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Evaluating the Effects of Moringa Tea on Lymphocyte Proliferation

Donja S. Pettiford[1], Joshua Hayes[2], and Radiah C. Minor[1]*

[1]*Department of Animal Sciences, North Carolina Agricultural and Technical State University, USA*
[2]*Department of Biology, North Carolina Agricultural and Technical State University, USA*

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***Corresponding author:**
Radiah C. Minor
Department of Animal Sciences
North Carolina Agricultural and Technical State University
1601 E Market St. Greensboro, NC. 27411
USA
E-mail: rcminor@ncat.edu

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Abstract

Moringa oleifera Lam (MOL) is a tree originating from tropical and subtropical regions in Asia and Africa that has been utilized in traditional medicine that is increasingly being investigated for its medicinal attributes. Moringa has been shown convincingly to have an immunomodulatory role in influencing innate immune cells such as macrophages, however, Moringa's role in modulating the adaptive immune response, particularly those of lymphocytes is emerging. The objective of this study was to investigate whether and how consumption of Moringa tea (MT) impacts T- and B-lymphocyte proliferation. For this study, mice were given fresh water or fresh Moringa Tea (MT) to drink for 14 days. On day 14, the spleens and lymph nodes were collected to compare the percentages of lymphocytes (T- and B-lymphocytes) between the two groups. Flow cytometric analysis showed no changes in the populations of CD3+, CD19+ cells, or CD4+ and CD8+ cells within the spleen or lymph nodes of mice that consumed MT compared to mice that consumed water. These data suggest that the consumption of MT did not alter the lymphocyte counts during non-challenge. Therefore, the effect of MT on lymphocyte proliferation in response to either Lipopolysaccharide (LPS), Concanavalin A (CON A), or anti-CD3 and anti-CD28 was evaluated ex vivo. MTT assays showed that lymphocytes within the spleen, but not the lymph node, of mice that consumed MT for 14 days had a trend toward decreased proliferation in response to activation with anti-CD3+ and anti-CD28+ (specific T lymphocyte coactivators), but not to CON A or LPS. Taken together, the data from this study suggests that Moringa tea consumption for 14 days has an impact on T cell and not B cell proliferative responses through a mechanism that may involve pathways associated with T cell and co-stimulation receptors.

Running Title: Role of Moringa Tea on Lymphocyte Proliferation

Keywords: Lymphocytes, T cells, proliferation, *Moringa oleifera*

1. Introduction

Moringa oleifera Lam (MOL) is a tree indigenous to the tropical and subtropical regions of Asia and Africa that is now cultivated worldwide and consumed by humans and animals for its nutritional and health benefits [1]. For centuries, Moringa has been used for nutritional purposes and in traditional medicine. MOL is known to have anti-microbial, anti-tumor, anti-diabetic, anti-ulcer, hepatoprotective, and anti-inflammatory capabilities [2-5].

Moringa oleifera extracts from its leaves, roots, and other sections have been shown to have immunomodulatory functions [6-8]. Much of the research has mostly focused on the impact of MOL on the regulation of macrophages, a key component of the innate immune response. Several studies show that Moringa consumption can impact hematological parameters. For example, it has been reported that Wistar rats that consume aqueous extracts from Moringa seed have significant increases in granulocytes with a concomitant significant decrease in lymphocyte counts [9]. In contrast, rats that consumed high doses (300 mg/body weight) of *M. oleifera* leaf extract had significantly higher blood lymphocyte counts compared to controls [10]. A study in Red Tilapia showed that replacing normal feed with feed containing at least 20% Moringa leaves significantly increased the number of circulating lymphocytes in the blood [11].

The influence of MOL on adaptive immune responses and lymphocytes is being increasingly investigated. For example, in poultry, while replacing canola meal with Moringa leaf powder had no impact on white blood cell counts in the birds, the Moringa leaf powder supplementation significantly increased antibody titer in Newcastle Disease and Infectious Bursal Disease vaccinated birds (12). Wuryandari, et al reported that fermented Moringa extracts administered to mice infected with *Salmonella typhi* intraperitoneally were shown to increase the number of CD11b+ (macrophages) and B220+ (B-cells) isolated from the abdominal space [13]. These studies support the potential for Moringa to regulate adaptive lymphocyte responses by impacting lymphocyte numbers and function, but the mechanisms remain to be elucidated.

Tea is reported to be the second most consumed drink, after water, globally [14]. Furthermore, the consumption of tea from a variety of plants and herbs has positive health benefits including immune modulation [15], and according to World Tea News the consumption of Moringa tea, for its health benefits, is rising [16]. A study aimed at comparing the efficacy of ethanolic extract of Moringa with a Moringa tea (infusion) for the treatment of a murine model of nonalcoholic fatty liver, hyperlipidemia, and hyperglycemia fed with a high-fat diet showed that there were better therapeutic effects with the infusion/tea as compared to the Moringa ethanolic extracts. Noteworthy was that this study also showed that neither the Moringa tea nor the extract made any difference in WBC counts in the blood of the mice in the study. Our lab has reported that mice that consume Moringa tea have reduced inflammation in their lungs post inhalation of swine confinement dust [17] and that Moringa tea consumption by mice induced variations in the levels of circulating neutrophils but not lymphocytes in response to stress [18]. Rachmawati, et al reported that the presence of both high (10ug/mL) or low doses (0.1ug/mL) of aqueous Moringa extracts in an *in vitro* splenocyte culture led to increased counts of CD4+, CD8+, and B220+ cells despite the cells not being activated [19]. The data from these studies suggest that Moringa extracts and tea can impact lymphocytes, but it is unknown what effect Moringa, particularly when consumed as a tea, has on lymphocyte proliferation. Therefore, the aim of the current study was to investigate the impact that consumption of *M. oleifera* leaf tea has on lymphocyte proliferation.

2. Materials and Methods

2.1. Animals:

All animal handling and experimental procedures for this study were conducted in accordance with applicable regulations and were approved by the Institutional Animal Care and Use Committee of North Carolina A&T State University. For this study, a total of 12 female Balb/c mice between the ages of 7-8 weeks were purchased from Harlan Laboratory (Indianapolis, IN. USA). Mice were maintained in the Laboratory Animal Resource Unit of North Carolina A&T State University on a 12 light/12 dark cycle and consistent temperature (68-70^{0}F) and humidity (58-60%). During the 14-day dosing period, all mice had ad libitum access to standard rodent chow (Purina 5001) and fresh water (100 ml) or Moringa tea (MT) (100 ml) daily and were minimally handled to record weights every 2 to 3 days.

2.2. Preparation of 2% (w/v) Moringa Tea and Dosing:

Moringa leaves were harvested from North Carolina Agricultural and Technical State University Farm at the mature stage of the growth (at least 24 inches in height), air dried, and used to prepare Moringa tea (MT). The tea was prepared as previously described [17, 18]. Briefly, 40 grams of dried Moringa leaves were steeped in 2000 ml of 95°C distilled deionized water for 30 minutes. After 30 minutes, the tea was filtered through sterile cheesecloth to remove large particles and then filtered through a sterile funnel lined with 3M filter paper to remove smaller particles. The tea was subjected to final filtration using a sterile 0.22µM PES vacuum filter system (Celltreat, Largo, FL.USA) under a laminar flow hood. The sterile filtered MT was aliquoted into sterile 50ml tubes under a laminar flow hood and stored at 40C until used. For dosing, mice were randomly divided into the control (water) (n=6) or experimental (tea) (n=6) groups. Every morning for 14 days, mice were provided with either 100ml of fresh distilled water (control) or Moringa tea (MT). The consumption of the water or tea was monitored every two to three days by measuring the amount of liquid remaining from the original (100ml) of liquid offered per cage.

2.3. Tissue Harvest and Cell Preparation:

On day 14 mice were humanely euthanized, and the spleen and the mesenteric, inguinal, brachial, axillar, and superficial cervical lymph nodes regions of the body were harvested. Single-cell suspensions from the lymph nodes and spleens were prepared via mechanical dissociation using the plunger of a sterile 3ml syringe. The cell suspension was passed through a 70µm cell strainer with complete Roswell Park Memorial Institute (RPMI) media composed of sodium

bicarbonate penicillin/streptomycin, beta-mercaptoethanol, and 10% Fetal Bovine Serum (FBS) into a sterile 50 mL tube. Cells were pelleted by centrifuged at 1200 rpm for 5 minutes. Cells were resuspended in red blood cell lysis buffer (eBiosciences, San Diego, CA. USA), incubated for 5 min then washed in complete RPMI media. Cells were resuspended ($1x10^7$/mL) and prepared for flow cytometry or proliferation assay.

2.4. Flow Cytometry:

To determine the amount and type of lymphocytes present in the spleen and lymph nodes cells were analyzed by flow cytometry. Briefly, $1x\ 10^6$ cells, in 100 µl, were seeded onto a 96-well-rounded bottom plate, the cells were pelleted by centrifuging the plate for 2 minutes at 2,000 rpm. The supernatant was discarded cells were treated with Fc-Block (rat IgG_2b anti-mouse CD16/CD32 monoclonal antibody) (eBiosciences, San Diego, CA. USA) to prevent non-specific binding. After a 20-minute incubation on ice, cells were washed in FACS buffer (PBS, 5% Fetal Bovine Serum, 0.1% Sodium Azide) then the resuspended cells were incubated for 30 minutes with an antibody cocktail containing (CD3-FITC, CD4-PE, CD8-APC, CD19- PerCPcy5, and CD49b-PEcy7 (eBiosciences, San Diego, CA. USA). Cells were washed, two times with FACS buffer (Phosphate buffered saline with 10% Fetal Bovine Serum and 0.1% NaN3 sodium azide), fixed with 2% paraformaldehyde, and resuspended in FACS buffer. Cell data was acquired with an Accuri C6 Flow Cytometer (BD Biosciences, San Jose, CA. USA) collecting 10,000 events per sample.

2.5. Proliferation Assay:

The proliferation of lymphocytes was determined using the CELLTITER 96 AQueous ONE proliferation assay (Promega Madison, WI. USA) per the manufacturer's protocol. Briefly, cells from the lymph nodes or spleen were prepared as described above to the concentration [$1x10^7$/mL] in fresh complete RPMI media and seeded into a sterile flat bottom 96-well plate. Lymphocytes from the spleen or lymph node were incubated in the presence or absence of T cell stimulators, Concanavalin A (CON A) [0.5 mg/mL], anti-CD3 and anti-CD28 antibodies [1.0 mg/mL each], or B cell stimulator Lipopolysaccharide (LPS) [1.0 µg/ml] at 37°C (5% CO_2) for 66-72 hours. After the incubation period, 20µl CELLTITER 96 AQueous ONE reagent was added to each well and incubated at 37°C for 4 hours in a humidified CO2 incubator (5%). The plate was read in a VersaMax spectrophotometer at an absorbance of 490 nm using a reference wavelength in the range of 650nm. The level of proliferation was determined by calculating the stimulation index (SI) using the following equation: SI = OD value of stimulated cells / OD value of unstimulated cells.

2.6. Statistical Analysis:

Graph Pad Prism, Inc. software version 10.1 (La Jolla, CA.USA) was used to create graphs and perform statistical analysis. Unpaired student t-test was used to determine significanceP values less than 0.05 were considered significant. Only data where significance was determined is indicated.

Results

Mice consume Moringa tea and water at a similar rate and have similar rates of weight gain.

As was reported previously by research conducted by our lab group [17,18], the mice in the control and Moringa tea groups gained weight at a similar rate (Figure 1) and consumed MT at a higher (but not significant) rate than those provided water (Figure 2).

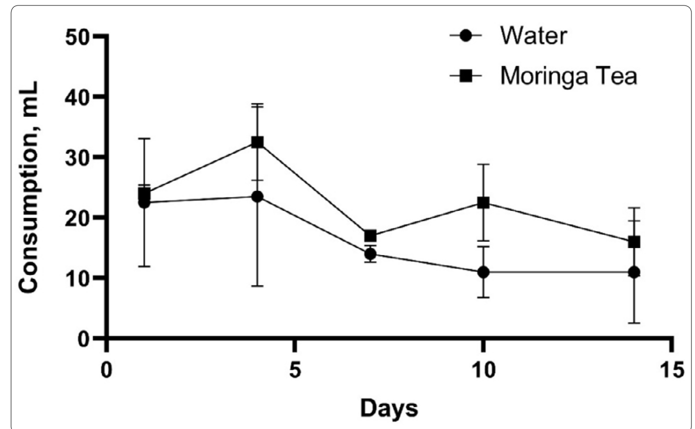

Figure 1. Change of weights over time taken every 2 to 3 days during the 14-day trial period. Data are an average of n=3 at each point.

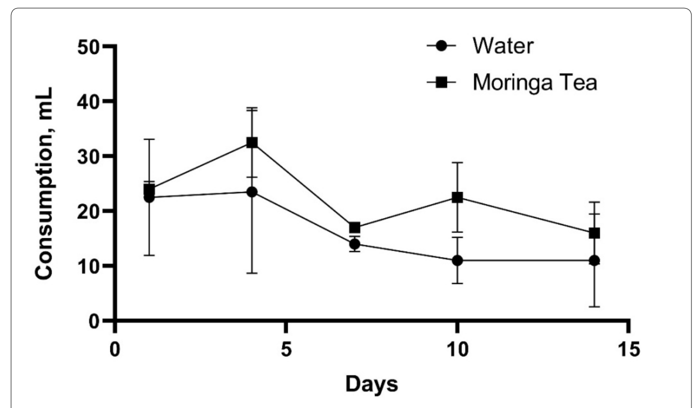

Figure 2. Comparison of Liquid Consumption. Data is an average of n=2 where an n equals a cage at each timepoint.

Consumption of Moringa tea does not result in changes to lymphocyte cell numbers in the spleen or lymph nodes.

Flow cytometric analysis shows that there are no changes in the percentages of T- and B-cells, within the spleen (Figure 3A) or lymph nodes (Figure 3B) of mice that drank fresh water compared to those that consumed Moringa tea. Furthermore, there were no changes to the percentages of CD4+ helper T-cells or CD8+ cytotoxic T-cells in either the lymph nodes (Figure 4A) or spleen (Figure 4B).

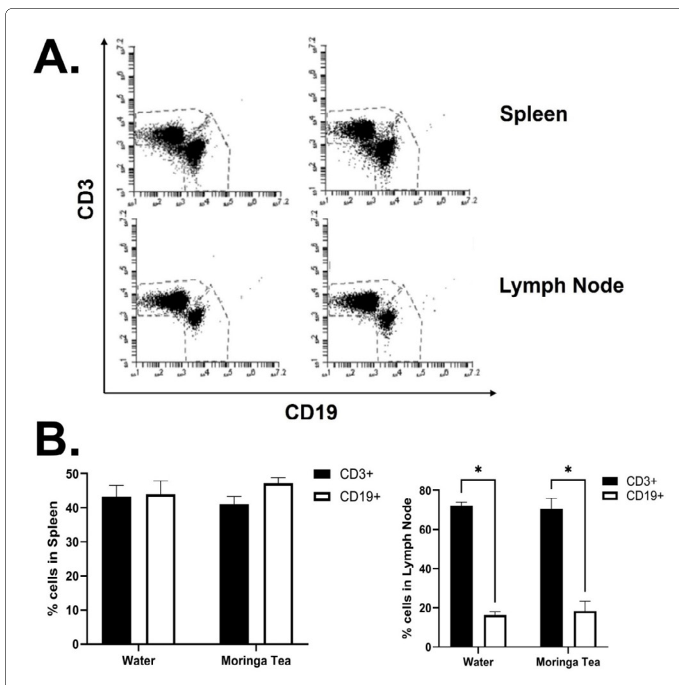

Figure 3. Flow Cytometric analysis of B and T-cell populations .**(A)** Representative dot plot chosen from an n= 3 in each group. **(B)**. Graphical representation of the flow cytometry data showing the percentage of CD3+ and CD19+ cells within the spleen and lymph nodes. (n=3) for each bar. Unpaired student t- tests indicate significant differences between CD3+ and CD19+ cells. * = $p < 0.05$.

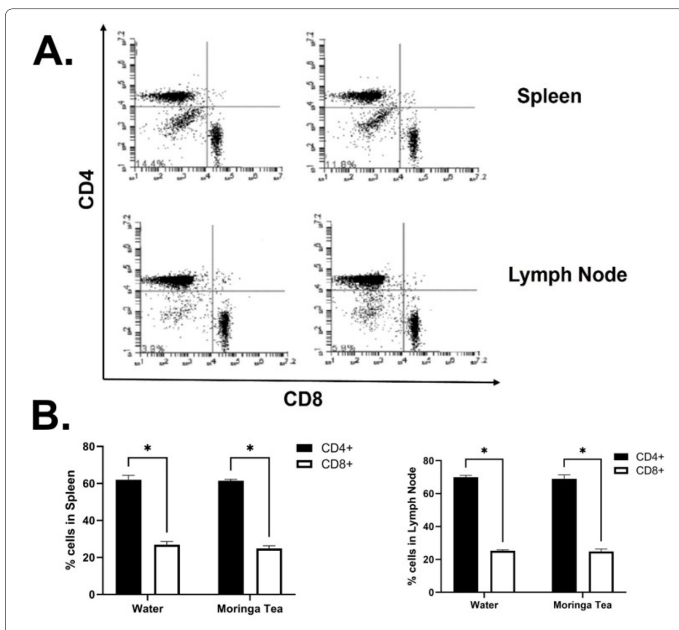

Figure 4. Flow cytometric analysis of T-cell populations. **(A)** Representative dot plot chosen from an n= 3 in each group. **(B)** Graphical representation of the flow cytometry data showing the percentage of CD4+ and CD8+ cells within the spleen and lymph nodes. (n=3) for each bar. Unpaired student t- tests indicate significant differences between CD4+ and CD8+ cells. * = $p < 0.05$.

Proliferation of T cells, but not B-cells is decreased by Moringa tea consumption.

In response to stimulation with anti-CD3 and anti-CD28, specific T cell stimulators, cells isolated from the spleen, but not the lymph nodes of mice that consumed MT showed a trend toward a lower proliferative response compared to those that consumed water (Figure 5A). In contrast, the proliferation of cells from the spleen (Figure 5A) or lymph nodes (Figure 5B) stimulated with CON A, a T cell mitogen, were not impacted by the consumption of Moringa tea. Similarly the proliferative responses of cells from the spleen and lymph node with the B cell mitogen, LPS, was similar between control mice and those that consumed Moringa tea.

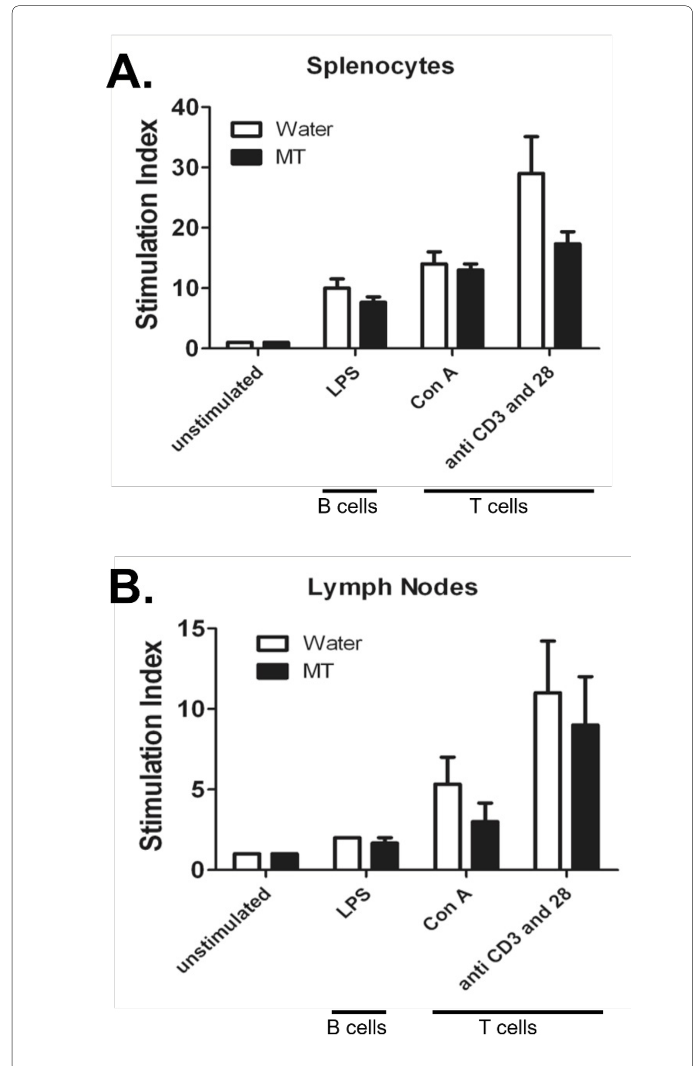

Figure 5. Proliferation of mouse B and T-cells. Cells isolated from the spleen (**A**) or lymph nodes (**B**) were stimulated with LPS [1 μg/ml] and CON A [0.5 μg/ml] and anti-CD3 and CD28 [1μg/ml] each. The level of proliferation was determined by calculating the stimulation index (SI) using the following equation. SI = OD value of stimulated cells / OD value of unstimulated cells. Data is an average of n=3.

Discussion

While there have been several reports investigating the impact of *Moringa oleifera* supplementation on the level of circulating white blood cells. For example, rats given Moringa for 12-weeks showed increases in circulating white blood cell counts, but the specific impact on circulating lymphocytes (T or B cells) was not reported [20]. Additionally, in studies where increases in the number of circulating leukocytes following Moringa treatment have been documented, the specific impact on lymphocytes was

either not investigated or not found. For example, Moringa supplementation provided to Wistar rats for 15 days led to increases in circulating white blood cell counts but not lymphocytes [21]. Similarly, Moringa supplementation provided to chickens for 13-weeks led to increases in white blood cell counts overall, but did not lead to significant increases in lymphocytes [22]. The current study specifically focused on determining whether consumption of tea from *Moringa oleifera* leaves impacted lymphocyte (T and B cells) counts within the spleen and lymph nodes, not cells in circulation. We found that mice consumed Moringa tea (MT) at a slightly better rate than mice provided water and gained weight at a similar rate during the 14 days, consistent with our previous studies (17,18). After the 14-day period, we report no differences in the percentages of T- or B-cells within the spleen or lymph nodes of mice that drank MT compared to those who drank water. These findings are consistent with a report by Katmawanti, et al which showed that Moringa supplementation of rats did not impact circulating lymphocyte numbers [23]. In a study conducted in rainbow trout, it was reported that while the percentage of circulating lymphocytes was not changed in unchallenged fish, fish that consumed Moringa had significant increases in circulating lymphocytes in response to infection compared to the control group [24].

The observational differences across these studies could be attributed to differences in the preparation of Moringa supplement (fresh, dried leaves, or extracts) or delivery methods i.e., concentration and length of delivery. They also suggest that Moringa does not impact lymphocyte numbers in naïve, unchallenged animals. A study investigating the impact of Moringa in *Plasmodium chabaudi* infection mice reported that infected mice fed Moringa in a pelleted form at doses of [60 or 30 mg/mouse] for 7 days had decreased CD4+ T cell numbers compared to infected mice that did not receive Moringa. In contrast, infected mice that consumed Moringa pellets [30 mg/mouse] for 3 weeks had increased levels of CD4+ T cells compared to control animals fed control pellets. This study highlighted that the length of Moringa administration and the dosage, as well as the timing of the Moringa (pre- or post-infection), have differential influences on T cells [25]. Anudeep et al, reported that proliferation of splenocytes was increased in the presence of low concentration (0.01 µg) of soluble fiber from Moringa seeds. In contrast, cells treated with high concentrations had lower response [26].

For the current study reported here Moringa was delivered as a tea (2% w/v) and delivered for 14- days and to further investigate the role of Moringa on stimulated lymphocyte populations we evaluated the proliferative response of lymphocytes (T and B cells) isolated from the spleen and lymph nodes of mice stimulated ex vivo with LPS, CON A, or anti-CD3 and anti-CD28. We report that Moringa tea consumption did not impact cell responses to LPS or CON A in either the spleen or lymph nodes, but in response to anti-CD3/28 stimulation, T cell proliferation within the spleen was decreased.

Lipopolysaccharide LPS is a potent mitogenic stimulator of mouse B-cells and has been shown to stimulate proliferation [27]. While we report no significant differences in the proliferative responses of cells treated with LPS between the control and MT groups, another study observed increases in B cell numbers in response to *S. typhi* infections in mice treated with Moringa [13], however specific proliferation was not assayed. Mechanistically, Moringa has been shown to impact the function of macrophages stimulated with LPS [6,28-30]. LPS stimulation of macrophages is induced through the CD14 stimulation activating TLR4 signaling cascades, which results in NF-κB translocation to the nucleus to induce the expression of proinflammatory signals (31). However, the activation of B cells by LPS is unique to that of macrophages. B cells do not express CD14 but do express TLR4 and LPS activation and proliferative response of B cells involves the phosphatidylinositol 3-kinase (PI3 kinase) signaling pathways [32]. Moringa has been shown to stimulate the PI3 kinase pathway in muscle cells in a model of diet-induced obesity [33], but the impact of Moringa on B-cell activation, proliferation, and signaling remains to be elucidated.

For this study, T cell proliferation was examined using either the mitogenic T cell stimulator Concanavalin A (CON A) or through the T cell receptor and costimulatory pathways using anti-CD3 and CD28 antibodies. We report that splenocytes from mice that consumed MT trended towards a decrease in proliferation in response to anti-CD3 and CD28 stimulation, but not CON A. This may be note-worthy since stimulation with anti-CD3 and CD28 is a more physiologically relevant way to mimic antigen-induced T cell activation than CON A which is a non-specific mitogen [34]. Our finding that Moringa impacted T cell proliferation in response to anti-CD3 and CD28 stimulation is consistent with a report by Attakpa et al, showing that T cell proliferation and IL-2 production were lowered in rats fed Moringa extracts in feed for 8 weeks, post- anti-CD3 stimulation [33]. We note that the studies into the impact of Moringa on T cell responses and function using *in vivo* systems do not delineate whether Moringa's influence is through a direct impact on T cells or through an indirect mechanism that involves Moringa's documented influence on antigen-presenting cells such as macrophages. It is well documented that the pathways activated in T cells (NF-κB, NFAT, and MAP kinases) are each shown to be inhibited by Moringa in macrophages [29,35-36], suggesting that Moringa may have a direct influence on T cells. Moreover, we note in this study that there were different responses by cells from the spleen stimulated with anti-CD3/28 but not the lymph nodes after similar stimulation. The structure, function, and micro-environments of the spleen and lymph nodes are unique and influence T cell activation, for example, there are varied numbers of antigen-presenting cells present. Therefore, since the T cells for this study were not purified before ex vivo stimulation, the contributions

that the other cells in the system made to the resulting T cell responses cannot be teased out [37]. It remains to be determined whether and how Moringa directly influences T cell responses and further investigation into the mechanism through which *Moringa* alters T cell activity is needed.

Conclusion

Having a better understanding of the immune modulating capacity is critical for characterizing the medicinal uses for Moringa and Moringa tea. This is the first study to investigate the impact of Moringa leaf tea on lymphocyte proliferation. These findings have implications for the use of Moringa and consumption of Moringa tea in persons with diseases where T cell activation and proliferation are at play.

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Author Contributions

RCM designed the study, DS performed experiments as part of the requirements of a master's thesis, and RCM and JH wrote the manuscript.

Conflict of Interest

There is no conflict of interest to declare.

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