THE EFFECTS OF MALNUTRITION AND *PLASMODIUM CHABAUDI* INFECTION ON INNATE MUCOSAL IMMUNITY IN THE GUT

A Thesis
by
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Abstract

THE EFFECTS OF MALNUTRITION AND *PLASMODIUM CHABAUDI* INFECTION ON INNATE MUCOSAL IMMUNITY IN THE GUT

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Plasmodium falciparum is a parasite that causes malaria in humans and results in more than 600,000 deaths annually as of 2020. These infections commonly occur in areas with high prevalence of malnutrition due to poverty, illiteracy, climate change, and poor governmental policies. Malnutrition is known to modulate immunity in a way that leads to increased susceptibility and severity of malaria and other coinfections. The overlap of malnutrition and malaria results in high morbidity and mortality, especially in children under the age of 5. Despite extensive research on *Plasmodium* infection and malnutrition independently, research on the relationship between the two is limited, especially in the gut. Since pathologies in the gut are associated with both malaria and malnutrition, we aimed to evaluate how malaria infection affects gut mucosal immunity during moderate malnutrition. To address this question, we hypothesized that *Plasmodium* infection decreases the dendritic cell population responsible for sampling the gut, thereby leading to possible peripheral organ damage in moderately malnourished mice.

To establish a moderately malnourished murine model, we used a specialized diet common in malaria endemic regions that is composed of 3% protein content and deficiencies in zinc and iron. This model was confirmed to induce moderate malnutrition with approximately

10% weight reduction after 4 weeks, and intestinal length shortening when compared to a calorie matched 17% protein control diet group. To investigate gut mucosal immunity, we focused on dendritic cell populations and function by evaluating expression of CD11b, CD11c, CD103, F4/80, MHC-II, and CD86 surface proteins along with IL-12/23 and IL-10 cytokine production. We found that malnutrition decreased overall myeloid cell, macrophage, and dendritic cell populations but differentially affected activation of dendritic cell subsets in the small and large intestines. Activated proportions of classical dendritic cells (cDCs) were increased in malnourished mice, resulting in similar total effector cell numbers when compared to well-nourished controls. The moderate malnutrition diet increased production of the IL-12/23 proinflammatory cytokines by cDCs while decreasing immunoregulatory cytokines as represented by a lack of IL-10 production. Most dendritic cell subsets in the large intestine produced undetectable levels of investigated cytokines, although more cDCs expressed co-stimulatory molecules which indicated elevated activation. Consistent with our prediction, innate mucosal immunity in the gut was skewed towards an inflammatory phenotype in the moderate malnourished mice. Because acute liver injury has been reported in malaria patients, we investigated the contribution of malnutrition to this damage by checking blood serum levels of hepatic enzymes. Alanine Transaminase (ALT) was elevated more during *Plasmodium* infection in moderately malnourished mice, indicating greater damage to peripheral organs.

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TLR: Toll-like Receptor

NLR: Nucleotide-binding Domain and Leucine-rich Repeat Containing Receptor

RLR: Retinoic Acid-inducible Gene-I-like Receptor

DC: Dendritic Cell

cDC: Classical Dendritic Cell

IL: Interleukin

Ig: Immunoglobulin

RBC: Red Blood Cell

TNF: Tumor Necrosis Factor

SEM: Standard Error of the Mean

IFN: Interferon

MHC: Major Histocompatibility Complex

MSP: Merozoite Surface Protein

MAdCAM-1: Mucosal Addressin Cell Adhesion Molecule-1

ILC: Innate Lymphoid Cell

WND: Infected well-nourished mice

cWND: Uninfected well-nourished mice

PDD: Infected malnourished mice

cPDD: Uninfected malnourished mice

PEM: Protein Energy Malnutrition

CD: Cluster of Differentiation

IEC: Intestinal Epithelial Cells

AST: Aspartate Transaminase

ALT: Alanine Transaminase

ALP: Alkaline Phosphatase

RA: Retinoic Acid

PAMP: Pathogen-associated Molecule Patterns

ALDH: Aldehyde Dehydrogenase

ERK: Extracellular Signal-regulated Kinase

Chapter 1: Introduction

Malaria

Malaria is a severe disease caused by infection from single-celled eukaryotic *Plasmodium* parasites transmitted through the bite of *Anopheles* spp. mosquitoes. Humans are hosts for five strains of *Plasmodium* parasite: *P. falciparum, P. vivax, P. malariae, P. ovale, and P. knowlesi,* of which *P. falciparum* and *P. vivax* are the most common and are responsible for the greatest public health burden (1). Malaria's historic presence has driven the evolution of human populations in malaria endemic regions with selection of unique genetic variants such as thalassemia and sickle-cell disease, both of which affect red blood cells (2). Today, *P. falciparum* malaria causes the bulk of malaria-associated morbidity and is prevalent despite modern vector control approaches and drugs for both treatment and prevention. This is especially true in economically poor countries, mostly on the African continent (1, 3). There were approximately 241 million cases of malaria reported in 2020 with an estimated 627,000 deaths of which 95% of cases and 96% of deaths came from the African region as reported by the World Health Organization (WHO). Children younger than 5 years old account for roughly 80% of all deaths in the WHO African region (4). Sub-Saharan Africa is home to most malaria cases due to its optimal climate, poor health care, lack of finance, political quarrel, and prevalence of malnutrition (5, 6). These factors contribute to the difficulty of dealing with an already complex parasitic life cycle that can evade anti-malarial treatments.

All *Plasmodium* parasites share a two-part life cycle. The parasite first infects a vertebrate host and then is transmitted from the infected host to another host by an *Anopheles* vector. This transfer has both sexual and asexual replication phases in the mosquito and host respectively. Human malaria is transmitted only by female *Anopheles* mosquitoes. When a vector

bites a human, sporozoites produced inside the vector enter the blood and lymphatics of the host and rapidly migrate to the liver, thus starting the asexual stage of the parasite's life cycle. During this migration to the liver, host innate immune cells like macrophages and dendritic cells are able to phagocytose and eliminate some of the sporozoites (7, 8). The remaining sporozoites selectively infect hepatocytes and begin to develop into merozoites. Thousands of merozoites per successful sporozoite are released into the bloodstream within days of infection (2-16 depending on *Plasmodium* species), and each merozoite is capable of invading an erythrocyte via recognition of red blood cell (RBC) receptors like Band 3 and Glycophorin A to initiate an exponential replication cycle (9, 10). This exponential replication leads to symptomatic malaria as pathology occurs during the blood stage of infection (11). Some merozoites develop into sexual gametocytes which can be taken up by *Anopheles* mosquitoes during blood meals. These gametocytes undergo sexual reproduction in the form of fertilization and maturation in the mosquito's midgut (9). This results in an infective ookinete which can migrate through the midgut and form oocysts in which sporozoites are formed (12). After fully maturing, oocysts burst and release sporozoites that travel via hemolymph to salivary glands of the mosquito for continued host infection (13). Only a portion of mature schizonts, or red blood cells infected with merozoites, will produce gametocytes required for sexual reproduction; the remaining schizonts release 4-36 daughter merozoites upon rupture to invade fresh RBC and perpetuate the asexual life cycle (11). Concurrently, schizont rupturing releases toxins and parasite by-products such as glycosyl-phosphatidyl inositol and hemozoin that activate innate immunity via TLRs and cytosolic receptors for subsequent inflammatory response and pathology (11, 14, 15). Symptomatic stages of malaria infection influence and damage multiple physiological systems, including the GI tract and liver (16-18).

Malnutrition

Malnutrition is a major issue in many regions of the world, and it is estimated that roughly a quarter of the world's population suffering from hunger reside in the Sub-Saharan Africa region. In addition to deaths caused by malaria disease, about 54% of deaths in children in developing countries are related to child malnutrition. This is broadly attributed to poverty, illiteracy, climate change, and governmental policy since current food aid lacks a mode of sustainability (19). The effects of malnutrition can create and maintain poverty, which further impacts health, economic, and social statuses. This is shown by increased hospitalizations and chronic illnesses in areas suffering from macro- and micro-nutrient deficiencies (20, 21). A common measure of macro-nutrient intake, or lack thereof, termed Protein Energy Malnutrition (PEM), involves looking at both protein and caloric intake. Two forms of PEM are classified; inadequate protein and caloric intake named marasmus, and sufficient caloric but inadequate protein intake named kwashiorkor. While both forms of PEM have distinct clinical differences, PEM in general has detrimental effects on growth, development, and disease susceptibility (22, 23). This is often compounded by micro-nutrient deficiencies like iron, zinc, vitamin A, folate, and iodine (24-26). As indicated by data from 53 developing countries, mild-to-moderate malnutrition accounts for 83% of child mortality (27). This is worrisome for malaria endemic regions where macro- and micro-nutrient deficiency is prevalent and is thought to contribute to pathogenic immune modulation and oxidative stress, especially in children under the age of 5 (28-30). These deficiencies contribute to changes in the gut microbiome which further increase disease susceptibility and may stunt growth (31).

Malaria and malnutrition

There is a large overlap in malaria endemic regions and areas suffering from malnutrition, specifically in sub-Saharan Africa and southeast Asia. Malaria and malnutrition have both been extensively studied independently, but the connection between them has been inconclusively characterized (32). Although no consistent association has been found between malaria and acute malnutrition, chronic malnutrition is generally related to more severe malaria with respect to parasitemia and anemia (32). Some studies report that chronic malnutrition is associated with higher risk of malaria (33, 34), while others suggest that this malnutrition may have a protective or irrelevant effect (35-37). There is also little information on the effect of malnutrition on anti-malaria treatments which prompts further investigation into possible influences between the two conditions (38, 39). To evaluate this, I will comment on individual influences of PEM and micronutrient deficiencies with respect to malaria susceptibility.

PEM has been shown to increase morbidity and mortality in African children who are infected with *Plasmodium* parasite (40, 41). In children with stunting, wasting, and underweight status, merozoite surface protein (MSP)-specific IgG concentrations are lower than in healthy individuals suggesting a defect in humoral adaptive immunity due to PEM (42). Supporting this, wasted and stunted children presented lower *P. falciparum*-specific IgG antibody response compared to well-nourished children which suggests that individuals facing PEM are at a higher risk for chronic infection and re-infection (43, 44). Protein deficiency has also been shown to affect the generalized host immunity that peripherally contributes to malarial defense, including dysfunctional modulation of innate mucosal immunity and decreased holistic adaptive immune response (45-47). This is attributed to changes in phagocytic/chemotactic activity of mononuclear leukocytes and changes to regulatory influences of T cells (48, 49). These changes

are consequences of bone marrow and mesenteric lymph node hypoplasia resulting from PEM (50, 51). This is triggered by decreases in IL-7 production responsible for lymphocyte proliferation, extracellular signal-regulated kinase (ERK) phosphorylation responsible for lymphocyte proliferation, and MAdCAM-1 expression responsible for lymphocyte homing to the gut (52).

Zinc deficiency is associated with similar pathological defects as PEM, including reduced circulating immune cells, reduced immune maturation, and impaired immune function (53). T cell activation relies on zinc for gene regulation, and B cell antibody production, specifically IgG production, is also compromised in zinc deficient individuals (54). The ability of macrophages to produce cytokines and phagocytose is adversely affected by zinc deficiency which leads to downstream immune dysfunction. DNA replication, RNA transcription, and cellular division all involve proteins with zinc-binding motifs which explains the drastic impact such a deficiency imposes on immune functionality (55, 56). A study of pregnant African women has shown increased *P. falciparum* associated morbidity during zinc deficiency (57). In contrast, in response to severe *P. falciparum* infection, zinc supplementation resulted in a significant reduction in parasitemia for the first 3 days (58) and reduced *P. falciparum*-attributable health problems for recipients (59). Children receiving zinc supplementation have also demonstrated a reduction in diarrhea, pneumonia, and malaria infection (60).

Iron is a key micronutrient for electron transfer and redox reactions within cells, and both iron deficiency and excess iron are detrimental for cellular function. Iron supplementation can increase the risk of infection for malaria and tuberculosis which is likely a reflection of iron's effect on pathogen growth (61). Iron deficiency is associated with reversible immune dysfunction which leads to increased susceptibility to infections (62). Iron deficiency is also

associated with higher proportions of lymphocytes producing inflammatory IL-6, IFN-γ, and TNF-α, which are consistent with stronger pathology during malaria infections (63). However, *Plasmodium* replication requires iron during both liver and symptomatic blood stages of infection. Iron chelators have been shown to inhibit pathogen growth *in vivo* and *in vitro* (64). Although iron deficiency has shown some reduction of clinical severity of malaria infection (65) which may be due to the development of anemia, patients with iron deficiency anemia had significantly lower IgG levels and phagocytic activity (66). With all of this in consideration, iron's influence on malaria infection is controversial and may depend heavily on other relevant factors such as its impact on gut microbiota.

Innate and adaptive immunity

The immune system is made up of two general groups of cells based on functionality: innate cells and adaptive cells. Innate immune cells, including dendritic cells (DC), macrophages, mast cells, neutrophils, basophils, etc., are responsible for rapid, non-specific responses to pathogen associated molecular patterns (PAMPs) and are considered the first line of immune defense (67). The PAMPs are conserved molecules on pathogenic bacteria, viruses, and other pathogens that bind to pattern recognition receptors (PRRs) like toll-like receptors (TLRs), nucleotide-binding domain and leucine-rich repeat containing receptors (NLRs), and retinoic acid-inducible gene-I-like receptors (RLRs) on innate immune cells (67, 68). Innate cells produce cytokines and express major histocompatibility complex (MHC) molecules that activate the adaptive immune response. Adaptive immunity, comprised of T and B cells, is based on clonal selection and expansion of antigen-specific receptors. B cells proliferate to increase antigen-specific antibody production and to generate long-term memory of antigens in

preparation of re-exposure. T cells differentiate into cytotoxic and helper T cells which are able to kill infected cells and secrete cytokines to further activate/differentiate other cells respectively (67).

Immunity crosstalk: Surveillance and tolerance

Regulated cross-talk of innate and adaptive immune cells is necessary for healthy steady-state and inflammatory responses. Cross-talk is often accomplished by cytokine production and direct contact of MHC molecules I and II with specific adaptive immune cells. MHC I presents intracellular antigens to cytotoxic T cells while MHC II presents exogenous antigen that has been phagocytosed to T helper cells and B cells (69). It is important to note that RBC do not express MHC I molecules.

Excessive immune responses in the form of unregulated inflammation are detrimental to surrounding cells, so commensal-induced signaling of innate immune cells allows for prevention of excessive adaptive immune response (70). Innate immune cells, specifically DCs, are able to regulate adaptive immune response to commensal and self-antigens with suppressor molecules like CTLA-4, IL-10, TGF-β, and IL-35 to avoid autoimmunity and prevent allergic reactions (71). DCs are also able to effectively prime naïve T cells by producing effector cytokines that promote different cell subtypes (e.g., IL-23 priming T_H17, IL-12/27 priming T_H1, IL-10 priming T_{reg}, etc.) (72, 73).

Since the gut is exposed to a high volume of exogenous antigens, the human immune system performs a regular surveillance of the gut lumen. This generally happens in gut associated lymphoid tissue (GALT), which acts as a frontline of defense. These tissues function as centers of non-specific pathogen recognition, innate immunity initiation, and presentation for

the downstream activation of adaptive immunity. GALTs are comprised of Peyer's patches in the small intestine, isolated lymphoid follicles, crypt patches in the colon, and mesenteric lymph nodes. Cell types within the GALT include microfold (M) cells that are capable of transferring antigens from the lumen to underlying immune cells, but are not capable of processing antigen for presentation to conventional lymphocytes and phagocytes in GALT such as T helper- (T_H), T regulatory- (T_{reg}), cytotoxic T-, IgA-producing B-, macrophages, and DCs (74-80).

DCs have a variety of functional differences that allow for unique potentials. Plasmacytoid DCs (pDCs) are found mostly in the blood and lymphoid tissues and express a narrow range of TLRs, namely TLR 7 and 9. These recognize foreign nucleic acids and prompt a massive type I IFN response against viral infection. Classical DCs (cDCs) populate both lymphoid and nonlymphoid tissues, including tissues on and around gut mucosa, and have a superior ability to capture and present phagocytosed antigens to T lymphocytes (81). cDCs are identified in gut tissue using the surface markers CD11c, CD11b, and CD103. CD11b+CD103- (CD11b+) cDCs are in both lymphoid and non-lymphoid tissue for T_H2/T_H17 induction, CD11b-CD103+ (CD103+) cDCs are primarily in GALT for T_{reg} induction, and CD11b+CD103+ (intestinal) cDCs are specific to the intestines and are primarily involved in T_H17 induction after luminal sampling although they can produce cytokines for polarization of most T_H subtypes (82). Intestinal cDCs specifically are able to metabolize dietary retinoic acid (RA) from vitamin A, which allows for gut trafficking of T- and B- effector cells (83).

cDCs are the best equipped cell type for migration to the T cell zone of lymph nodes while loaded with antigen. After migrating, these DCs can utilize variable antigen processing mechanisms to effectively prime naïve T cells (81). Both lymphoid- and nonlymphoid-resident cDCs subsets express similar TLR, C-type lectin receptor, and chemokine receptor profiles with

the exception of intestinal cDCs expressing a double-stranded viral RNA sensor and TLR3 (84). Both cDC subsets express CD36 for binding to dead cells and C-type lectin receptor Clec9A for binding to necrotic bodies in damaged tissue (85, 86). Nonlymphoid tissue cDCs constantly migrate through afferent lymphatics to lymph nodes while loaded with antigen in a CCR7-dependent manner during steady state conditions, and their migration increases significantly in response to inflammation (82). The MHCII and costimulatory molecule expression required for driving adaptive immunity is upregulated on cDCs with inflammation. Furthermore, upon maturation, migratory cDCs acquire the ability to produce proinflammatory cytokines like type I IFN, IL-1, TNF-α, IL-6, IL-12, and IL-23 (87).

Lymphoid-resident cDCs are found in Peyer's patches and are phenotypically immature in steady state conditions; however, activation and maturation can occur with microbial product stimulation leading to greater production of CD4+ T cell attractant chemokines (88). Peyer's patch cDCs can capture translocated IgA immune complexes and extend dendrites through M cell-specific transcellular pores. Moreover, transport of soluble material by small intestine goblet cells and retro-transport of IgG immune complexes across the epithelium delivers antigens to cDCs (89-91). Lymphoid-resident CD11b+ cDCs are the primary source of IL-12 and IL-15 cytokines, which are involved in CD8+ T cell differentiation and Th1 polarization (92, 93). In terms of tolerance, cDCs contribute via induction of T_{reg} cells and deletion of self-reactive T cells via the capture of dying cells (94). Migratory lamina propria cDCs have a better ability to induce peripheral T_{reg} differentiation *in vivo* due to their expression of aldehyde dehydrogenase, an enzyme that metabolizes dietary vitamin A into retinoic acid (95). pDCs are involved in steady- and inflamed-state tolerance induction via stimulation of regulatory CD4+ T cells (96).

Malnutrition and gut mucosal immunity

Malnutrition has a strong influence on gut mucosal immunity. Human intestines maintain an environment that supports a symbiotic microbiome with normal, adequate nutrient intake. This microbiome in turn supports microbicidal and antiviral molecule production such as lysozymes, α-defensins, and secretory phospholipase A2 by innate immunity. This is paired with adaptive immune support in the form of secretory IgA production to defend against intraluminal pathogens (52). The largest and most diverse microbial community of a human resides in the gut, and its importance in the development of gut-associated lymphoid tissues has been well characterized thus far (97, 98). As much as the microbiome helps to shape human immunity and aid in digestive processes, a variety of host factors including environmental, dietary, and host immune responses can impact the microbiome reciprocally. This two-way relationship represents the "host-microbiome" axis that maintains homeostatic conditions and leads to pathogenesis when disrupted. This axis is critically important and is often overlooked. Xenobiotic surveillance may be disrupted during infection and/or nutritional alteration which leads to downstream immune modulation.

As the first line of defense against exogenous antigens, mucosal innate immunity is supported by additional innate lymphoid cells that are divided into three main groups: ILC_1s, ILC_2s, and ILC_3s. ILC_1s consist of non-cytotoxic NK-like cells that produce inflammatory IFN-γ and TNF-α (99). ILC_2s secrete IL-4, IL-5, IL-9, and IL-13 to promote inflammation in the context of helminth immunity, allergic reactions, and tissue remodeling (99). ILC_3s are able to secrete IL-17A and IL-22 to promote T_H17 cell development and to reinforce epithelial barriers. Additionally, ILC_3s sample the gut lumen and present self-antigens to adaptive immune cells to

induce tolerance and prevent autoimmunity (100). During steady-state conditions, ILC_3s are dominantly present in the gut. All ILCs utilize PRRs like other innate immune cells. Additionally, TLR signaling is important in other cell types for the maintenance of epithelial homeostasis and protection from injury because these receptors have been shown to be involved in epithelial cell proliferation, IgA production, tight junction maintenance, and antimicrobial peptide production (101). Paneth cells and intestinal epithelial cells (IECs) specifically express TLR2, 4, 5, and 9 to induce secretion of antimicrobial peptides (101, 102). Recognition of PAMPS by these receptors induce signal transduction cascades that also ultimately produce NF-kB, IFN molecules, and phosphorylate various protein kinases to promote production of other proinflammatory cytokines, antimicrobial peptides, and chemokines for cell recruitment.

PEM has been reported to decrease gut microbial diversity, specifically the abundance of beneficial *Bifidobacteria, Lactobacilli, Bacteroides,* and *Bilophila* (103). This corresponds with a reduction in short-chain fatty acids which are required to strengthen the epithelial barrier and prevent pathogen translocation. This microbial shift also contributes to increased inflammatory cytokines like TNF-α, IL-2, and IL-6, which are hallmarks of severe malaria pathology. PEM has also been shown to have negative impacts on epithelial barrier integrity as a result of faulty tight junction protein expression (104, 105). This leads to decreased nutrient absorption and increased disease susceptibility and severity.

Micronutrient deficiency in children has resulted in similar increases in plasma inflammatory cytokines and decreases in short-chain fatty acids, further contributing to decreased epithelial barrier integrity and exacerbated inflammation in the gut (106). Vitamin A deficiency leads to a reduction in ILC_3 cells and an increase in ILC_2 cells with IL-4, IL-5, and IL-13 production increased consequentially (107). This suggests that the immune system shifts

away from a tolerogenic, bacterial-oriented phenotype towards a protective anti-helminth phenotype. Zinc and iron deficiencies have been associated with leaky gut syndrome, dysbiosis, shallow mucosal depth, and downregulation of tight junction proteins that are essential for proper epithelial barrier functioning (108-110). Micronutrient malnutrition leads to decreased immune cell populations but greater activated proportions of T and B cells since zinc, iron, and many vitamins are required for adaptive immune cell proliferation, activation, and induction of immunotolerance (111-113). This gut status leads to faulty PAMP recognition and increased risk of comorbidity (114).

In this study, we were particularly interested in mucosal DCs as they have a critical role in sampling tissue and blood antigens in nonlymphoid tissues, a superior ability to process and present antigens, and a predominant ability to migrate to lymph nodes while loaded with antigen to prime naïve T cells for adaptive immune response (81). During malaria infection, mucosal tissue has increased susceptibility to pathogen translocation across a weakened epithelial barrier and may suffer from unregulated inflammatory signaling (115). Tight regulation of DC populations and polarization is required for inflammatory antimicrobial protection while controlling other effector cell populations. DCs may be inducing adaptive immunity against malarial pathogens as well as consequential luminal pathogens which emphasizes the importance of these cells. Loss of DC function may result in intestinal dysbiosis and autoimmunity, which would impact the severity of infection (116).

Protein, zinc, and iron deficiencies all contribute to decreased immune function and increase subsequent morbidity in the face of *Plasmodium* infection. Furthermore, these macro- and micro-nutrient deficiencies affect mucosal immunity and pathophysiology (62, 117, 118).

During malnutrition-induced dysbiosis, malaria may have increased disease severity and further modify the microbiome (119).

Increased intestinal permeability has been reported in patients facing *P. falciparum* infection (120). Supporting evidence has been found when looking at FITC-dextran leakage in a murine model challenged with the *P. falciparum* murine counterpart; *P. chabaudi* (121).This is paired with gastrointestinal bleeding that has been found in *P. falciparum* patients suggesting significant damage to the epithelial barrier (122). A breach in the epithelial barrier may allow for dissemination of pathogens into the bloodstream resulting in peripheral organ damage (18, 123). In addition to evidence of morphological damage, case studies have shown *P. falciparum* to induce bowel ischemia and intestinal necrosis which correlated with parasite load, suggesting that infected RBC cytoadherence to the microvasculature of the intestines may prevent oxygen delivery to villi (124, 125). This damage prevents proper nutrient absorption and thus exacerbates the compounding effects of concurrent malnutrition and *Plasmodium* infection (126).

To investigate how a combination of protein, zinc, and iron deficiencies in the presence of *Plasmodium* infection affect mucosal innate immunity, we used *P. chabaudi* to evaluate *in vivo* DC activation and function within a murine model.

Our first aim was to evaluate how a combination of protein, zinc, and iron deficiencies affect dendritic cell populations and function in the small intestine during a *P. chabaudi* infection. More specifically, we sought to evaluate DC subpopulations and their polarizing cytokine productions.

Our second aim was to evaluate how protein, zinc, and iron deficiencies compound with *P. chabaudi* infection to affect DC populations and function in the large intestine with emphasis on differentiation of DC subpopulations and their cytokine production profiles.

Our third aim was to determine if the mucosal damage caused by malnutrition and *Plasmodium* infection resulted in increased serum protein levels indicative of peripheral organ damage. More specifically, we sought to determine the extent of liver damage in terms of bloodstream hepatic enzyme presence and relate it to the result of a weakened epithelial barrier.

Chapter 2: Materials and Methods

Mice and parasite

Adult C57BL/6 mice were purchased from Harlan labs and a breeding colony was maintained in the vivarium at Appalachian State University. The rodent strain of malaria, *P. chabaudi*, was received as a gift from Dr. Robin Stephens at the University of Texas Medical Branch Galveston. Authorization to use the parasite was given by Dr. Jean Langhorne from the Francis and Crick Institute, UK. All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at Appalachian State University (Opata Protocol 20-10). Male mice aged 8-16 weeks were used for experiments with consistency in both the malnourished and well-nourished control groups.

Malnutrition and infection

Mice were fed either a moderately malnourished diet (PDD; TD.99075) with 3% protein content, 74% carbohydrate content, 9% fat content, and deficient in iron and zinc, or a well-nourished diet (WND; TD.99103) with 17% protein content, 59% carbohydrate content, 9% fat content, and all necessary micronutrients from Envigo/Teklad (Indianapolis, IN). Both diets have similar caloric content (3.9 vs 3.8 Kcal/g), which is compensated for by carbohydrates in the moderately malnourished diet. The diets were administered to each group of mice at 3 grams per mouse daily for 4-6 weeks to induce moderate malnutrition. After 4-6 weeks, both the control and malnourished mice were infected with $1x10^5$ *P. chabaudi* iRBC intraperitoneally and other groups of control and malnourished mice were left uninfected to serve as uninfected controls. At 9 days post-infection, all mice were euthanized via cervical dislocation as approved by the IACUC (Opata Protocol 20-10). The small and large intestines were harvested and placed in ice-cold 1X PBS supplemented with 0.02% EDTA in preparation for tissue cleaning.

Preparation of gut tissues for flow cytometry

The large and small intestine tissues were cleaned by manually removing all residual adipose tissue using scissors and forceps. The tissues were cut longitudinally and flushed with ice cold 1X PBS supplemented with 0.02% EDTA using a wash bottle to remove fecal matter. Residual fecal matter was removed by lightly scraping with curved forceps before rinsing with more ice-cold 1X PBS supplemented with 0.02% EDTA. The tissues were cut into approximately 0.2 cm sections, then placed in their own well in a 24-well plate with 1 mL of ISCOVES culture media (Corning #10-016-CV) supplemented with 2mM L-glutamine (Atlanta Biologicals B21210), 5mM sodium pyruvate (Gibco 11360-070), non-essential amino acids (Gibco 11140-050), 10mM HEPES (Gibco 15630-080), 100 U/mL penicillin-streptomycin (Atlanta Biologicals, #B21210), and $2e^{-5}$M β-mercaptoethanol (Gibco 21985-023). Type I collagenase (ThermoFisher #17018029) at a concentration of 100 U/mL, DNAse (Sigma Aldrich #10104159001) at a concentration of 60 μg/mL, and Liberase (Sigma Aldrich #05401020001) at a concentration of 60 μg/mL were all added to the wells containing the cells, followed by a 1.5-hour incubation in a HERAcell 150i incubator at $37^{\circ C}$ and 5% CO_2 for extraction of lamina propria cells. Cells were agitated every 15 minutes during the incubation to ensure homogeneity.

Flow Cytometry (surface staining)

After the 1.5-hour incubation, the 24-well plates were removed from the incubator and the tissues were quantitatively transferred to 70-μm nylon filters using 1 mL ice-cold 1X PBS. These filters were sitting on petri dishes to collect the flow-through media. Tissues were mashed through the nylon filters using a plunger, and the resulting media with cell suspension was quantitatively transferred to a: A) 5-mL polypropylene round-bottom tube using 500 μL ice-cold

1X PBS for large intestine samples and B) 15-mL polypropylene round-bottom tube using 500 µL ice-cold 1X PBS for small intestine samples. The tubes were centrifuged at 1200 rpm for 8 minutes (4$^{\circ C}$). The tubes were decanted, vortexed, then RBCs were lysed using 1X RBC lysis buffer for 1 minute at room temperature. RBC lysis was stopped by adding 2 mL ice-cold 1X PBS followed by 8 minutes centrifugation at 1200 (4$^{\circ C}$). The cells were washed further with ice-cold FACS buffer (PBS, 2% FBS, 0.1% NaN_3 sodium azide), then resuspended in 1 mL of FACS buffer for enumeration at a 1:10 dilution in 0.1% trypan blue on a hemocytometer. After determining cell counts, an aliquot of $\leq 3x10^6$ cells/mL for each sample was added to a new 5-mL polypropylene round-bottom tube and washed with 1 mL FACS buffer.

The Fc receptors were blocked using a CD32/CD16 antibody followed by a 20-minute incubation in the dark at 4$^{\circ C}$ to prevent non-specific binding. Fluorescent antibodies were used to label cells of interest: FITC-conjugated anti-CD103 (Clone 2E7, BioLegend, San Diego CA), FITC-conjugated anti-CD11b (Clone M1/70, BioLegend), PE-eFluor610-conjugated anti-MHCII (Clone M5/114.15.2, ThermoFisher, Waltham MA), PE-Cy7-conjugated anti-CD86 (Clone GL1, Tonbo, San Diego CA), APC-conjugated anti-CD11c (Clone N418, BioLegend), Brilliant Violet 421-conjugated anti-CD11c (Clone N418, BioLegend), Brilliant Violet 605-conjugated anti-CD103 (Clone 2E7, BioLegend), and PE-conjugated anti-F4/80 (Clone BM8.1, Tonbo). Fluorescently stained cells were washed (1200 rpm, 5 minutes, 4$^{\circ C}$) twice with 1 mL of FACS buffer, then resuspended in 500 µL of FACS buffer and filtered through 70-µm nylon filters into a 5 mL round-bottom polystyrene tube before being analyzed on an Attune NxT acoustic flow cytometer (ThermoFisher).

Flow cytometry (intracellular cytokine staining)

Aliquots of cells for each sample equating to $\leq 3x10^6$ cells/mL were added to a sterile 24-well plate with 1 mL of ISCOVES culture media (Corning #10-016-CV) supplemented with 2mM L-glutamine (Atlanta Biologicals B21210), 5mM sodium pyruvate (Gibco 11360-070), non-essential amino acids (Gibco 11140-050), 10mM HEPES (Gibco 15630-080), 100 U/mL penicillin-streptomycin (Atlanta Biologicals, #B21210), and $2e^{-5}$M β-mercaptoethanol (Gibco 21985-023). The cells were stimulated *in vitro* with 2 µL of 500X cell activation cocktail with brefeldin A (BioLegend #423303). The negative control was not stimulated with cell stimulation cocktail. The 24-well plate was placed in a HERAcell 150i incubator set at 37°C and 5% CO_2 for 4-5 hours. After incubation, cells were quantitatively transferred into 5-mL polypropylene round-bottom tubes. The cells were washed (1200 rpm, 5 minutes, 4°C) twice with FACS buffer, then incubated with fluorescent antibodies used against surface markers to label cells of interest: FITC-conjugated anti-CD103 (Clone 2E7, BioLegend), FITC-conjugated anti-CD11b (Clone M1/70, BioLegend), PE-eFluor610-conjugated anti-MHCII (Clone M5/114.15.2, ThermoFisher), PE-Cy7-conjugated anti-CD86 (Clone GL1, Tonbo), Brilliant Violet 605-conjugated anti-CD103 (Clone 2E7, BioLegend), PE-conjugated anti-F4/80 (Clone BM8.1, Tonbo), and Brilliant Violet 421-conjugated anti-CD11c (Clone N418, BioLegend) at 4°C in the dark for 40 minutes. After incubation, cells were washed (1200 rpm, 5 minutes, 4°C) with 1 mL FACS buffer. Without resuspension, cells were fixed with 300 µL of 2% paraformaldehyde at 4°C for 25 minutes. The cells were spun out of the paraformaldehyde then permeabilized with 1 mL of 1X permeabilization buffer diluted from 10X stock (Tonbo #TNB-1213-L150) at 4°C for 30 minutes. The cells were then washed followed by incubated with F_c block diluted in 1X permeabilization buffer at 4°C for 20 minutes. After F_c block incubation, without washing, cells were stained with

fluorescent antibodies against cytokines of interest: FITC-conjugated anti-IL-10 (Clone JES5-16E3, BioLegend) and PE-Cy7-conjugated anti-IL-12/23 (Clone C15.6, BioLegend). Isotype controls were stained with FITC-conjugated anti-rat IgG2b, κ (Clone RTK4530, BioLegend) and PE-Cy7-conjugated anti-rat IgG1, κ (Clone RTK2071, BioLegend). The cells were incubated at 4℃ for 40 minutes, then washed (1200 rpm, 5 minutes, 4℃) twice with 2 mL of 1X permeabilization buffer and once with FACS buffer before resuspending in a final volume of 500 µL. The cells were filtered through a 70-µm nylon filter into a 5 mL round-bottom polystyrene tube and analyzed on an Attune NxT acoustic flow cytometer (ThermoFisher).

ALT Assay

Whole blood was extracted, and serum was collected following centrifugation at 13,200 rpm for 30 minutes. A commercially available kit was used to measure alanine aminotransferase activity (Sigma; MAK052). Samples were prepared according to the manufacturer's instructions and absorbance at 570 nm was taken every 5 minutes using a SpectraMax M3 spectrophotometer until the most active sample reached the same reading as the highest standard.

Data analysis

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

Chapter 3: Results

Malnutrition lowers innate immune cell numbers in the small intestine during P. chabaudi *infection*

While a lot of studies have been done to understand the immune response to malaria and how the disease affects mucosal immunity, the influence of malnutrion has not been explored extensively. Therefore, we sought to investigate how a diet consisting of low protein and deficiencies in zinc and iron would affect mucosal innate immune cells during the peak of *Plasmodium* infection. To establish these conditions, mice were fed either a well-nourished, control diet or a moderately malnourished diet for 4-6 weeks and were then infected with $1x10^5$ *P. chabaudi* iRBCs intraperitoneally. Mice were weighed daily while on the diet and during infection, revealing that mice fed the moderately malnourished diet had approximately 10% weight loss compared to their well-nourished controls over the course of the diet which is consistent with literature ((127) and data not shown). After confirming that the diet induces moderate malnutrition, lengths of the intestine from pyloric to anal sphincter were measured using a ruler to confirm whether intestinal shortening was occuring in this model. As we previously reported, the intestinal lengths of infected malnourished mice were shorter, although not significantly, compared to both the uninfected malnourished and infected well-nourished controls ((121) and data not shown).

With confirmation of published physiological changes induced by protein, zinc, and iron deficiency, we sought to investigate the impact of these nutrient deficiencies on innate immune cell populations. In order to evaluate this, we performed flow cytometric analysis on the small and large intestine during the peak of infection; day 9 post-infection. The tissues were harvested from the mice and prepared for analysis on the flow cytometer by staining with fluorescently-

labeled antibodies specific for surface markers on innate immune cells. We found that moderate malnutrition reduces the number of general CD11c+ myeloid cells (**Figure 1A&B)** and specifically reduces CD11b+F4/80+ macrophages in the small intestine (**Figure 1C).** Moreover, challenge with *P. chabaudi* infection tends to increase the number and proportion of both

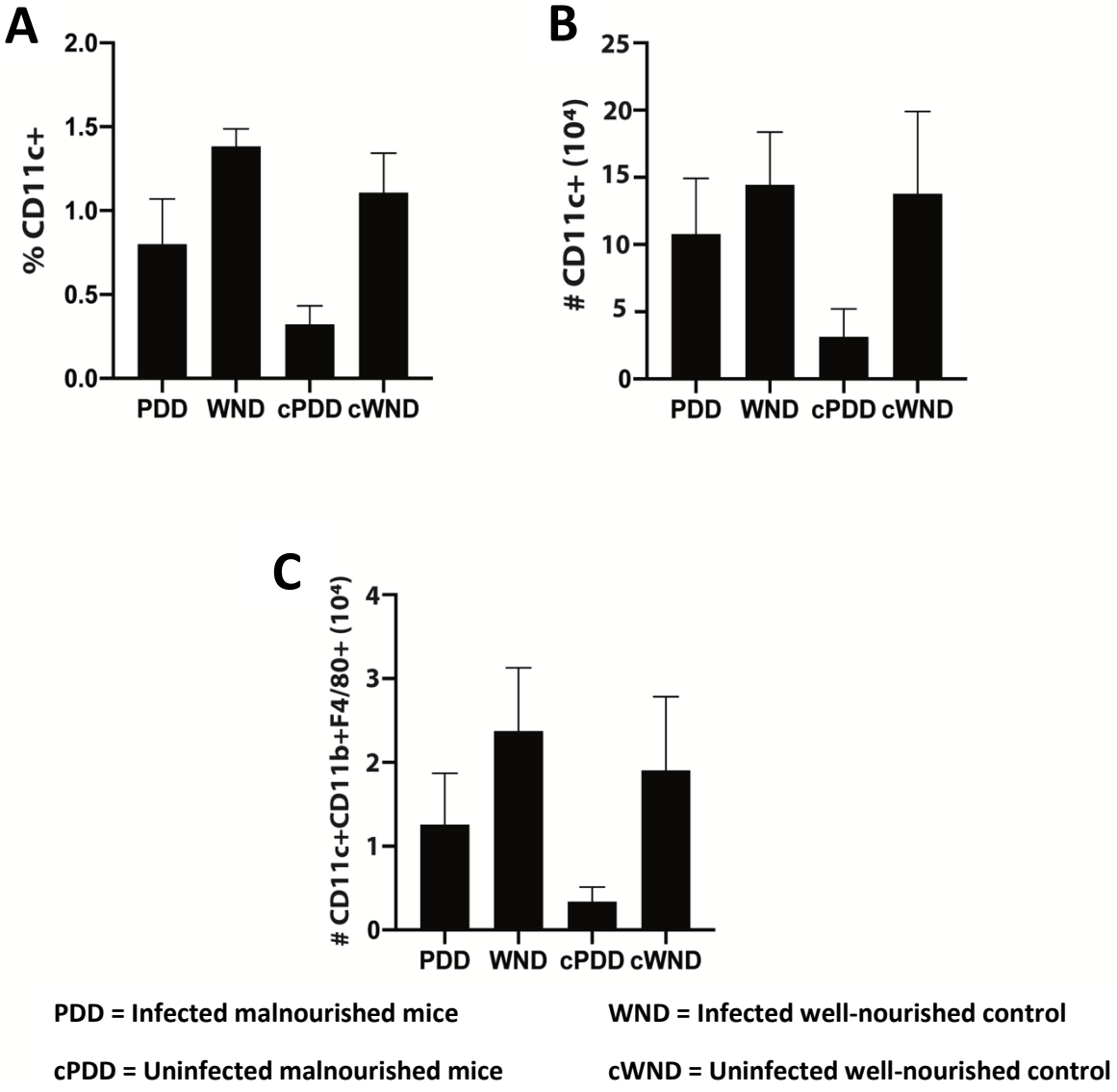

Figure 1. **Malnutrition lowers innate immune cell numbers in the small intestine during *Plasmodium* infection.** C57BL/6 mice were fed either a control diet or a moderately malnourished diet. All infected mice were interperitoneally injected with $1x10^5$ iRBCs of *P. Chabaudi*. At 9 days post-infection, mice were euthanized, and the small intestines were collected for FACS analysis. Graphs show **A**) CD11c+ proportion, **B**) CD11c+ number, and **C**) macrophage number. All data are shown as means ± SE, n=4 male mice per group, representative of 3 independent experiments. Statistical analysis was performed using a two-way ANOVA with Tukey's test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, indicates a statistically significant difference between treatments.

CD11c+ myeloid cells and specifically macrophages during malnourishment but not in the well-nourished controls (**Figure 1A-C**).

Malnutrition leads to increased percentage of activated intestinal cDCs, but reduced cell numbers in the small intestines of* P. chabaudi *infected mice.

Intestinal cDCs are able to produce RA, imprint gut tropism on activating T cells, and generate inducible T_H17 or T_{reg} polarization, and are required for optimal protection against exogenous antigens and autoreactivity. We sought to investigate how protein, zinc, and iron deficiencies affect intestinal cDC populations and activation status in the presence of *P. chabaudi* infection. We observed no difference in the proportion of intestinal cDCs in mice without *Plasmodium* infection and only a slight reduction in the proportion within infected mice **(Figure 2A**). However, since we observed decreased numbers of myeloid intestinal cDCs in the small intestines of moderately malnourished mice as shown by the CD11c+CD103+CD11b+F4/80- cells (**Figure 2B),** this data suggests that cell survival may be altered in an inflammatory gut setting. The proportion of activated intestinal cDC as represented by MHC-II upregulation was significantly higher in the small intestine of the infected and moderately malnourished mice compared to their well-nourished and uninfected controls (**Figure 2C**) which may contribute to further inflammation during *Plasmodium* infection. This translated to similar activated cell numbers of these intestinal cDC in infected but not uninfected mice (**Figure 2D**). This activation is indicative of antigen loading for subsequent priming of adaptive immunity.

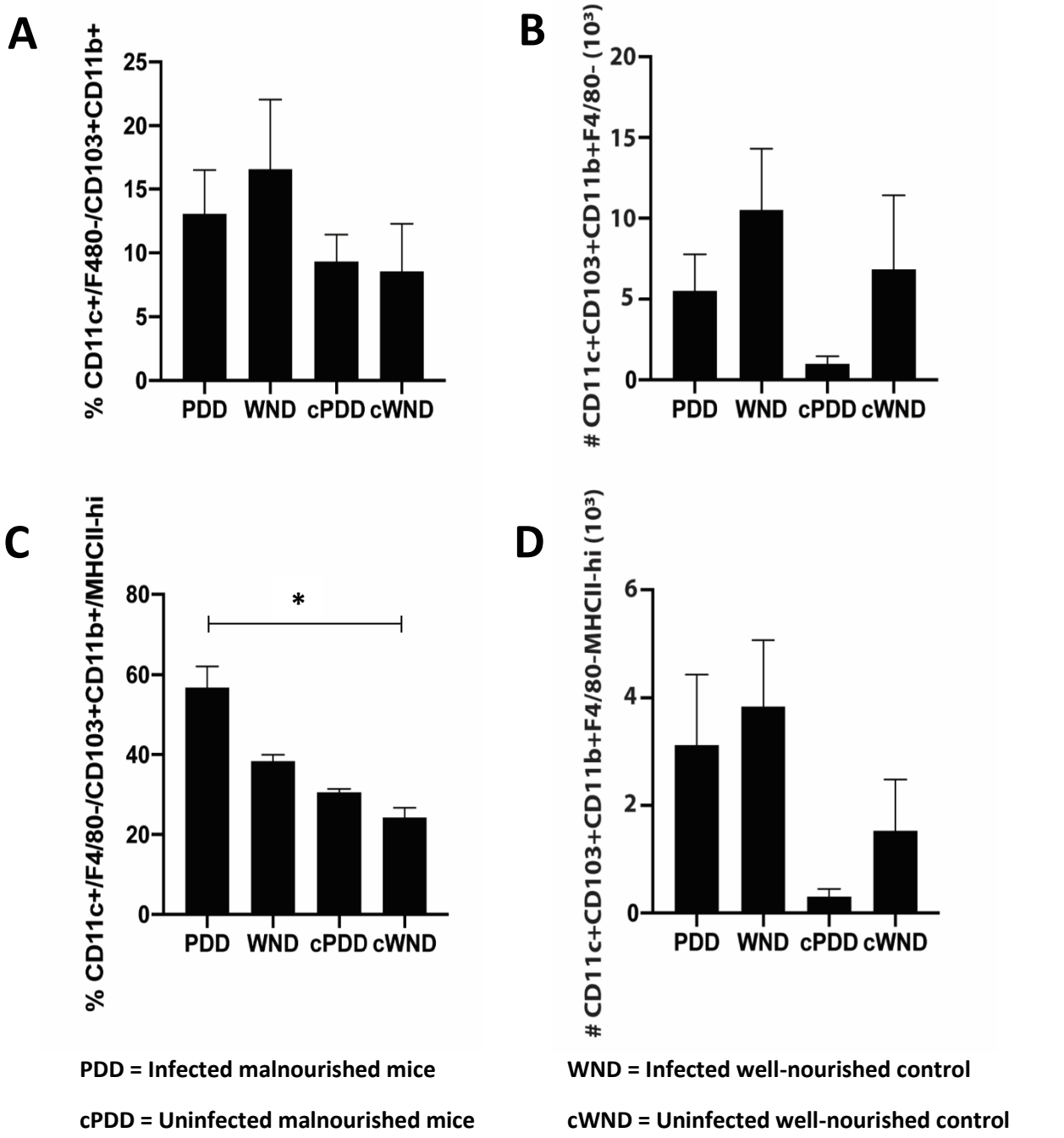

Figure 2. ***Malnutrition leads to increased percentage of activated intestinal cDCs, but reduced cell numbers in the small intestines of*** **P. chabaudi** ***infected mice.*** C57BL/6 mice were fed either a control diet or a moderately malnourished diet. All infected mice were interperitoneally injected with $1x10^5$ iRBCs of *P. Chabaudi*. At 9 days post-infection, mice were euthanized, and the small intestines were collected for FACS analysis. Graphs showing **A**) Intestinal cDC proportion of non-macrophage myeloid cells, **B**) Intestinal cDC number, **C**) Activated proportion of intestinal cDCs, **D**) Number of activated intestinal cDC. All data are shown as means ± SE, n=4 male mice per group, representative of 2 independent experiments. Statistical analysis was performed using a two-way ANOVA with Tukey's test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, indicates a statistically significant difference between treatments.

Malnutrition increases activated, co-stimulatory molecule-expressing intestinal cDC proportion but lowers IL-10-producing intestinal cDCs number in the small intestine during* Plasmodium *infection

Since cDCs may change expression of MHCII in response to inflammation and the activation of cDCs can lead to both inflammatory and regulatory T cells, we next determined the effect of moderate malnutrition and malaria infection on cytokine production and co-stimulatory molecule expression of these intestinal cDCs. We evaluated the tolerogenic response initiated by intestinal cDCs by investigating the T_{reg}-polarizing cytokine IL-10 and evaluated the expression of the co-stimulatory molecule CD86. We found that the moderately malnourished mice have a higher proportion of activated cells expressing the costimulatory molecule CD86. For both dietary groups, infection seems to decrease the proportion of activated, costimulatory intestinal cDCs (**Figure 3A**). Conversely, we found that moderately malnourished mice have less cells capable of producing IL-10 compared to their well-nourished counterparts and do not seem to increase IL-10-producing cells even in response to *P. chabaudi* infection unlike the well-nourished mice that had enhanced IL-10 production after infection (**Figure 3B**).

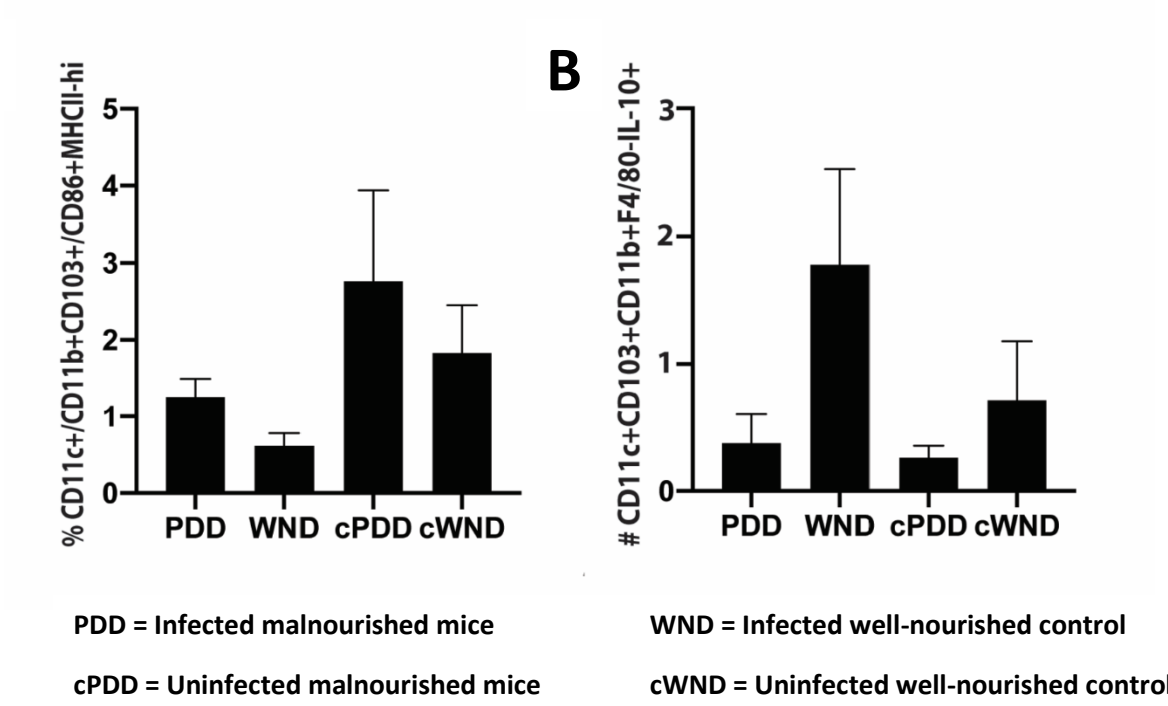

Figure 3. Malnutrition increases activated, co-stimulatory molecule-expressing intestinal cDC proportion but lowers IL-10-producing intestinal cDCs number in the small intestine during *Plasmodium* infection. C57BL/6 mice were fed either a control diet or a moderately malnourished diet. All infected mice were interperitoneally injected with $1x10^5$ iRBCs of *P. Chabaudi*. At 9 days post-infection, mice were euthanized, and the small intestines were collected for FACS analysis. Graphs show **A**) Intestinal cDC expressing high levels of MHC-II and co-stimulatory CD86, **B**) Intestinal cDC secreting IL-10. All data are shown as means ± SE, n=4 male mice per group, representative of 2 independent experiments. Statistical analysis was performed using a two-way ANOVA with Tukey's test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, indicates a statistically significant difference between treatments.

Malnutrition increases IL-12/23-producing intestinal cDCs number and proportion in the small intestine during Plasmodium *infection*

Since malnourished mice were expressing surface molecules for T cell activation, but with lower T_{reg}-inducing cytokines, we hypothesized that malnourished mice are skewing T cell populations towards an inflammatory phenotype in the gut. To evaluate pro-inflammatory influence from intestinal cDCs, we investigated intestinal cDCs producing IL-12/23. IL-12 would promote T_H1 inflammatory response and IL-23 is associated with the generation of T_H17

cells and downstream IL-17. Consistent with our predictions, malnourished mice had greater cell numbers and proportions of intestinal cDCs producing IL-12/23 (**Figure 4A&B**). The differences of these inflammation-promoting cells were more enhanced in the proportions as opposed to cell numbers.

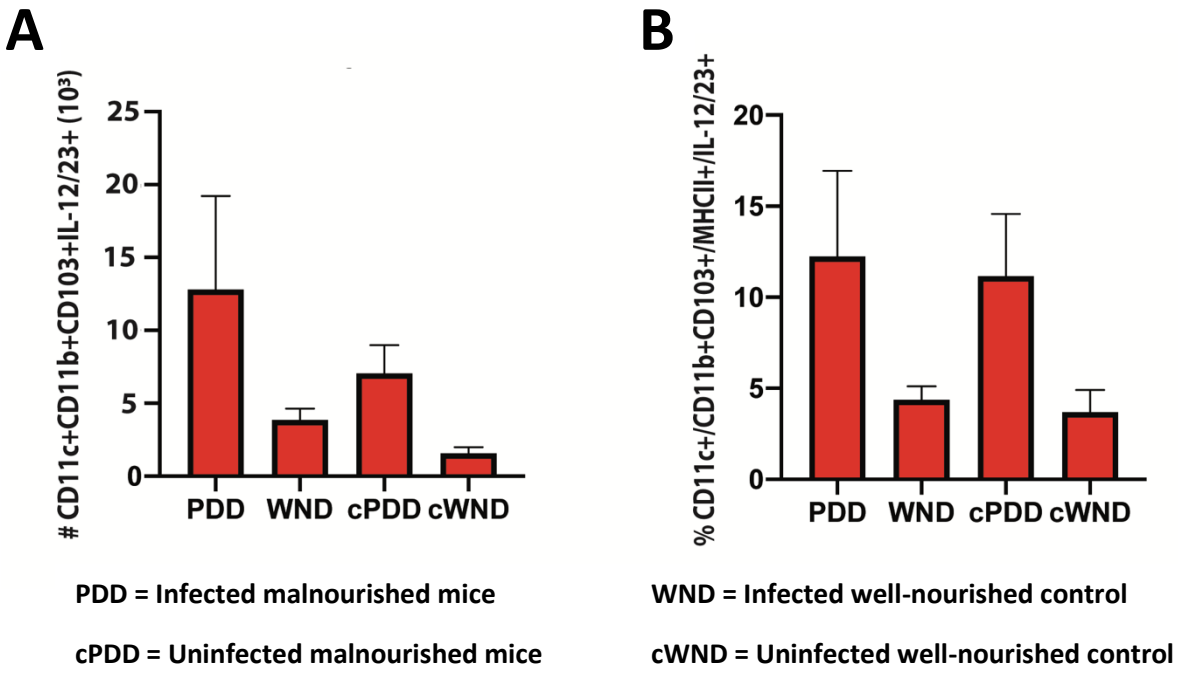

Figure 4. Malnutrition increases IL-12/23-producing intestinal cDCs number and proportion in the small intestine during *Plasmodium* infection. C57BL/6 mice were fed either a control diet or a moderately malnourished diet. All infected mice were interperitoneally injected with $1x10^5$ iRBCs of *P. Chabaudi*. At 9 days post-infection, mice were euthanized, and the small intestines were collected for FACS analysis. Graphs show **A**) Intestinal cDC secreting IL-12/23, **B**) Activated Intestinal cDC secreting IL-12/23. All data are shown as means ± SE, n=4 male mice per group, representative of 2 independent experiments. Statistical analysis was performed using a two-way ANOVA with Tukey's test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, indicates a statistically significant difference between treatments.

Malnutrition increases IL-12/23-producing and reduces IL-10-producing CD103+ cDC in the small intestine during* Plasmodium *infection

Trends in intestinal cDC functionality may have been due to a combination of localization in the lamina propria and response to pathogenic challenge as a consequence of malaria infection and/or dysbiosis induced by moderate malnutrition. cDCs also localize in lymphatic areas of the small and large intestine, including Peyer's patches as the keystone of GALT tissue in the small intestine, which more directly facilitates interaction between activated cDCs and T cells. We sought to investigate how CD103+ Peyer's patch-resident cDCs influence T cell differentiation with respect to cytokine production. To achieve this, we evaluated CD103+ cDC cellular proportions (**Figure 5A**), activation status (**Figure 5B**), IL-12/23 production (**Figure 5C, 5D**), and IL-10 production (**Figure 5E**). There seems to be no clear proportional disparity between total CD103+ cDC in infected, malnourished mice and their control counterparts (**Figure 5A**). However, well-nourished and infected individuals had slightly higher CD103+ activated proportion compared to the moderately malnourished (**Figure 5B**). The effect of this activation is conserved from intestinal cDCs to CD103+ Peyer's patch cDCs with malnourished and infected mice having greater IL-12/23-producing cell populations and proportions (**Figure 5C, 5D**). Consistent with our earlier observations, the well-nourished mice had higher production of IL-10 (**Figure 5E**).

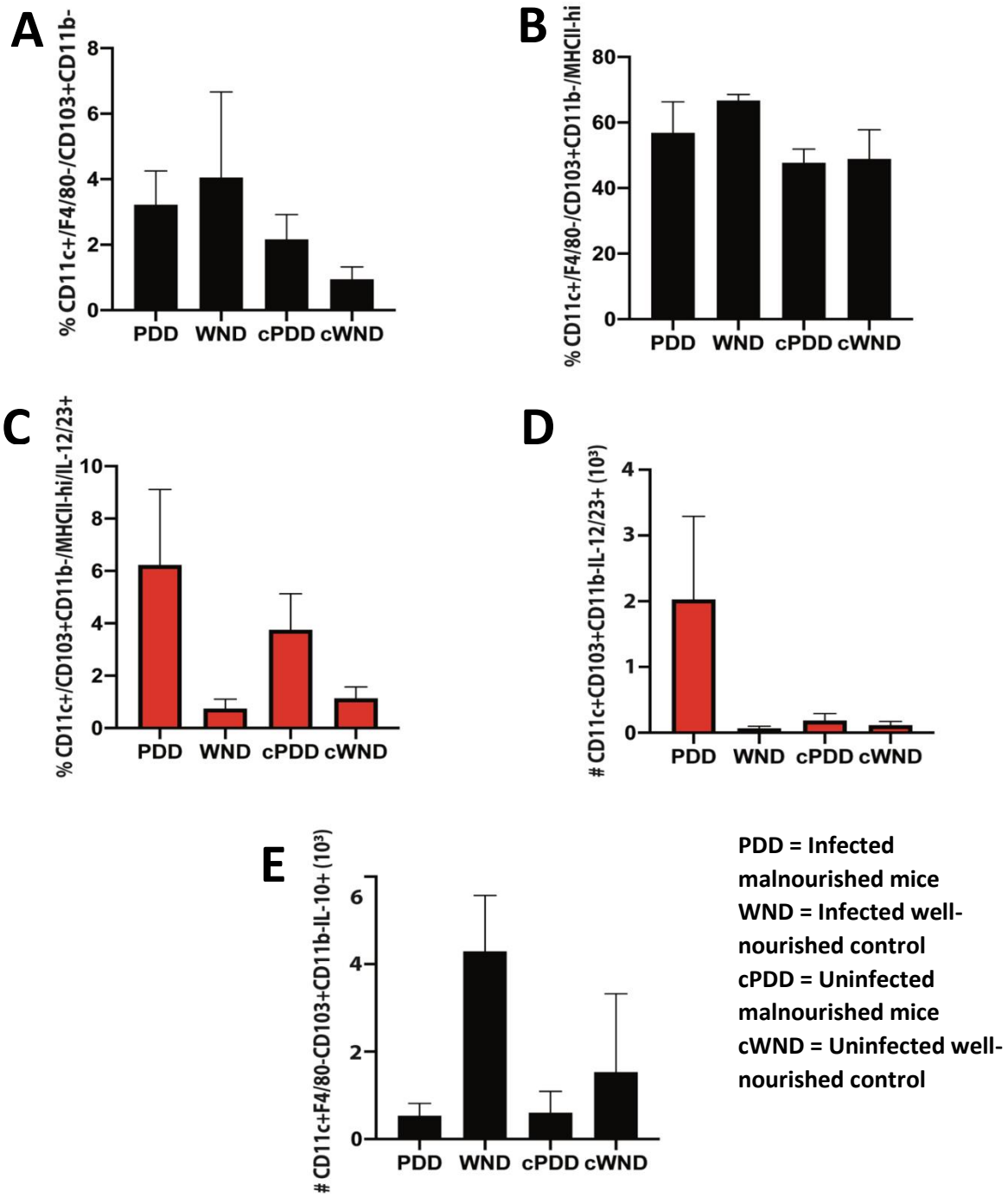

Figure 5. Malnutrition increases IL-12/23-producing and reduces IL-10-producing CD103+ cDC in the small intestine during *Plasmodium* infection. C57BL/6 mice were fed either a control diet or a moderately malnourished diet. All infected mice were interperitoneally injected with $1x10^5$ iRBCs of *P. Chabaudi*. At 9 days post-infection, mice were euthanized, and the small intestines were collected for FACS analysis. Graphs show **A**) CD103+ cDC proportion of myeloid cells, **B**) Activated CD103+ cDC, **C**) Proportion of activated CD103+ cDC producing IL-12/23, **D**) Number of activated CD103+ cDC producing IL-12/23, E) Number of CD103+ cDC producing IL-10. All data are shown as means ± SE, n=4 male mice per group, representative of 2 independent experiments. Statistical analysis was performed using a two-way ANOVA with Tukey's test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, indicates a statistically significant difference between treatments.

Malnutrition decreases CD11b+ cDC population, activation, and IL-10 production in the small intestine during* Plasmodium *infection

Dendritic cells selectively expressing CD11b but not CD103 inhabit both lymphoid and non-lymphoid tissues and constantly migrate through lymphatics to prime T cell differentiation. To evaluate the influence of malnutrition and malaria on CD11b+ cDCs, we used MHC-II upregulation, IL-12/23 production, and IL-10 production as an indication of activation and functional capacity. We found that CD11b+ populations stayed relatively consistent across the different groups with a trend towards lower proportions and cell numbers in uninfected, well-nourished controls (**Figure 6A, 6B**). Malnutrition seemed to decrease the number of activated CD11b+ cDCs in the face of infection when compared to the well-nourished control. Both uninfected groups showed roughly the same number of activated CD11b+ cDCs (**Figure 6C**). Although there is a trend in infected groups having higher numbers of IL-10-producing cells, there is significant error observed in IL-10-producing CD11b+ cDC cell counts for infected groups (**Figure 6D**) which was also observed in IL-12/23 data across all replicated experiments (data not shown).

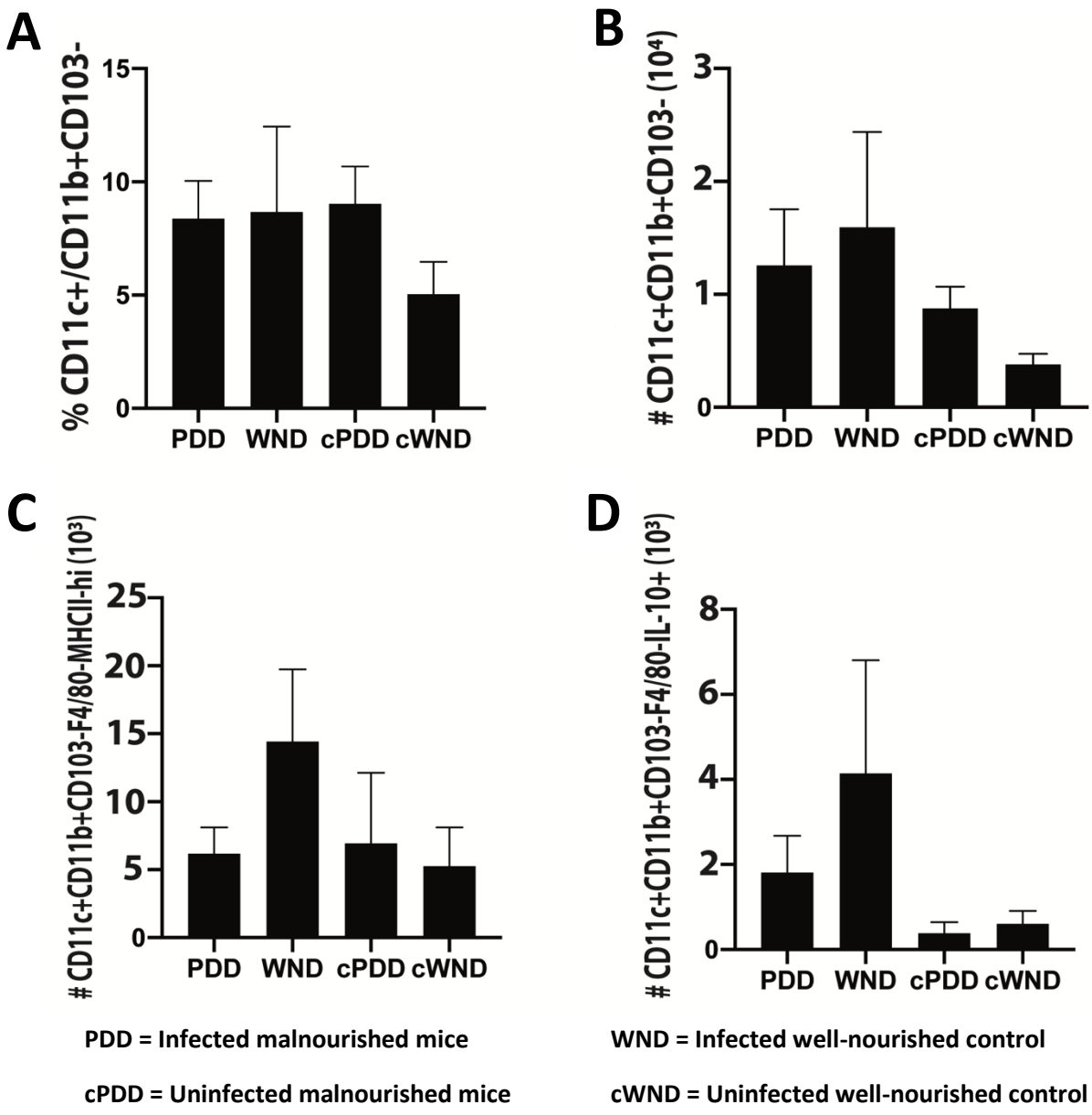

Figure 6. Malnutrition decreases CD11b+ cDC population, activation, and IL-10 production in the small intestine during *Plasmodium* infection. C57BL/6 mice were fed either a control diet or a moderately malnourished diet. All infected mice were interperitoneally injected with $1x10^5$ iRBCs of *P. Chabaudi*. At 9 days post-infection, mice were euthanized, and the small intestines were collected for FACS analysis. Graphs show **A**) CD11b+ cDC proportion of myeloid cells, **B**) CD103+ cDC Population, **C**) Number of activated CD11b+ cDC, **D**) Number of CD11b+ cDC producing IL-10. All data are shown as means ± SE, n=4 male mice per group, representative of 2 independent experiments. Statistical analysis was performed using a two-way ANOVA with Tukey's test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, indicates a statistically significant difference between treatments.

Malnutrition lowers innate immune cell populations in the large intestine during* Plasmodium *infection

Since most microbes are found in and some nutrient absorption takes place in the large intestine, we wondered if the defects seen by the cDCs in the small intestine due to malnutrition

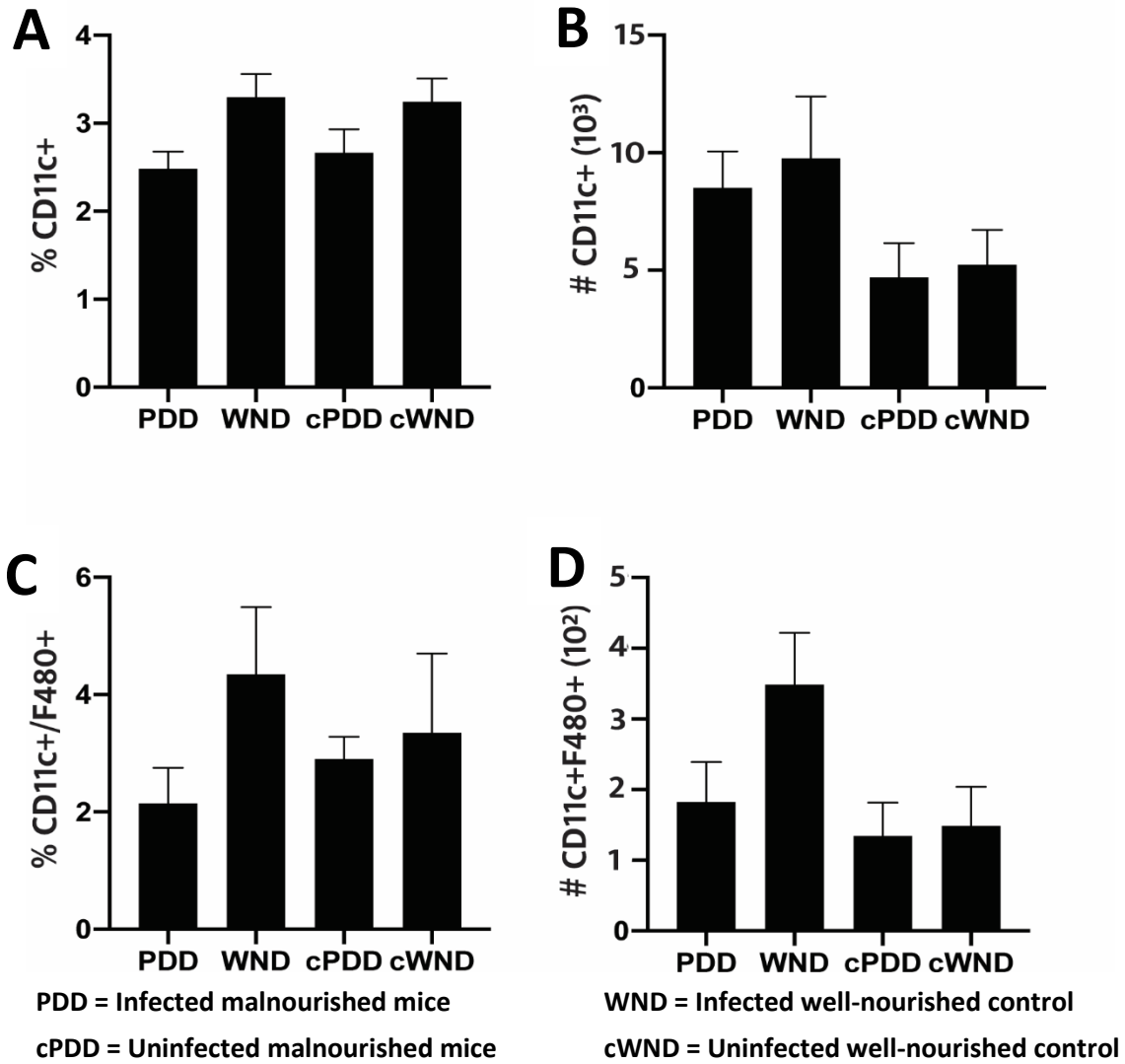

Figure 7. Malnutrition lowers innate immune cell populations in the large intestine during *Plasmodium* infection. C57BL/6 mice were fed either a control diet or a moderately malnourished diet. All infected mice were interperitoneally injected with $1x10^5$ iRBCs of *P. Chabaudi*. At 9 days post-infection, mice were euthanized, and the large intestines were collected for FACS analysis. Graphs show **A**) CD11c+ cDC proportions, **B**) CD11c+ cDC number, **C**) Macrophage proportion of CD11c+ cells, **D**) Number of CD11c+ macrophages. All data are shown as means ± SE, n=4 male mice per group, representative of 2 independent experiments. Statistical analysis was performed using a two-way ANOVA with Tukey's test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, indicates a statistically significant difference between treatments.

and malaria were replicated in the large intestine as well. Therefore, we used flow cytometry to investigate myeloid cell populations, cDC subset distributions, cDC activation status, and cytokine production capacity. We found that moderate malnutrition reduces the proportion and number of general CD11c+ myeloid cells (**Figure 7A&B)** and specifically reduces CD11b+F4/80+ macrophages (**Figure 7C&D)** in the large intestine during *Plasmodium* infection**.** Similar CD11c+ myeloid cell numbers were observed among both dietary statuses without challenge from *Plasmodium* infection (**Figure 7C)**, which was also reflected in macrophage populations within the large intestine (**Figure 7D)**.

Malnutrition lowers intestinal cDC populations but increases IL-12/23 production and activation status in the large intestine during* Plasmodium *infection

Malnutrition appears to decrease intestinal cDC cell number and proportion in the large intestine as indicated by the CD11c+CD11b+CD103+ population (**Figure 8A&B**). This change is dependent on challenge with *Plasmodium* infection, since intestinal cDC proportions and numbers appear consistent across both diets in uninfected controls (**Figure 8A&B**). Intestinal cDCs capable of producing inflammatory cytokines increase in proportion in moderately malnourished mice regardless of infection status, although infection seems to reduce this cell proportion selectively in the malnourished group (**Figure 8C**). Moreover, well-nourished mice trend towards an increase in inflammatory cDCs during infection whereas malnourished mice's cell populations are consistent across infection status (**Figure 8D**). To evaluate whether this was a result of lowered activation status, we investigated intestinal cDC upregulation of MHC-II and costimulatory molecule CD86. We found that malnourished mice had a higher proportion of fully activated intestinal cDCs in the large intestine, and infected mice tended to have lower

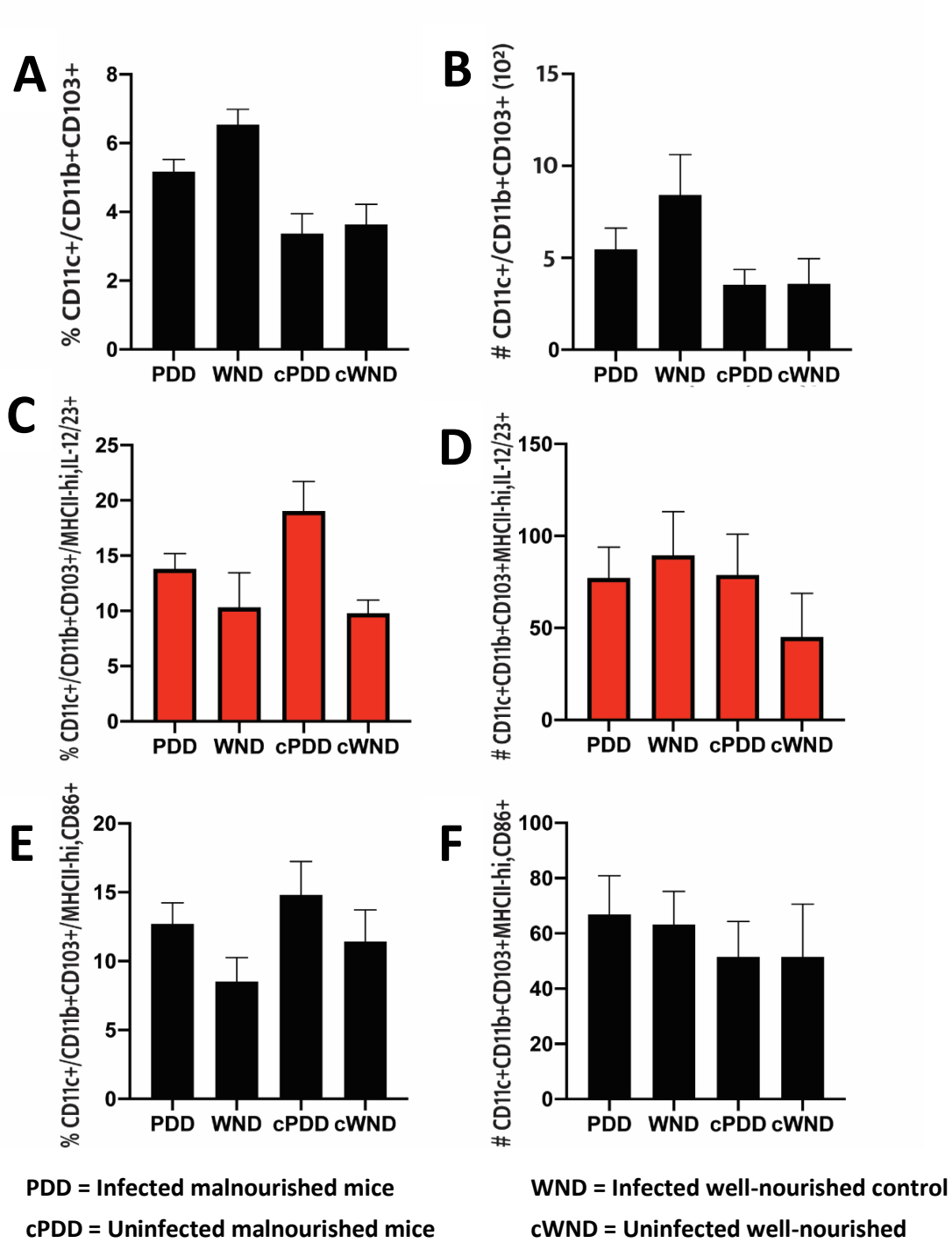

Figure 8. Malnutrition lowers intestinal cDC populations but increases IL-12/23 production and activation status in the large intestine during *Plasmodium* infection. C57BL/6 mice were fed either a control diet or a moderately malnourished diet. All infected mice were interperitoneally injected with $1x10^5$ iRBCs of *P. Chabaudi*. At 9 days post-infection, mice were euthanized, and the large intestines were collected for FACS analysis. Graphs show **A**) intestinal cDC proportions, **B**) intestinal cDC number, **C**) activated intestinal cDC proportion of IL-12/23 production, **D**) activated, IL-12/23-producing intestinal cDC number, **E**) proportion of fully activated intestinal cDCs, **F**) number of fully activated intestinal cDC. All data are shown as means ± SE, n=4 male mice per group, representative of 2 independent experiments. Statistical analysis was performed using a two-way ANOVA with Tukey's test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, indicates a statistically significant difference between treatments.

proportions of co-stimulatory capacity when compared to uninfected mice on the same diet (**Figure 8E**). Contrary to this, both infected groups tended to have higher fully activated intestinal cDC cell numbers compared to the uninfected controls (**Figure 8F**), suggesting that total effector activation may be regulated by inflammation. None of the groups exhibited IL-10 production above levels observed in negative and isotype controls (data not shown).

Malnutrition increases CD103+ cDC cell numbers and activation in the large intestine with and without* Plasmodium *infection

Lymphoid-resident CD103+ cDCs are important in luminal sampling of microbes and other pathogens. This is especially important in the large intestine because of the diversity of microbes, so we aimed to determine how moderate malnutrition and *Plasmodium* infection affected CD103+ cDCs. We found that CD103+ cDC proportions and cell numbers were increased in malnourished mice compared to their well-nourished controls (**Figure 9A&B**), and *Plasmodium* infection did not seem to affect cell proportions regardless of diet (**Figure 9A**) but did lead to a trend of increasing cell numbers (**Figure 9B**). Among these, a higher proportion and number of CD103+ cDCs were found to be activated in malnourished mice while infection led to a similar increase when compared to uninfected controls (**Figure 9C&D**). Surprisingly, none of the groups of mice produced IL-10 or IL-12/23 above what was observed in negative and isotype controls (data not shown).

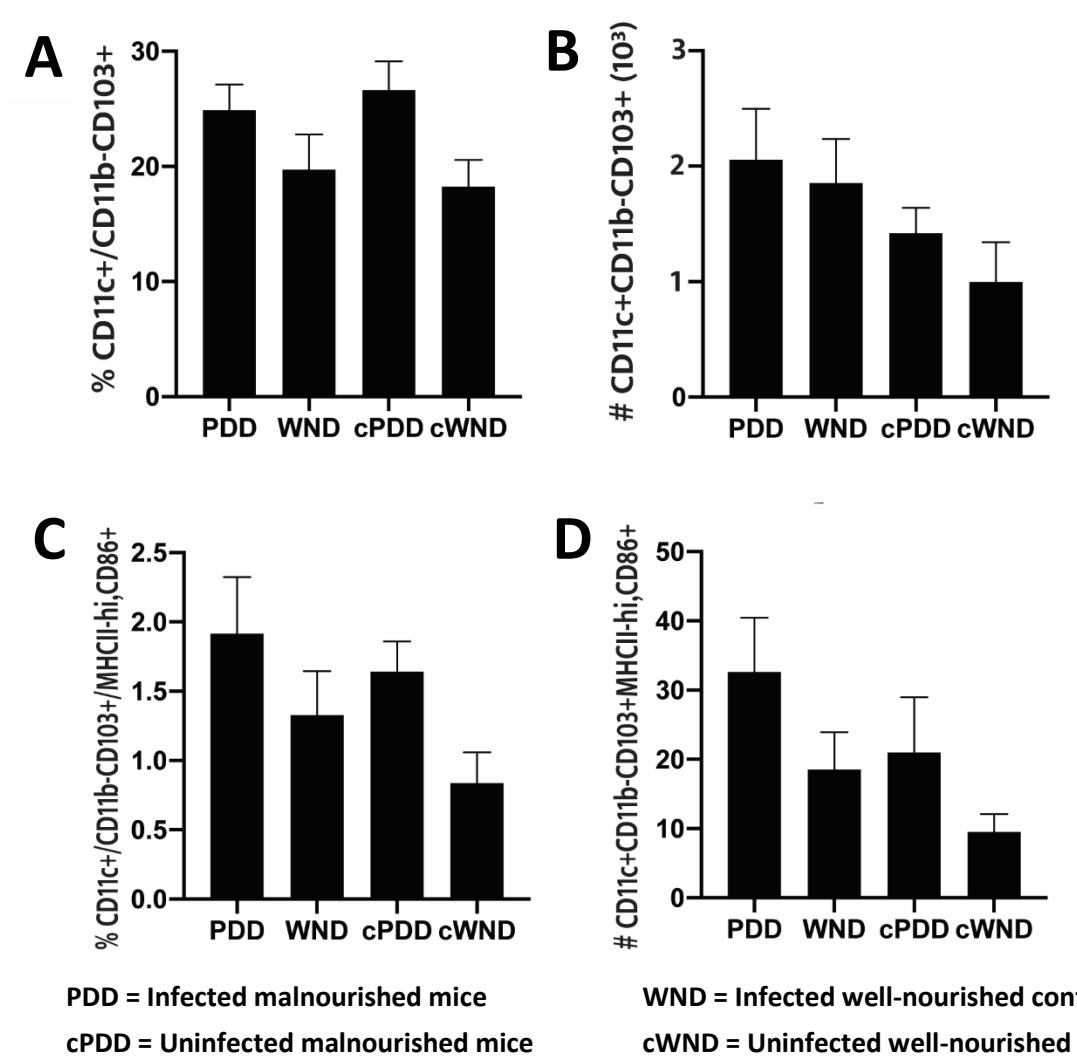

Figure 9. Malnutrition increases CD103+ cDC cell numbers and activation in the large intestine with and without *Plasmodium* infection. C57BL/6 mice were fed either a control diet or a moderately malnourished diet All infected mice were interperitoneally injected with $1x10^5$ iRBCs of *P. Chabaudi*. At 9 days post-infection, mice were euthanized, and the large intestines were collected for FACS analysis. Graphs show **A**) CD103c+ cDC proportions, **B**) CD103c+ cDC number, **C**) proportion of activated CD103c+ cDCs, **D**) Number of activated CD103c+ cDCs. All data are shown as means ± SE, n=4 male mice per group, representative of 2 independent experiments. Statistical analysis was performed using a two-way ANOVA with Tukey's test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, indicates a statistically significant difference between treatments.

Malnutrition increases the proportion of activation, but decreases CD11b+ cDC cell numbers in the large intestine during* Plasmodium *infection

Tolerance is induced by CD11b+ cDCs specifically in the colon (128), so we evaluated large intestine CD11b+ cDC population, activation, and cytokine production changes during moderate malnutrition and *Plasmodium* infection. We found that malnutrition decreases the proportion of CD11b+ cDCs during infection but does not change the proportion of these cells with diet alteration alone (**Figure 10A**). This trend was reflected in CD11b+ cell numbers with a slight but insignificant decrease in cell numbers attributable solely to malnutrition (**Figure 10B**). Malnourished mice had higher proportions of activated CD11b+ cDCs, and activation proportion was generally consistent across diets regardless of infection status (**Figure 10C**). This proportional difference resulted in increased activated cell numbers in both infected groups (**Figure 10D**). None of the groups contained cells that produced IL-10 or IL-12/23 at a level greater than what was observed in negative and isotype controls (data not shown).

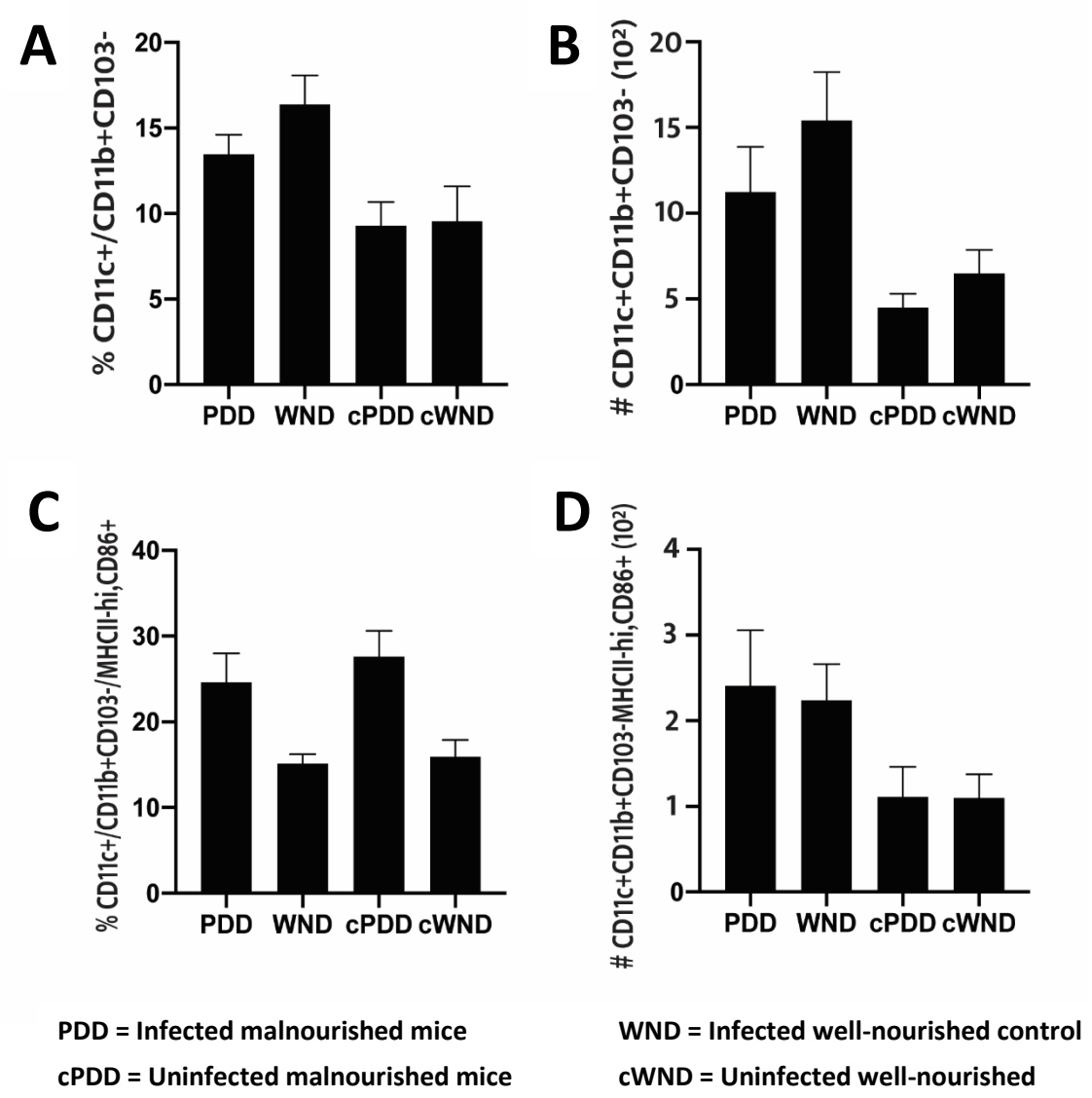

Figure 10. Malnutrition decreases CD11b+ cDC cell numbers but increases activation proportion in the large intestine during *Plasmodium* infection. C57BL/6 mice were fed either a control diet or a moderately malnourished diet. All infected mice were interperitoneally injected with $1x10^5$ iRBCs of *P. Chabaudi*. At 9 days post-infection, mice were euthanized, and the large intestines were collected for FACS analysis. Graphs show **A**) CD11b+ cDC proportions, **B**) CD11b+ cDC number, **C**) proportion of activated CD11b+ cDCs, **D**) Number of activated CD11b+ cDCs. All data are shown as means ± SE, n=4 male mice per group, representative of 2 independent experiments. Statistical analysis was performed using a two-way ANOVA with Tukey's test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, indicates a statistically significant difference between treatments.

Malnutrition increases serum levels of ALT during* Plasmodium *infection

Liver injury occurs in both severe and uncomplicated cases of malaria (129). Because of altered mucosal DC sampling and our previous data showing mucosal damage in malnourished mice (121), we wondered if this would be associated with alterations in liver enzyme proteins. Therefore, we determined blood serum levels of alanine aminotransferase (ALT) as an indicator of liver damage. We found normal (20-60 u/L) (130) levels of ALT in the sera of uninfected controls. For both malnourished and well-nourished groups, we noticed a significant increase in serum ALT levels during *Plasmodium* infection (**Figure 11A**). The fold-change in ALT activity during infection differed significantly between diets with roughly a 3-fold increase observed in well-nourished mice and a 5.5-fold increase in moderately malnourished mice (**Figure 11B**).

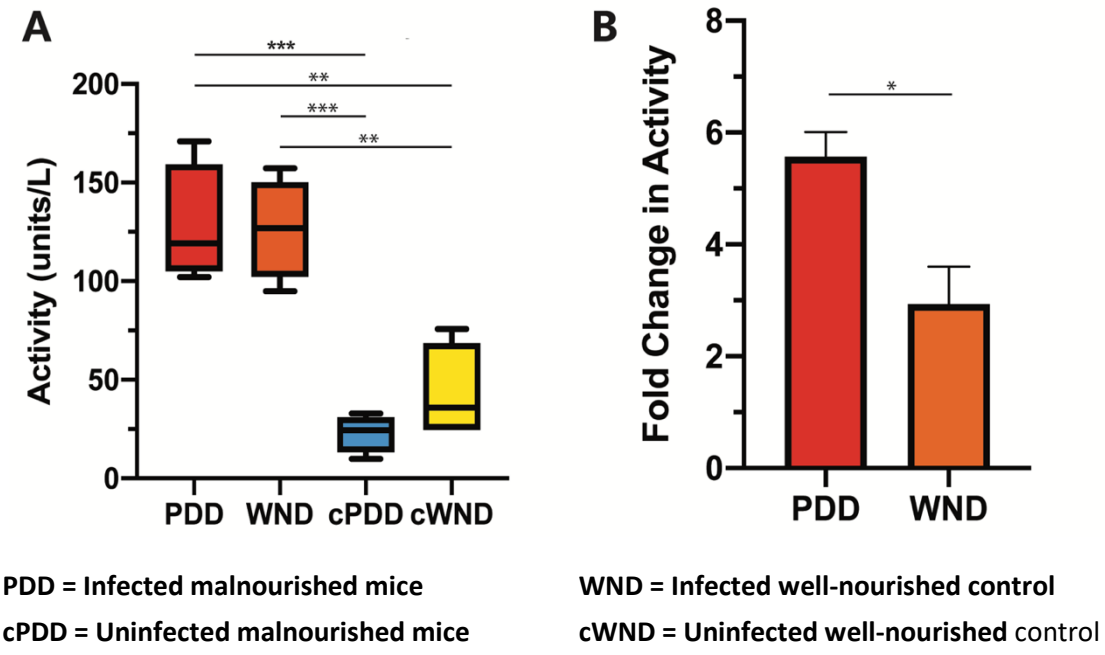

Figure 11. Malnutrition increases serum levels of ALT during *Plasmodium* infection. C57BL/6 mice were fed either a control diet or a moderately malnourished diet. All infected mice were interperitoneally injected with $1x10^5$ iRBCs of *P. Chabaudi*. At 9 days post-infection, mice were euthanized, and the large intestines were collected for FACS analysis. Graphs show **A**) ALT activity normalized to international units per liter, **B**) fold change in ALT activity from uninfected controls. n=4 male mice per group, each sample run in duplicate, representative of 2 independent experiments and assays. Statistical analysis was performed using A) a one-way ANOVA with Tukey's test, and B) a two-tailed T test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, indicates a statistically significant difference between treatments. One unit of ALT is the amount of enzyme that generates 1 µmole of pyruvate per minute at 37°C.

Chapter 4: Discussion

Even though malaria and malnutrition are common in the tropical and subtropical regions, research focused on how malaria affects gut immunity during moderate malnutrition is limited. To expand on this, we investigated how mucosal innate immunity specifically in the gut is affected by these two conditions. In this study, we have shown that moderate malnutrition and malaria infection holistically decrease mucosal innate immunity in both the small and large intestines. This is reflected by decreased CD11c+ general populations, specifically reduced F4/80+ macrophages (**Figures 1&7**), and differential impacts on dendritic cell subsets. Differences in the activation and cytokine production of the DC subsets can be explained by the changes in localized environment and compensatory responses induced by moderate malnutrition and malaria. This is reflected by changes in MHC-II upregulation, CD86 expression, IL-12/23 and IL-10 production by CD11b+, CD103+, and intestinal cDCs. These changes may further lead to epithelial barrier breakdown and defective adaptive immune cell activation which is necessary to effectively combat *Plasmodium* infection.

Both malaria and malnutrition have been shown to induce dysbiosis thus leading to activation of mucosal innate immunity (119, 131). Previous research using the same diet as the one used in this study revealed increased gut leakage and production of inflammatory IFN-γ by the macrophages (121). Antigen sampling in the gut is primarily handled by M cell antigen delivery and cDC (both intestinal and CD103+) dendrite extension into the lumen. With the presence of foreign bacteria and the breakdown of the epithelial barrier allowing the bacteria to invade subsequent tissues, innate immunity has compounding signals for activation. Both local inflammatory signaling from macrophages and TLR-7 stimulation from bacterial lipopolysaccharide recognition via PRRs on DCs lead to cDC maturation. This was shown by

increased proportions of intestinal cDCs in small intestines of malnourished mice having MHC-II upregulation and co-stimulatory molecule CD86 expression during *Plasmodium* infection. Intestinal cDCs are able to produce a variety of effector cytokines to influence T cells, so we investigated which polarizing direction these cDCs were working toward. We found that small intestine intestinal cDC in the moderately malnourished mice promoted inflammatory T_H17 differentiation and induced IFN-γ through T_H1 polarization and natural killer cell activation via IL-12/23 production (132) while well-nourished controls had cDCs producing anti-inflammatory IL-10. This diet-specific polarization was shown similarly in CD103+ cDCs found in the small intestine but to a lesser extent in terms of IL-12/23 production and a greater extent with respect to IL-10 production. The promotion of T_H1 and T_H17 in malnourished mice may be associated with mucosal damage due to increased inflammation whereas IL-10 production is indicative of immunomodulation and regulation. Our data suggests that malnourished mice may have a loss of immunoregulatory function especially in the small intestines that further skews the gut associated cells toward an inflammatory phenotype.

Since intestinal cDCs and CD103+ cDCs are primarily responsible for luminal sampling which is especially important in microbe-rich areas like the large intestine, we evaluated the activation and cytokine production of these subsets in both intestinal tissues. We found increased activation and IL-12/23 production in large intestine intestinal cDCs of moderately malnourished mice, consistent with the influence of an already inflammatory localized environment (133). Interestingly, we found no significant production of IL-12/23 in any of the other cDC subsets in addition to no significant production of IL-10 in any cDC subset within the large intestine. It has been shown that malnourished children produce lower levels of secretory IgA which lowers their surveillance of pathogenic antigens (134), so cytokine production may be triggered primarily by

disseminating pathogens as opposed to proper luminal sampling. This may have also been due to the difference in cell numbers since the large intestine has significantly fewer total cells compared to the small intestine and any detectable signal may have been equivalent to background noise observed in isotype and unstimulated controls.

Regarding activation, CD103+ cDC stimulatory capacity was upregulated selectively in the large intestines of moderately malnourished mice. This is explained by the differential role of CD103+ cDCs in the different tissues. CD103+ cDCs in the small intestine largely contribute to induction of tolerance whereas CD11b+ cells in the large intestine are able to induce tolerance in a way that is independent of DC-generated retinoic acid (128). In this sense, large intestine CD103+ cDCs are geared more toward antigen loading and migration to secondary lymph tissue for T cell priming, and increased activation agrees with a migratory response to inflammation (135). These cells are also able to influence CD8+ cytotoxic T cells with expression of the chemokine receptor XCR1 and production of IL-12 and IL-15 (81, 82). Due to an increased demand for adaptive immunity priming in response to dysbiosis and dissemination associated with malaria, malnutrition, and subsequent epithelial barrier breakdown, malnourished mice have CD103+ cDC populations that are increased in proportion, cell number, and activation compared to well-nourished controls.

In addition to their ability to prime tolerance in the large intestine, CD11b+ cDCs play a dominant role in MHC-II expression and consequentially effector CD4+ T cell activation (81). This multi-faceted capacity to induce different T cell responses is critically important in the gut, especially since the PRR profile of intestinal cDCs more closely resembles CD103+ cDCs (136). The necessity for such cells to exist in the lamina propria of the large intestine explains why a decreased proportion and cell number is compensated by an increased proportion of activation

that leads to comparable activated cell numbers among both diet types of the same infectious status.

Another explanation for the apparent diet-specific pattern of these cDCs toward pro- or anti-inflammatory effector function is the possibility of ontogeny differences. Dendritic cells derived from DC-restricted precursors are capable of becoming phenotypically mature without production of inflammatory cytokines which differs from monocyte-derived inflammatory cDCs (81) and the proportion of these precursors may differ among our treatment groups. In addition to this, the zinc finger transcription factor zbtb46 is expressed specifically in the cDC lineage and acts as a negative regulator of cDC activation (81); reduced zbtb46 as a result of micronutrient deficiency may lead to upregulated activation.

Malaria has been associated with multi-organ damage which further impacts all bodily systems *in vivo* (137). Some of the pathologies associated with *Plasmodium* infection are kidney and liver failure, which was observable as darkened urine especially in the moderately malnourished mice of this study. Our results are consistent with other studies that have reported elevation of hepatic enzymes in patients with malaria indicating liver damage (138, 139), suggesting that the blood stage malaria infection by *P. chabaudi* negatively impacts the livers of moderately malnourished C57BL/6 mice. People with *Plasmodium falciparum* infection have shown up to approximately 3-fold increase in aspartate aminotransferase (AST) and ALT serum levels depending on the severity of infection (123). Well-nourished mice experienced the expected 3-fold increase in ALT levels during *P. chabaudi* infection, which was exacerbated to a 5.5-fold increase in moderately malnourished mice. This data is particularly valuable since evaluation of ALT in human patients suffering from *Plasmodium* infection may be affected by alcoholic liver diseases, and AST/ALT ratios are not uniquely attributable to alcoholic liver

disease or cirrhosis alone (140). AST is represented in the heart, skeletal muscle, kidneys, brain, and RBCs whereas ALT is primarily in liver cells, therefore ALT is more specific for liver damage (141). Moreover, time-based AST/ALT index can be used to discriminate the type of liver damage that is observed. This would be useful in understanding how *Plasmodium* infection is impacted by malnutrition to lead to fibrosis or cirrhosis. Since RBCs contain AST, elevated levels may be representative of haemolysis that is increased in severe cases of malaria. We found higher levels of ALT in uninfected, moderately malnourished mice when compared to their well-nourished controls since serum liver enzymes are generally increased in patients with PEM (142); however, decreased ALP activity has been reported in cases of PEM, zinc, and magnesium deficiencies too (143-145). This helps emphasize the necessity for studies with compound nutrient deficiencies to model real-world circumstances. Further investigation into AST and ALP may help elucidate the relationship between nutritional status and liver damage during *Plasmodium* infection (123).

Future studies surrounding this detrimental relationship between malaria and moderate malnutrition will help to clarify the mechanisms that lead to severe gastrointestinal disruption. Our current understanding of how epithelial barrier weakening results from these conditions prompts an analysis of tight junction protein synthesis, both at a transcriptional and translational level (146). Evaluation of the tight junction proteins can be accomplished with molecular techniques like RT-PCR and western blotting. A loss of tight junction protein expression may be leading to bacteremia and the subsequent acute liver damage that was observed in our study.

Since dysbiosis plays a role in immune modulation and the dendritic cells involved in this regulation are impacted by the conditions of this study, shifts in the resident bacterial communities as a result of *Plasmodium* infection and moderate malnutrition may be leading to

increased disease severity and coinfection susceptibility (147). In addition to this, bacterial dissemination is a factor in multi-organ failure which is made possible by a loss of epithelial barrier integrity (148). Identifying taxa shifts in microbial communities during *Plasmodium* infection and moderate malnutrition would help explain pathologies that have been observed. This can be accomplished by extracting DNA from stool samples and comparing them to microbial DNA found in the blood of afflicted and control mice. Characterizing the impacts of protein, zinc, and iron deficiencies during *Plasmodium* infection on microbial diversity and dissemination will help to explain bacteremia and peripheral organ damage that has been identified (18). Additional peripheral organ damage evaluation in the form of AST and ALP assays to further characterize liver damage and histological analysis of kidney and liver tissue with hematoxylin and eosin (H&E) staining would be useful to fully characterize impacted physiology.

In accordance with changes in microbial diversity regulating differential immune response, surveillance of these microbes is critical. A key component of luminal surveillance is secretory IgA binding to antigens and allowing for trans-epithelial passage (149). To evaluate how immune surveillance may be affected in a way that is distinct from direct DC sampling, levels of IgA in the gut can be measured in our moderately malnourished model during *Plasmodium* infection. This can be measured by ELISA, and the results would be indicative of B cell and epithelial cell functionality. Further investigation into effector T cell populations and cytokine production would allow for hypothesis of diet-based changes in immunoregulation during *Plasmodium* infection to be supported wholistically or characterized distinctly in terms of innate and adaptive immune affects. Dendritic cell production of IL-6, IL-12, and IL-15 could also be evaluated to support conclusions of cDC anti-microbial defense during *Plasmodium*

infection and nutrient deficiency (82). Intestinal cDCs have a superior ability to induce peripheral tolerance due to their expression of aldehyde dehydrogenase (ALDH), an enzyme that metabolizes vitamin A into retinoic acid (81). Investigating expression of ALDH via RT-PCR and western blotting would be useful in understanding the mechanism by which tolerance is affected in our model.

Lastly, the deficiencies within our moderately malnourished model are all suspected to have both individual and synergistic effects on gut mucosal immunity and integrity. To better understand how a lack of protein, iron, and zinc impact the host, new diets that are specifically deficient in a combination of these nutrients can be utilized (i.e., protein/iron-deficient, protein/zinc-deficient, and iron/zinc-deficient). This will elucidate where efforts toward supplementation should be focused for maximum restoration of gut mucosal immunity and integrity since supplementation of zinc and iron have shown promising results of reducing pathological severity thus far (150-152).

Chapter 5: Conclusions

Malaria and moderate malnutrition result in pathophysiological conditions that influence one another in a way that complicates the understanding of possible remedies, especially with regards to the underlying immunological mechanisms. In our current study, we investigated how mucosal innate immunity in the gut is impacted by malaria in a malnourished condition. Our data suggest that moderate malnutrition and *Plasmodium* infection lead to fewer general CD11c+ innate immune cells with specific reductions in macrophages and certain cDC subsets. Moderate malnutrition differentially affects cDC maturation in the small intestine, increases activation of all cDC subsets in the large intestine, and influences the cytokine production profiles of cDCs towards an inflammatory phenotype. Protein, zinc, and iron deficiencies exacerbate peripheral organ damage induced by *Plasmodium* infection as indicated by blood serum levels of hepatic enzyme ALT. Improper inflammatory regulation resulting from cDC dysfunction may contribute to the hallmark pathologies seen commonly in malaria cases around the world. Malaria and moderate malnutrition result in phenotypic differences in gut mucosal innate immunity which may contribute to susceptibility to coinfections and consequential comorbidity.

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Vita

Tyler Bernard Olender was born in Greensboro, North Carolina in 1999. From a young age, he was always interested in medicine and science. This fascination originated from his mother and extended family working in hospitals as nurses. At the start of his undergraduate degree, Tyler intended to work in the health care field. After taking classes in the biochemistry and molecular biology concentrations, he realized that he had a particular interest in microbes and the intricacies of human physiology at a molecular level. The passion for furthering his understanding of these topics along with his love for the Appalachian Mountains led to him pursuing a master's degree in cellular and molecular biology at Appalachian State University as a part of the Opata lab (Team Malaria).

During his first year in the master's program, Tyler was published as a second author for the article "*Plasmodium chabaudi* infection Alters Gut Morphology and Mucosal Innate Immunity in Moderately Malnourished Mice". He gave an oral presentation at Appalachian State's Celebration of Student Research in 2022 after writing and receiving several competitive university sponsored research grants for funding of his projects. Additionally, Tyler will be presenting his thesis data in the Biology Department's Seminar Series at Appalachian State University. He is currently working on a collaborative project with the North Carolina Research Campus' Human Performance Laboratory in Kannapolis. Tyler hopes to work as a research scientist in the microbiology and/or immunology fields. Tyler has taught Introductory biology lab for non-biology majors and the junior level Molecular Biology labs. He has also trained several students in lab techniques. As a leader, he is the president of the Biology Graduate Students Association (BGSA).

In his free time, Tyler likes to enjoy the Appalachian Mountains by hiking and snowboarding. He also enjoys spending time with his friends and family whether it be sharing a meal or going to events like concerts and sporting games. He is a part of a university-based recreational soccer team and tries to stay active when time permits.