+$ T cells identified as CD4$^+$CD44^{hi}CD62L^{lo}, **(c)** numbers of IFNγ and **(d)** TNFα production by CD4$^+$ T cells determined using intracellular cytokine staining and analyzed using flow cytometry. **(e)** Proportions and **(f)** number of Tbet expression on CD4$^+$ T cells was also determined using flow cytometry. Data represents an average of 5 mice per group and error bars represent SEM. Significance was determined by two-tailed t test with 95% confidence, *p<0.05. Pre = Prophylactic Moringa treatment before and throughout infection, Post = Curative Moringa treatment after infection, Cnt = Control with no Moringa treatment.

Long term prophylactic Moringa treatment lead to increased central memory CD4$^+$ T cell proportions but reduced pro-inflammatory cytokine secretion

Given the results observed in the low dose prophylactic versus curative effector populations observed above, we next investigated the effect of Moringa treatment on the generation of T effector memory (Tem) and T central memory (Tcm). These are especially important in malarial immunity as they are necessary to fight subsequent infections [24]. We accomplished this by feeding the mice low dose (30mg) or high dose (60mg) Moringa pellets prophylactically for 3 weeks prior to and throughout infection or curatively after infection. We sacrificed the mice at day 60 post infection with *P. chabaudi* to determine these memory populations. We observed a reduction in the proportions of Tem in post-infection treated mice, but no difference in cell numbers (**Figure 7a&b**). We also observed an increase in the proportions of Tcm in the pre-treated mice, compared to post-treated and control groups (**Figure 7c&d**). There was an increase in IFNγ (**Figure 7d**), and slightly higher TNFα (**Figure 7e**) in the mice fed Moringa after infection for cure compared to the prophylactic and control groups.

In the high dose we observed similar results, but the reduction in Tem proportion was observed in the prophylactically treated mice compared to the curatively fed mice and controls but no difference in the numbers (**Figure 8a&b**). Similar to the low dose we observed increased proportions of Tcm in the prophylactic compared to the curative and controls but no difference in numbers (**Figure 8c&d**). This was accompanied by an increase in Tbet expression in the prophylactic treated mice, but no statistical significance in cell numbers (**Figure 8i&j**). Surprisingly, pro-inflammatory cytokines were not statistically different but a trend towards increased IFNγ in curatively fed mice (**Figure**

8g&h). We decided to investigate IL-10 and IL-2 expression to determine if this could be due to anti-inflammatory cytokine IL-10 or a defect in IL-2.

Interleukin 10 (IL-10) is an anti-inflammatory cytokine which is secreted during malaria infection to protect against tissue damage by systemic inflammation. Interleukin 2 (IL-2) is an autocrine stimulatory cytokine produced by T cells upon interaction with antigen presenting cell (APC) such as a macrophage or dendritic cell [69]. This interleukin is necessary for the differentiation and proliferation of *Plasmodium* specific Teff cells. We observed increased IL-2 expression in prophylactic mice and a trend towards increased IL-2 in curatively fed mice compared to controls. We observed similar trends in the IL-10 production between the groups although there were no significant differences (**Figure 8 e&f**).

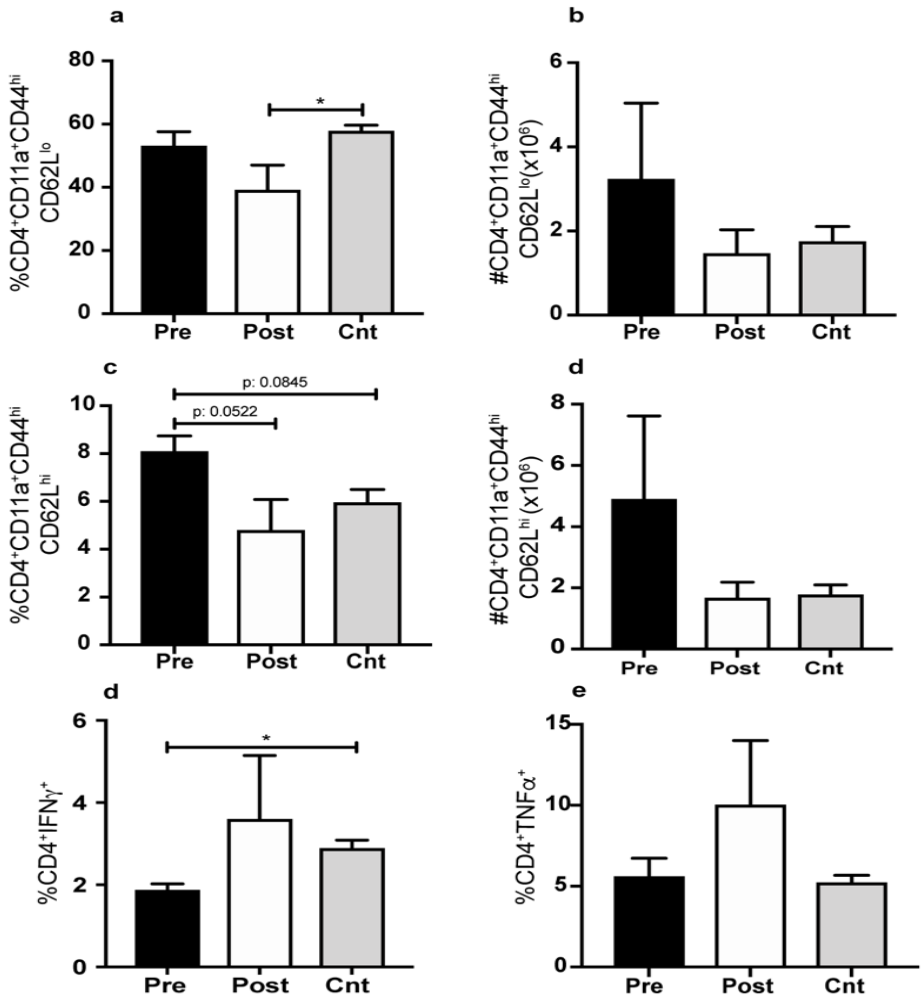

Figure 7: Low dose curative treatment with Moringa decreased the number of activated Tmem proportions and pro-inflammatory cytokines in long term treatment. Mice were fed low dose Moringa pellets prophylactically for 3 weeks prior to and throughout infection or curatively after infection with appropriate controls. All mice were infected with a $1x10^5$ dose of *P. chabaudi AS* and sacrificed at day 60 post-infection. Summary graphs show **(a)** proportions and **(b)** numbers of effector memory $CD4^+$ T (Tmem) cells identified as $CD4^+CD44^{hi}CD62L^{lo}$, **(c)** proportions and **(d)** numbers of central memory $CD4^+$ T (Tcm) cells **(e)** IFNγ and **(f)** TNFα production by $CD4^+$ T cells determined using intracellular cytokine staining and analyzed using flow cytometry. Data represents an average of 3 mice per group and error bars represent SEM. Significance was determined by two-tailed t test with 95% confidence, *$p<0.05$. Pre = Prophylactic Moringa treatment before and throughout infection, Post = Curative Moringa treatment after infection, Cnt = Control with no Moringa treatment.

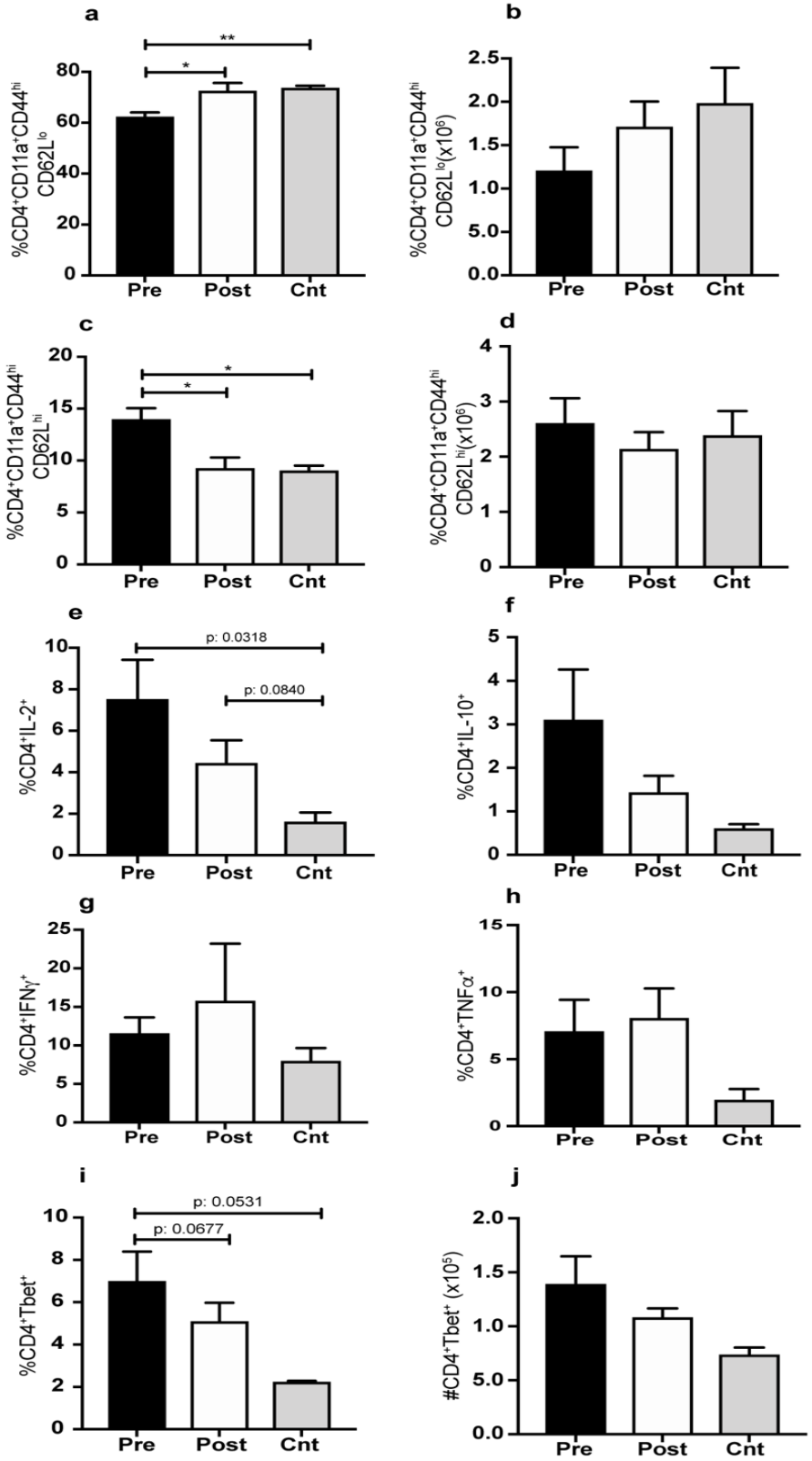

Figure 8: High dose prophylactic and curative treatment with Moringa decreased Tmem proportions but increased Tbet and Il-2 expression in long term treamtent. Mice were fed low dose Moringa pellets prophylactically for 3 weeks prior to and throughout infection or curatively after infection with appropriate

controls. All mice were infected with a $1x10^5$ dose of *P. chabaudi AS* and sacrificed at day 9 post-infection. Summary graphs show Tem **(a)** proportions and **(b)** numbers identified by $CD4^+CD44^{hi}CD62L^{lo}$. Tcm **(c)** numbers and **(d)** proportions $CD4^+CD44^{hi}CD62L^{hi}$. Proportion of IL-2 **(e)**, IL-10 **(f)**, IFNγ **(g),** and TNFα **(h)** production by $CD4^+$ T cells determined using intracellular cytokine staining and analyzed using flow cytometry. **(i)** Proportions and **(j)** number of Tbet expression on $CD4^+$ T cells was also determined using flow cytometry. Data represents an average of 5 mice per group and error bars represent SEM. Significance was determined by two-tailed t test with 95% confidence, *$p<0.05$. Pre = Prophylactic Moringa treatment before and throughout infection, Post = Curative Moringa treatment after infection, Cnt = Control with no Moringa treatment.

3.2 Malnutrition Experiments

Malnutrition induced Tbet expression but reduced effector $CD4^+$ T cell activation

Malnutrition is a common issue in many low income, malaria endemic areas and children are the most affected by malnutrition and malaria infection, as they comprise the highest number of deaths and morbidity observed in many malaria endemic areas [59,70,71]. To understand how malnutrition affects $CD4^+$ T cell immunity against malaria infection, we developed a malnutrition model by food limitation. As malnourished people are exposed to a poor diet from childhood, we utilized 3-week-old mice that weighed 10-13g and limited their access to standard lab mouse chow (4g per mouse) to 4 hours daily, while control mice had unlimited access. The mice were exposed to this pattern of feeding for 4 weeks to induce malnutrition, which was followed by infection with a $1x10^5$ dose of *P. chabaudi* in the third week of limited food feeding. The mice were sacrificed at d9 post-infection and $CD4^+$ T cell, and cytokine secretion profiles were determined. We observed reduced percentages and numbers of effector $CD4^+$ T cells in the malnourished group compared to control mice **(Figure 9a&b)**. There was a slight reduction in TNFα production but no difference in IFNγ (**Figure 9c-f)** . Surprisingly, these mice exhibited increased expression of Tbet compared to controls **(Figure 9 &h)**.

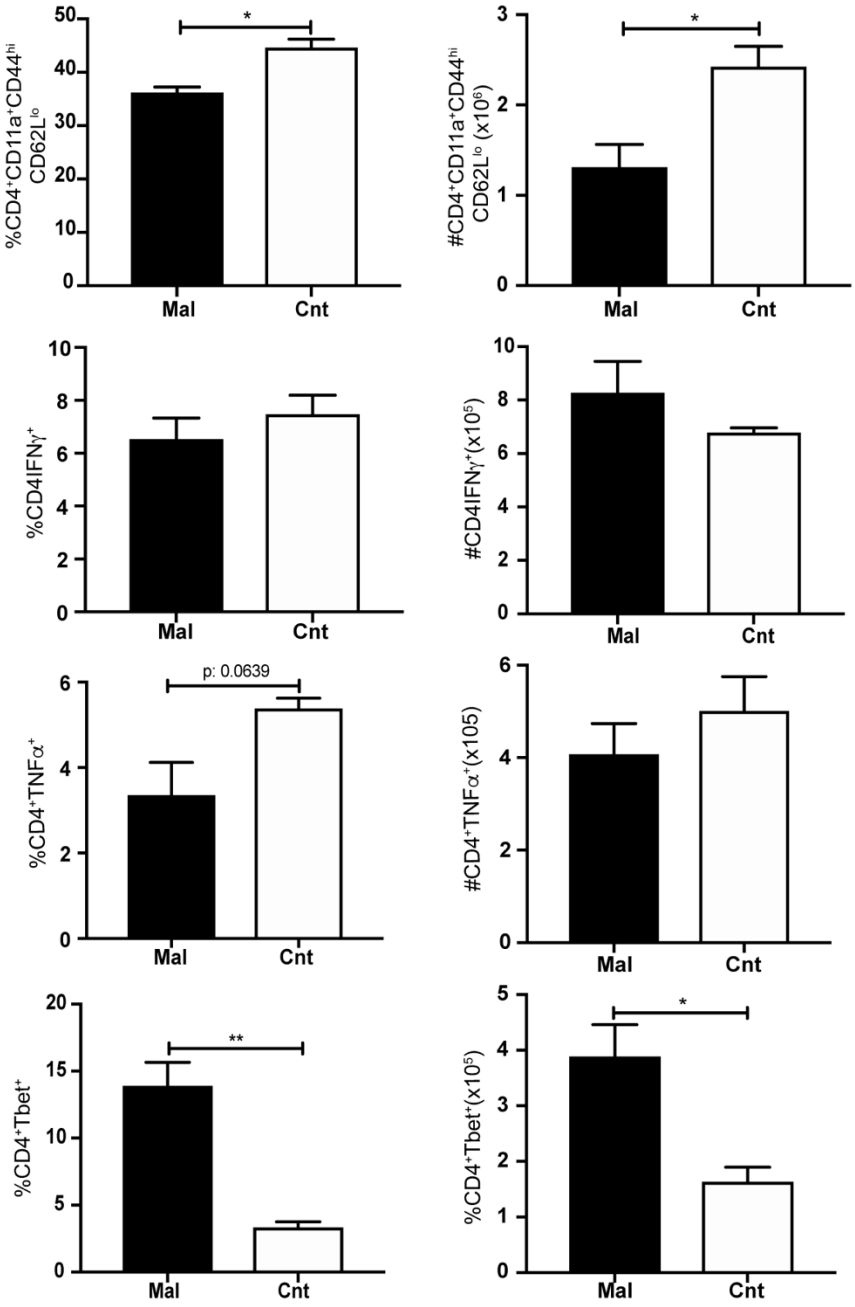

Figure 9: Malnourished mice express high Tbet but reduced effector CD4$^+$ T cells. Young mice (3 weeks old and 10-13g of body weight) were malnourished through food limited by only allowing mice access to food for 4 hours daily for 3 weeks, while controls had unlimited access to food. All mice were infected with a 1x10^5 dose of *P. chabaudi AS* and sacrificed at day 9 post-infection. Summary graphs show **(a)** percent and **(b)** numbers of effector CD4$^+$ T cells identified as CD4$^+$CD44^{hi}CD62L^{lo}. **(c)** Percent and **(d)** numbers of IFNγ, **(e)** Percent **(f)** and numbers of TNFα producing CD4$^+$ T cells determined using intracellular cytokine staining. **(g)** Percent and **(h)** number of Tbet expression on CD4$^+$ T cells was also determined using flow cytometry. Data represents an average of 5 mice per group and error bars represent SEM. Significance was determined by two-tailed t test with 95% confidence, *p<0.05. Lim = Malnourished and Con = Control

Malnutrition induced Tbet expression and reduced pro-inflammatory cytokine secretion by effector memory and central memory CD4$^+$ T cells

Since we observed reduced numbers of effector CD4$^+$ T cells in the malnourished mice, we reasoned that this may affect the generation and survival of memory CD4$^+$ T cells as seen in malaria infection. Therefore, we induced malnutrition in already adult mice by food limitation, as described above, with littermate controls having unlimited access. After 4 weeks of inducing malnutrition, mice were infected with a 1x10^5 dose of *P. chabaudi*. The mice were kept on same pattern of feeding until d60 post-infection at which time they were sacrificed to determine effector (Tem) and central (Tcm) memory T cell proportions and numbers, cytokine secretion profiles, and Tbet expression. We observed no difference in the Tem or Tcm numbers (**Figure 10a&b**), but there was a slight reduction in the proportions of IL-2, and no difference in IL-10 in the malnourished group compared to controls (**Figure 10c&d**). Both IFNγ and TNFα were reduced in the malnourished groups as well, although no statistical difference in IFNγ was observed (**Figure 10e&f**). Consistent with the effector time point, Tbet proportions and numbers were statistically higher in the malnourished group compared to the controls (**Figure 10g&h**).

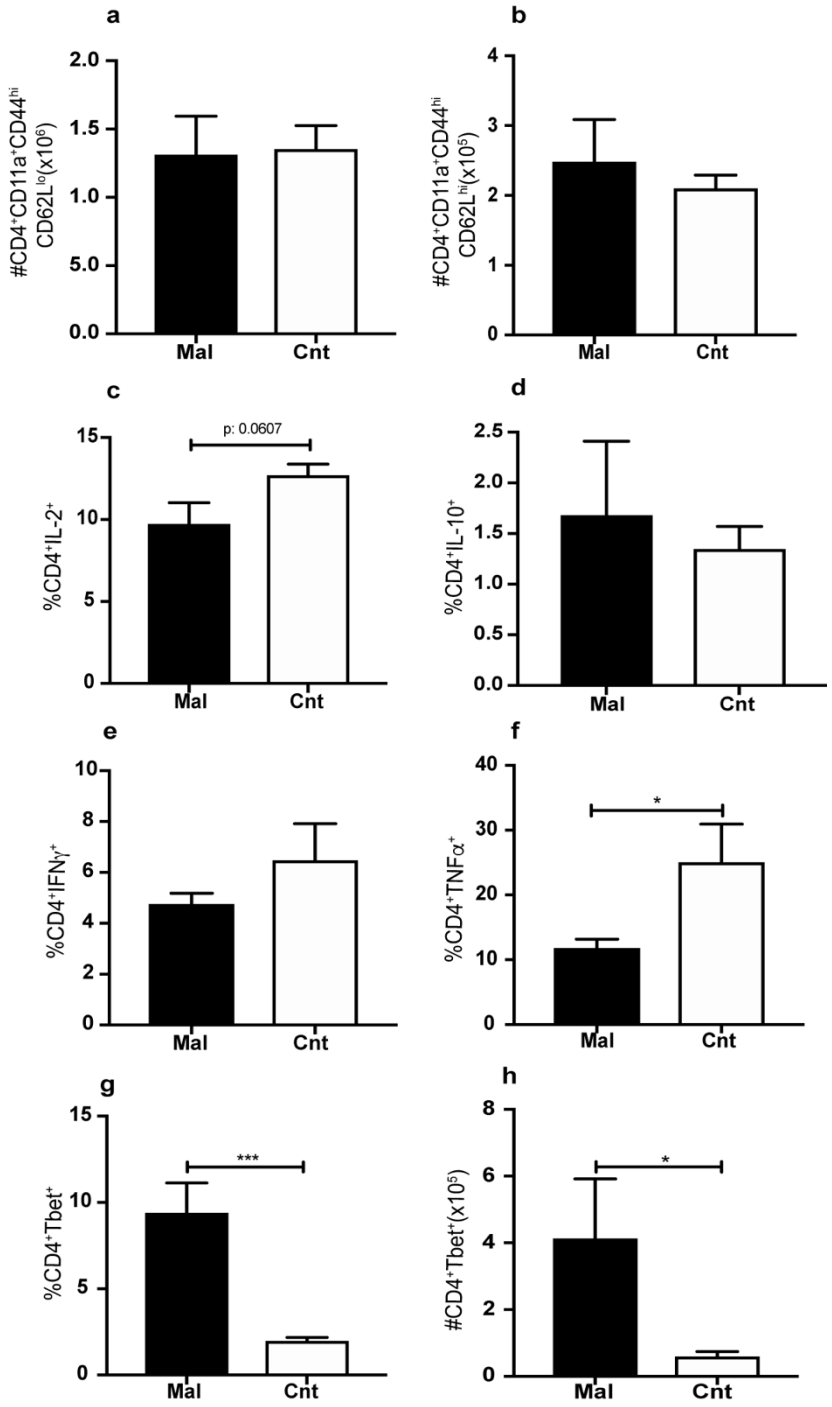

Figure 10: ***Malnutrition did not affect memory T cells, but induced Tbet expression and decreased cytokine secretion.*** Adult mice (6 weeks) were malnourished by 4 hour food limitation daily for 4 weeks or controls given access to unlimited food supply. All mice were infected with a $1x10^5$ dose of *P. chabaudi AS* and sacrificed at day 60 post-infection. Summary graphs show **(a)** numbers of Tem and **(b)** Tcm $CD4^+$ T cells identified as $CD4^+CD44^{hi}CD62L^{lo}$ and $CD4^+CD44^{hi}CD62L^{hi,}$ respectively. **(c-f)** Percent of IL-2, IL-10, IFNγ TNFα producing $CD4^+$ T cells determined by intracellular cytokine staining and analyzed using flow cytometry. **(g)** Percent and **(h)** number of Tbet expressing $CD4^+$ T cells determined using flow cytometry.

Data represents an average of 6 mice per group and error bars represent SEM. Significance was determined by two-tailed t test with 95% confidence, *p<0.05. Mal = Malnourished and Cnt = Control.

3.3 Malnutrition and Moringa

Nutritional supplementation with Moringa increased CD4$^+$ T cell activation in malnourished food limited mice

The reduced numbers of effector CD4$^+$ T cells in malnourished mice was expected, as studies suggest that malnutrition has detrimental effects on immune response [59,67]. Given that Moringa is rich in protein [57] and our results have shown it to increase CD4$^+$ T cells numbers and proportions, we sought to determine if it could remediate the immune suppressive effects of malnutrition. Using the malnutrition model of 4 hours food restriction, we malnourished 2 sets of mice. One group of the malnourished mice was given nutritional Moringa pellets upon food removal for the remaining 20 hours. We observed that Moringa increased Teff proportions compared to limited mice, with trends towards increased cell numbers that did not reach statistical significance (**Figure 11a&b**). Surprisingly we did not observe increased IL-2 secretion in the Moringa supplemented mice, but did observed slightly increased IL-10 secretion (**Figure 11c-f**). IL-10 has been shown to be critical for regulating pathology in malaria infection. In regard to pro-inflammatory cytokine secretion we observed reduced secretion of IFNγ in the Moringa supplemented mice compared to controls, but no difference in TNFα. We also observed increased Tbet expression in the malnourished mice compared Moringa fed mice and controls (**Figure 11i-j**). Taken together these results suggest that Moringa supplementation may ameliorate some of the immune defects induced by malnutrition upon malaria infection.

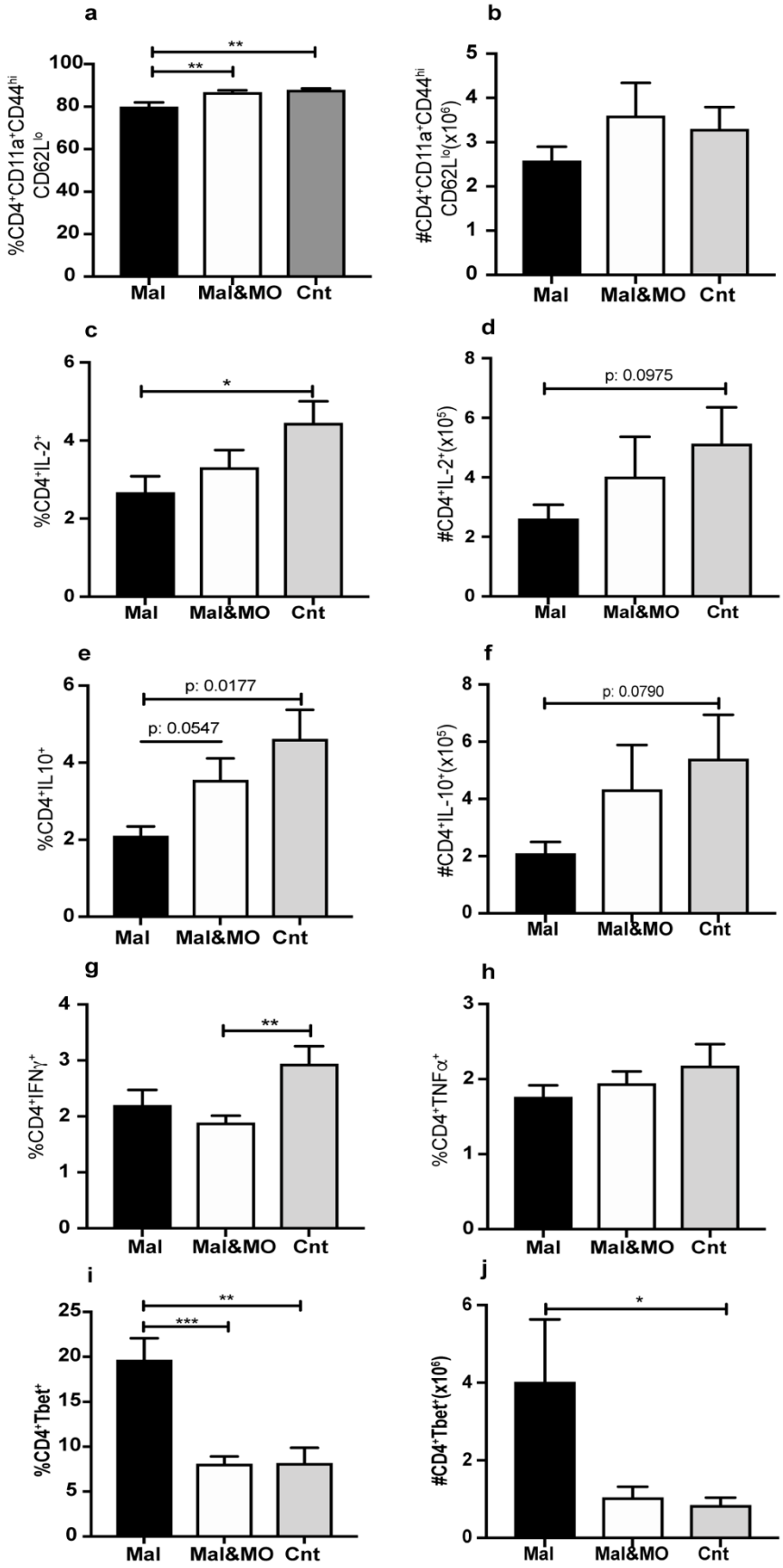
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Figure 11: N*utritional supplementation with Moringa increased Teff proportions in malnourished mice*. Adult mice (6 weeks) were malnourished by food limitation by allowing mice access to food for 4 hours daily at for 4 weeks prior to infection. A second group of malnourished mice were supplemented with Moringa (500mg per mouse), while controls had constant supply of food. All mice were infected with a $1x10^5$ dose of *P. chabaudi AS* and sacrificed at day 9 post-infection. Summary graphs show Teff **(a) Percent** and **(b)** numbers of effector $CD4^+$ T cells identified by $CD4^+CD44^{hi}CD62L^{lo}$. **(c-f)** Percent and numbers of IL-2. and IL-10 producing $CD4^+$ T cells. **(g)** Percent of IFNγ and TNFα **(h)** producing $CD4^+$ T cells determined using intracellular cytokine staining. **(i)** Proportions and **(j)** number of Tbet expression on $CD4^+$ T cells was also determined using flow cytometry. Data represents an average of 5 mice per group and error bars represent SEM. Significance was determined by two-tailed t test with 95% confidence, *$p<0.05$. Mal = Malnourished and Cnt = Control.

Chapter 4

DISCUSSION

Drug resistance to malaria infections has been observed for years and combination therapies with artemisinin have become the normal course of treatment. Quinine and artemisinin combination therapies as well as other anti-malarial drugs are currently used to effectively treat malaria resistant strains of *P. falciparum*[29]. These drugs and adjuncts are plant derived; quinine is derived from the cinchona tree while artemisinin is derived from the sweet wormwood plant [72,73]. But despite their effectiveness, the investigation of other potential antimalarial drugs or adjuncts is greatly needed, as there are new cases of resistance reported to the combination therapies [74]. Therefore, low cost, effective anti-malarial adjuncts will be needed to combat this growing resistance and Moringa is a good candidate as it is already widely used in southern Nigeria for its medicinal and nutritional properties [75]. Moringa is frequently consumed raw, as a vegetable, or added into various foods as a supplement [57]. It is also commonly boiled in water, to make an extract, and used to treat a variety of ailments including: malaria, stomach pains, high blood pressure, stroke, rheumatism, and to ease labor symptoms with a greater than 70% fidelity level [75]. Given that this plant is low cost, easy to grow, and is already commonly used to treat malaria [35,50], we investigated its potential to improve the immune response to *P. chabaudi* as well as remediate the detrimental effects of food limitation induced malnutrition, as studies on this topic are limited.

In our current report we observed that when mice were given a low dose of Moringa for 3 weeks after infection, the number of activated $CD4^+$ T cells in the spleens of the mice increased in response to the *Plasmodium* infection. This was accompanied by increased

proportions of TNFα (**Figure 5).** When we treated the mice with a high dose for a short time, there was no effect on activated effector $CD4^+$ T cells in the treated mice, but long-term treatment increased $CD4^+$ T cell activation and cytokine secretion capability. Surprisingly, while there were no significant variabilities in cell activation or cytokine secretion in the mice treated before infection with high dose pellets, Tbet expression was increased in the effector T cells. Higher Tbet expression in this case promotes Th1 cytokine secretion, which could explain the similar proportions of cytokines secretion seen among the groups as seen by others [36]. In all cases, the treated mice consistently had reduced parasitemia. In some instances, we got a decrease in IFNγ secretion. In our case, we speculate that the timing and dose of treatment may influence the cytokine inhibition and stimulatory effects of Moringa.

Moringa has long been suggested to possess some anti-inflammatory properties [36] and murine studies using Moringa leaves have shown a significant reduction in experimentally induced inflammation [76,77]. The immune response to malaria is characterized by a robust Th1 induced inflammatory response [9]. This robust response is triggered by the expression of transcriptional regulator Tbet [10] which is upregulated when the T cells are primed to secrete IFNγ and other Th1 related cytokines. Unlike most parasitic infections, such as helminth, which rely on eosinophils, *P. falciparum* is capable of suppressing eosinophilia [16], and is characterized by secretion of IFNγ and TNFα [4]. Recent studies have shown production of IL-10 by Th1 cells to regulate the inflammatory response [9]. In some cases, a surge in inflammation due to malaria infection leads to severe and moderate malaria, specifically higher levels of circulatory pro-inflammatory cytokines, including TNFα and IL-6 were indicators for severe malaria, but IL-10 regulates the

outcome [78]. As Moringa is reported to have anti-inflammatory effects [79], it could reduce this surge alleviating the effects that cause severe malaria.

When we tested for curative or prophylactic treatment to mimic field scenarios where people take Moringa continuously, we observed that Moringa treatment inhibited parasitemia in both cases, whether administered for cure or for prophylaxis. This was accompanied by significant activation of $CD4^+$ T cells, cytokine secretion and Tbet expression, a master regulator for Th1 $CD4^+$ T cell subset. Consistently higher Tbet in pre-infection treated mice indicates that early treatment with Moringa may program the cells to be more Th1 biased.

Some of the discrepancies observed in our pre versus post-infection treatment in our study may be due to accumulation of macromolecules. A biochemical safety study on the micro and macro nutrients present in Moringa was performed by I. J. Asiedu-Gyekye and colleagues using an *in vivo* murine model [80]. In their studies to access the macromolecules, they treated mice with a subacute single dose of 5000 mg/ kg and a range of 0 mg/kg to 1000/kg (40, 80, 200, and 1000 mg/kg) for 14 days. White blood cell counts increased by 52.5% compared to controls in their single high dose as well as in their 40mg/kg and 80 mg/kg dosages [80].

In comparison, we performed our studies at 30 mg/g (low dose) and 60mg/g (high dose) and only treated for 7 days or 9 days, compared to 14 days by the other researchers. We believe that immune suppression may occur early in Moringa treatment at suboptimal levels (as our results show), but when treated for longer periods of time at a dosage of 40mg/kg to approximately 80mg/kg there is immune stimulation as shown by I. J. Asiedu-

Gyekye. It is also possible that the content of macromolecules may be higher as we used already processed Moringa from a commercial vendor. Overall, our data, along with the results shown by I. J. Asiedu-Gyekye [80], lead us to believe that there is a delicate balance between the immune system and Moringa dosage that must be maintained to improve the benefits from Moringa's documented immune stimulating and anti-plasmodial properties.

These immune stimulating properties prompted us to determine if prophylactic and curative treatments would stimulate the generation of effector memory and central memory $CD4^+$ T cells. Since both *P. falciparum* and *P. chabaudi* cause a chronic infection, research has shown that these memory T cell subsets are necessary for protection against severe malaria as the infection persists over a long time [81,82]. Central memory T cells are thought to regenerate more of the functional effector memory subsets [24,83]. We observed that both long term low and high dose curative Moringa treatment reduced the proportion of Tem. In both studies we also observed an increase in Tcm cells in mice treated prophylactically. This was accompanied by a reduction in the proportions of IFNγ and a trend towards reduced TNFα (**Figure 7&8**). This was not surprising as there are less parasites at day 60, hence less proinflammatory cytokines produced. As $CD4^+$ T cells persist in chronic infection, this may induce inflammation. Therefore, we sought to determine levels of IL-2 which promotes T cell proliferation and IL-10 which has been shown to down regulate the effects of inflammation in chronic infections [69]. We observed increased IL-2 and a trend towards increased IL-10 in the mice fed prophylactically (**Figure 8**). This suggests that Moringa not only increases Teff numbers and proportions early but also stimulates the production of central memory $CD4^+$ T cells which could be beneficial in subsequent *Plasmodium* infections.

In our second aim we assessed the contribution of malnutrition by food limitation and malaria immunity to mimic field scenarios and determined how Moringa could contribute to alleviating these defects. It has been shown that children who are malnourished suffer more clinical episodes of malaria with greater morbidity [63]. Given that Moringa is commonly consumed as a protein supplement in areas where malnutrition is prevalent [84], we investigated its effect on food limitation induced malnutrition. Many rural areas suffer from malnutrition and lack of access to basic health services [85]. We observed that malnourished mice exhibited reduced Teff proportions and numbers accompanied by a slight reduction in TNFα. We did not observe any difference in IFNγ, expression of Tbet in the food limited mice. As B cells produce antibodies to protect against malaria, we determined the activation of B cells, as they are reliant on $CD4^+$ T cells to perform their function [12]. There was a reduction in the number of activated B cells (data not shown). We theorize that without proper nutrition the immune system is suppressed leading to reduced Teff cells which results in reduced pro-inflammatory cytokine secretion. This would explain the slightly reduced TNFα proportions compared to the control mice. We believe that Tbet is highly expressed as the immune system tries to compensate for the generation of Teff to control the infection (**Figure 9**).

While malnutrition reduced Teff cells, we observed no difference in Tem or Tcm proportions or numbers. There was a reduction in TNFα, but not IFNγ, similar to the effects observed in the Teff at day 9 post infection. As there was no increase in inflammatory cytokines, there was no significant difference in IL-10 production, but IL-2 was slightly reduced (**Figure 10**). To assess the contribution of Moringa in alleviating the decreased immunity induced by malaria in malnourished mice, we supplemented a group of

malnourished mice with Moringa when food was removed. We observed that Moringa supplementation increased T cell proportions compared to the malnourished mice without supplementation as well as increased IL-10 expression compared to the malnourished group. There was also a reduction in IFNγ expression compared to the controls (**Figure 11**).

All these data together suggest that Moringa treatment improves the immune response and remediates immune suppression by malnutrition in *P. chabaudi* infected mice. We believe Moringa does this by both acting on the parasite directly , thereby reducing parasite burden, and activating the immune system. We believe this immune stimulation occurs by activation of Tbet and secretion of IL-2 which promotes the differentiation of Th1 cells into Teff. An increase in these cells increases IFNγ and TNFα production leading to greater parasite control. We observed immune suppression in malnourished mice by a reduction in Teff cells leading to an increase in Tbet expression in this group. We observed that malnourished mice that were supplemented with nutritional Moringa exhibited increased Teff proportions and trends towards increased IL-2, and IL-10 expression. This suggests that Moringa supplementation ameliorates the immune suppression induced by malnutrition as this group exhibits a phenotype similar to control mice. Overall, we believe that Moringa may be useful as an adjunct in the treatment of malaria as well as a nutritional supplement in malnourished populations.

Chapter 5

CONCLUSIONS

While Moringa is extensively believed to have anti-plasmodial properties by inhibiting parasite growth, our data suggest that it may enhance $CD4^+$ T cell activation as well. Increased T cell numbers are important for helper function and parasite clearance by the host's immune system. We observed decreased parasitemia in Moringa treated mice which was accompanied by increased cytokine secretion and Tbet expression in mice that were treated with Moringa before (prophylactic) or after (curative) infection. We also observed that this increases Tcm in prophylactically treated mice at both high and low doses. Moringa remediates the immune suppressing effects of food limitation induced malnutrition by increasing Teff proportions and IL-10 expression. We propose that use of Moringa prophylactically or curatively is beneficial in the control of malaria disease and may aid in the treatment of malnutrition and malaria infection.

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Vita

Jennifer Pilotos was born in Havana, Cuba in 1993 and immigrated to the United States in 1995. She has always had a passion for helping others and therefore early in her life made the decision to pursue medicine. She attended Appalachian State where she received a bachelor's degree in cell and molecular biology. During this time, she received her certified nurse aid (CNA) certification and worked as a CNA and Med Tech in dementia care, assisted living, and rehabilitation. This interest in biology convinced her to pursue a master's degree in cell and molecular biology with a concentration in immunoparsitology.

Due to the quality of her work she has attended a variety of immunology conferences including: Woods Hole Immunoparsitology conference (WHIP) 2016, North Carolina American Association of Microbiology (NC-ASM) 2017&2018, and Immunology 2018 hosted by the American Association of Immunologists (AAI). She also received a variety of awards including Student Faculty and Excellence (SAFE) Fund award, Office of Student Research travel and research awards, AAI travel award, and the Federation of American Societies for Experimental Biology Diversity Resources for Enrichment, Access &mentoring (FASEB-DREAM) travel award. This research culminated in the submission of a manuscript titled "Long Term *Moringa oleifera* Treatment Induces Tbet expression and Activation of Effector CD4 T cells in *P. chabaudi* Infected mice" to the International Journal of Environmental Sciences and Public Health with another manuscript in the works. This love for immunology has inspired her to pursue

a career in medicine, with a specialty in Allergy and Immunology. She hopes to open her own non-profit clinic to help uninsured individuals once out of medical school.

Outside of academia she is an avid painter who specializes in charcoal portraits and acrylic landscape painting. She also enjoys gardening as it has been a tradition between her and her father for years. They set up a garden every year and enjoy growing odd varieties of vegetables, herbs, and fruits.