The role of dietary choline on the immune system through different life stages

by

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Abstract

Choline is an essential nutrient that can be found in the diet as water- (i.e., free choline (FC)) and lipid-soluble forms. Animal studies have demonstrated that choline is needed for optimal maternal immune function and immune system development in offspring. Moreover, the forms of choline modulate the development of the immune system differently, with diets high in phosphatidylcholine (PC; lipid-soluble form of choline) enhancing the development of the immune system to a greater extent than diets containing only FC in rodent. Despite evidence suggesting a greater impact of PC on immune responses, little is known about the role of sphingomyelin (SM), another lipid-soluble form, on immune system development. In addition, the benefits observed of dietary PC on immune function are consistently accompanied by an increased T cell proliferation as measured by higher interleukin (IL)-2 production. Obesity is characterized by the presence of immune dysfunction in both rodents and humans where a lower IL-2 production upon T cell stimulation is typically observed. To date, it is unknown whether dietary choline, especially the lipid-soluble forms, would counteract the obesity-related immune dysfunction observed in the state of obesity. Therefore, the objective of this thesis was to determine the impact of feeding different forms of choline on the immune system through different life stages (i.e., early in life and obesity).

In a series of experiments, we aimed to determine the impact of feeding diets high in SM and/or PC on immune responses of lactating dams and local and peripheral immune systems development in offspring. Dams were fed one of three experimental diets all containing the same amount of total choline but differing in the form of choline: 1) a control diet containing only FC (water-soluble form); 2) a 50%PC diet (50% PC, 25% GPC and 25% FC); 3) a diet high in SM and PC (SMPC, 34-36% each). We observed that the forms of choline in the maternal diet were able to modulate the proportion of choline forms in breastmilk. Moreover, diets containing a high

proportion of choline in the form of SM, improved T cells response in lactating dams by increasing *ex vivo* IL-2, TNF-α, and IFN-γ production by splenocytes. Meanwhile, growth was improved in offspring from the SMPC group. In addition, both diets containing a high proportion of lipid-soluble forms (at least 50%) improve the maturation of peripheral immune responses in suckled pups by increasing the production by splenocytes of TNF-α, and IFN-γ following T cells mitogen stimulation. In weaned pups (10 weeks of age), peripheral immune responses were enhanced by both diets high in lipid-soluble forms of choline. There was a higher production of IL-2 by splenocytes along with IFN-γ after T cell mitogen stimulation. Moreover, the lipid-soluble forms of choline positively modulated T cell responses in the gut by increasing IL-2, TNF-α, and IFN-γ production in mesenteric lymph nodes (MLN) and the proportion of B cells in Peyer's patches. In addition, upon dietary antigen stimulation, there was a lower production of IL-2, TNF-α, and IL-6 and higher IL-10 production by immune cells from both spleen and MLN suggesting that the lipid-soluble forms of choline could potentially favor the development of oral tolerance.

In a parallel study, we examined the effect of dietary PC in the context of a high-fat diet (HFD) feeding. Male Wistar rats were randomized to consumed one of three experimental diets: a control low-fat diet (FC only); a control HFD (FC only) and a HFD with PC as the main source of dietary choline. We observed that when fed the control HFD, Wistar rats had a lower *ex vivo* IL-2 and TNF-α production by splenocytes when compared to the control low-fat diet. When adding PC to a HFD these changes were no longer observed.

Overall, we concluded that the lipid-soluble forms of choline provide more benefits for the development of both local and peripheral immune responses and the establishment of oral tolerance early in life. In addition, these results were also translated to a different model (HFD

feeding) where we observed that the lipid-soluble forms (PC) can attenuate to some extent the obesity-related immune dysfunction.

Preface

This thesis is original work by Jessy Azarcoya Barrera. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta's Institutional Animal Care Committee and is listed as "Modulation of T cell function and development through the manipulation of diet-derived long chain polyunsaturated fatty acids" with protocol number AUP00000125, June 2015 and "High-fat diet and T cell function" with protocol number AUP00002859, August 2018. The contributions made by the candidate, Jessy Azarcoya Barrera, and the co-authors to the completion of this work are described here.

Chapter 3 of this thesis has been published as Azarcoya-Barrera, J., Goruk, S., Lewis, E.D., Pouliot, Y., Curtis, J. M., Steele, R., Wadge, E., Field, C. J., Jacobs, R. L., & Richard, C. (2020). Feeding buttermilk-derived choline forms during gestation and lactation modulates *ex vivo* T-cell response in rat dams. *The Journal of Nutrition*, 150(7): 1958–1965. JAB, SG, EDL, RS, and EW were responsible for conducting the research analyzing the data. JAB performed statistical analysis of the data and prepared the manuscript. CR, CJF, RLJ, YP, and JMC designed and obtained funding for this study. JAB, CR and RLJ wrote the manuscript. CR and CJF had primary responsibility for final content.

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List of Abbreviations

Adequate intake - (AI)

Alberta Pregnancy Outcomes and Nutrition - (APrON)

Antigen presenting cells - (APC)

B-cell receptor - (BCR)

Cardiovascular diseases - (CVD)

C-C Motif Chemokine Ligand - (Ccl)

CDP-choline:1,2-diacylglycerol choline/ethanolamine phosphotransferase - (CEPT)

CDP-choline:1,2-dyacylglycerol cholinephosphotransferase - (CTP)

C-junk terminal kinase - (JNK)

Cluster of differentiation - (CD)

Concanavalin A - (ConA)

C-reactive protein (CRP) - (CRP)

Cytotoxic T cells - (CTL)

Dendritic cells - (DC)

Dextran sodium sulfate - (DSS)

Diacylglycerol - (DAG)

Docosahexaenoic acid - (DHA)

Eicosapentaenoi acid - (EPA)

Endoplasmic reticulum - (ER)

Free choline - (FC)

Glycerophosphocholine - (GPC)

Gut-associated lymphoid tissue - (GALT)

High-fat diet - (HFD)

Immunoglobulins - (Ig)

Institute of Medicine - (IOM)

Interferon-gamma - (IFN-y)

Interleukin - (IL)

Isolated lymphoid follicles - (ILF)

Lamina propria - (LP)

Lipopolysaccharide - (LPS)

linker for activation of T cells - (LAT)

Lysophosphatidylcholine - (Lyso-PC)

Macrophage inflammatory protein-1beta - (MIP-1β)

Major histocompatibility complex - (MHC)

Mesenteric lymph nodes - (MLN)

Milk fat globule membrane - (MFGM)

Monocyte chemoattractant protein-1 - (MCP-1).

Natural killer cells - (NK)

Nitric oxide synthase (iNOS)

NLR family pyrin domain containing 3 - (NLRP3)

Nonalcoholic fatty liver disease - (NAFLD)

Nuclear factor kappa B - (NF-κB)

Peyer's patches - (PP)

Phosphatidylcholine - (PC)

Phosphatidylethanolamine - (PE)

Phosphatidylethanolamine N-methyltransferase - (PEMT)

Phosphocholine - (Pcho)

Protein tyrosine kinase - (LCK)

Regulatory T cells - (Treg)

S-adenosylmethionine - (SAM)

Sphingomyelin - (SM)

Sphingomyelin synthase 2 - SMS2

T cell receptor - (TCR)

T folicullar helper cells (Tfh)

T helper - (Th)

Toll-like receptor 4 - (TRL-4)

Transforming growth factor beta - (TGF-β)

Trimethylamine - (TMA)

Trimethylamine-N-oxide - (TMAO)

Tumor necrosis α - (TNF-α)

Type 2 diabetes - (T2D)

United States Department of Agriculture - (USDA)

Very low-density lipoprotein - (VLDL)

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Introduction and literature review

1.1 Overview of the immune system

The immune system protects the body from infectious agents and the damage they produce by the action of a variety of effector immune cells and molecules (Calder 2007). Most of the immune cells emerge from the bone marrow where some of them develop and mature. Once mature, immune cells are localized circulating in the bloodstream and lymphatic system, and within peripheral tissues (Murphy, Travers *et al.,* 2012). The immune system has also the ability to suppress responses and develop tolerance towards antigens considered as "self" to the host, in addition to commensal antigens which do not confer a threat to the host. The immune system is divide into two components the innate and acquired (also termed adaptive) immune systems that coordinate responses through the action of effector cells and, anatomical and chemical barriers, among others (Yaqoob & Calder 2011).

1.1.1 Innate immune system

The innate immune system is the initial line of defense against foreign agents. Anatomic and physical barriers, such as the skin and mucosal surface, provide the initial line of defense against infection and protect internal tissues from exposure to pathogens. In addition, they provide further resistance by producing microbial proteins that function as natural antibiotics. Physiological barriers includes body temperature, low pH in stomach and chemical mediators. When foreign agents evade the host's anatomic, physical and chemical barriers, the cellular defenses of the innate immunity come into play (Goldsby, Kindt *et al.,* 2003). Cellular defenses are mainly provided by phagocytic cells including monocytes/macrophages, neutrophils,

basophils, eosinophils, mast cells, dendritic cells (DC), and natural killer (NK) cells. Macrophages, DC, and neutrophils express a number of receptors that are able to recognize specific molecules or molecular structures of the pathogens. The recognition of these molecules by phagocytic cells will initiate immune responses and led to the production of pro-inflammatory cytokines (Calder 2007). NK cells are lymphocyte-like cells that do not contain an antigen specific-receptor commonly found in cells of the adaptive immune system. NK cells can recognize and kill certain tumor cells and other infected cells (Calder 2007).

1.1.2 Adaptive immune system

The immune cells of the adaptive immune system (lymphocytes) can recognize specific molecules on invading pathogens called antigens. Lymphocytes contain antigen receptor molecules on their surface that allow them to recognize and bind to antigens that are presented to them by antigen presenting cells (APC) that express major histocompatibility complex (MHC) I or II molecules (Goldsby, Kindt *et al.,* 2003). Unlike the innate immune system, the adaptive immunity is highly specific and is affected by previous encounters with foreign agents. This unique feature is known as immunological memory and will provide a stronger response to an infectious agent if a subsequent exposure occurs (Yaqoob & Calder 2011). The adaptive immune responses require lymphocytes and APC cooperation to be effective. Lymphocytes are a type of white blood cells that are produce in the bone marrow by a process called hematopoiesis. Once outside the bone marrow, lymphocytes circulate in the bloodstream and the lymphatic system. They also reside in various lymphatic organs such as the spleen and mesenteric lymph nodes (MLN) (Murphy, Travers *et al.,* 2012). B and T lymphocytes are the two major lymphocyte populations in charge of orchestrating an adaptive immune response and will be described in more detail below.

1.1.2.1 B lymphocytes

B lymphocytes or B cells arise and maturate within the bone marrow. Once mature, B cells express a unique antigen-binding receptor called B-cell receptor (BCR) that allows them to produce antibodies, also known as immunoglobulins (Ig), that targets a specific antigens in circulation and neutralize them before entering and infecting cells (Pieper, Grimbacher *et al.,* 2013). The ability to eliminate foreign antigens by the production of antibodies is known as humoral immune response. Ig are protein molecules composed of two identical heavy chains and two identical light chains. Five major classes of heavy chain Ig have been identified, including, IgM, IgE, IgA, IgG, and IgD (Calder 2007). The binding of a specific antigen to the BCR will result in B cell proliferation and differentiation into plasma cells. Plasma cells secrete antibodies that are specific to the antigen that was recognize in first place by the BCR. Some B cells activated by antigens will give rise to memory B cells, which are responsible for long-lasting immunity and will differentiate into effector cells on a secondary exposure to their specific antigen (Chaplin 2010, Murphy, Travers *et al.,* 2012). B cells also elicit immune responses in a T-cell independent manner to certain antigens (i.e. bacterial lipopolysaccharide (LPS), polymeric proteins, polymeric polysaccharide) that contain repetitive epitope structures that allows cross-linkage on the BCR. This type of immune responses by B cells does not result in the develop of memory B cells (Chaplin 2010).

1.1.2.2 T lymphocytes

T lymphocytes or T cells arise in the bone marrow and migrate to the thymus for further maturation. During its maturation, T cells come to express a T cell receptor (TCR) on their surface that will be specific for certain antigen base of their extensive repertoire. TCR can only recognizes

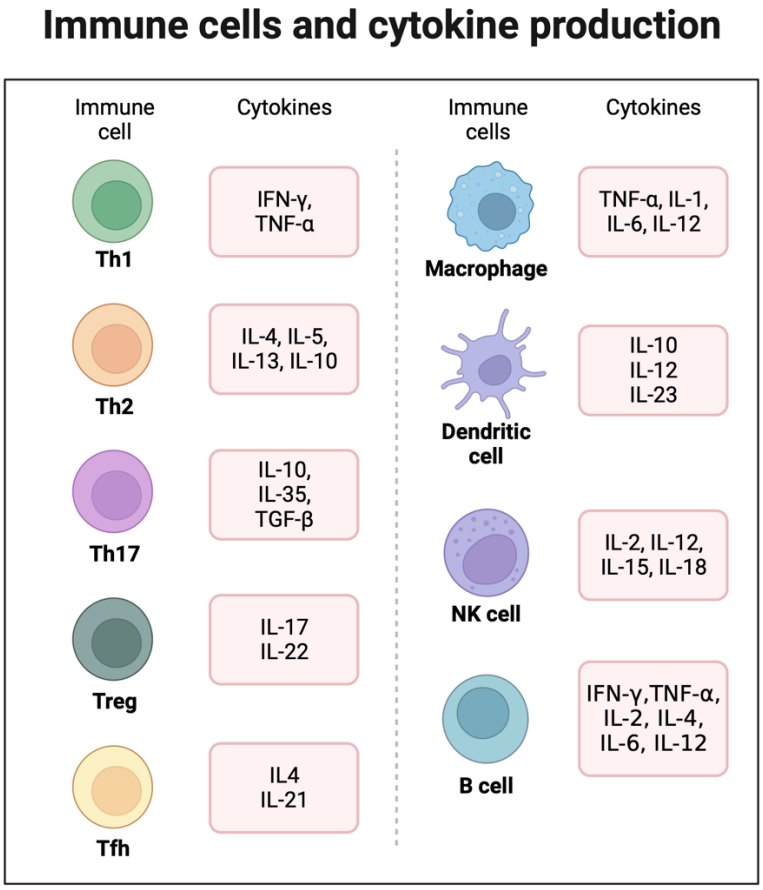
antigens that are presented to it by APC expressing MHC molecules and elicit a cell-mediated immune response (Calder, Krauss-Etschmann *et al.,* 2006). Activation of T cells by the stimulation of the TCR results in the secretion of interleukin (IL)-2 that will further promotes cell proliferation and differentiation.

There are two main well-defined type of T cells: T helper (Th) and cytotoxic T cells (CTL). Th and cytotoxic T cells are distinguished by the markers they express on their membrane surface. Cluster of differentiation (CD) 4 is generally found on the surface of Th cells whereas cytotoxic T cells express the CD8 marker (Yaqoob & Calder 2011). Th cells are activated by recognizing an antigen-class II MHC complex presented to them by an APC. Naïve CD4+ T cell activation leads to the production of interleukin (IL)-2 and further proliferation of cells. If proliferation is dominated mostly by the production of IL-12 it will results in the generation of a specific population called Th1 cells that promote a pro-inflammatory environment and produce cytokines such as Interferon-y (IFN-y), Tumor necrosis-α (TNF-α), and IL-2. Th1 cells stimulate the activation of macrophages and DC and are important for antiviral and antimicrobial immune responses (Murphy, Travers *et al.,* 2012). If, on the other hand, the proliferation of Th cells is led by the production of IL-4, a Th2 population will emerged instead and will promote an anti-inflammatory environment characterized by the production of cytokines such as IL-10, IL-5, IL-13 which downregulates Th1 responses and upregulates humoral responses by activating B cells (Murphy, Travers *et al.,* 2012). Another specific Th subset in charge of anti-fungal and anti-bacterial host defenses are the Th17 cells. Th17 cells secrete cytokines such as IL-17 and IL-22. In addition, Th17 cells are present in the gut to help regulate the gut microbiota (Saravia, Chapman *et al.,* 2019). Regulatory T cells (Treg) are a subset of Th cells that mainly promotes immunosuppressive responses. Treg are characterize by constitutively expressing CD4+ and

CD25+ along with the intracellular marker FoxP3 (Josefowicz, Lu *et al.,* 2012) and secrete a number of inhibitory cytokines such as IL-10, transforming growth factor beta (TGF-β) and IL-35 (Hoeppli, Wu *et al.,* 2015). T follicular helper cells (Tfh) are a specialized CD4+ subset that produces cytokines, such as IL-4 and IL-21, and helps with B cell activation and differentiation. They are abundant in Peyer's patches and are important for the generation of IgA responses (Crotty 2019, Saravia, Chapman et al., 2019). The major cells of the immune system and the cytokines they secrete are illustrated in **Figure 1-1**. CD8+ cells recognize MHC class I molecule and, unlike CD4+ cells, they do not secrete many cytokines. CTL exhibit, instead, a cytotoxic or "killing" activity. CTL are capable of eliminate virus-infected cells and tumor cells and secrete, primarily, IFN-y and TNF-α.

(Calder 2007).

Figure 0-1 Immune cells response and the cytokine production to an inflammatory response. Created with http:BioRender.com.



Abbreviations: Il- interleukin; IFN-y, interferon gamma; NK, natural killer; Tfh, T follicular helper cells; Th, T helper; TNF-α, tumor-necrosis alpha; Treg, T regulatory cells.

1.1.3 The gut-associated immune system

A vast diversity of immune cells is found through the intestinal tract in well-organized immune compartments that can be dived into two different inductive sites: MLN and the gut-associated lymphoid tissue (GALT) where cells of the innate immune system undergo initial priming and differentiation (Forchielli & Walker 2005). Effector sites such as the intestinal lamina propria (LP) and the epithelium can be also found in the immune system of the gut. Effector sites

contain cells from the adaptive immune system that promote barrier integrity and protection (Morbe, Jorgensen *et al.,* 2021). The GALT comprises the Peyer's patches (PP) and isolated lymphoid follicles (ILF) which are located within the intestinal wall. The MLN and PP are connected through lymphatic vessels and together serve as sites for antigen presentation to T and B cells (Morbe, Jorgensen *et al.,* 2021). One of the key roles of the intestinal immune system is provide active protection against foreign agents that are a treat while maintaining a tolerogenic response to the intestinal flora. The disruption of this balance can result in intestinal diseases such as intestinal bowel disease, Crohn's disease, celiac disease and ulcerate colitis (Koboziev, Karlsson *et al.,* 2010). At birth, the GALT is considered to be relatively naïve due to lack of antigen exposure, which ca be modulated by diet (breastmilk mostly) and the environment (Blumer, Pfefferle *et al.,* 2007). Maturation of the gut immune responses also depends on early microbial colonization of the mucosal surface. The microbiome plays an important role in maintaining immune homeostasis in the gut and perturbation of the microbial population (dysbiosis) can disrupt immune homeostasis and lead to inflammatory and/or autoimmune diseases (Zheng, Liwinski et al., 2020).

1.1.4 Oral tolerance

During early developmental period, immune responses in the GALT need to maturate and learn to discern between what is harmful and what is not for the host. This is a process known as oral tolerance. Oral tolerance consists in the active suppression of immune responses towards an antigen that it is not considered to be a threat to the host (i.e. food proteins, commensal bacteria) (Hornef, Pabst *et al.,* 2021). Typically, the establishment of oral tolerance occurs during the first years of life in humans and during the suckling/weaning periods in rats. In the absence of oral

tolerance, immune responses to food antigens and gut microbes can lead to the development of food allergies and inflammatory bowel disease (Pabst & Mowat 2012). Treg play an important role in modulating and controlling responses to allergens. Treg can promote the secretion IL-10, TGF-β and IL-35 (figure 1-1) that inhibit immune responses. It has been shown that FoxP3+ cells are necessary for the establishment of oral tolerance in both human and mice (Wawrzyniak, O'Mahony *et al.,* 2017). IgA is the dominant antibody in the mucosal immune system and has several functions. IgA can neutralize pathogens and stop them from reaching tissues. In addition, IgA can induce the production of IL-10 by DC, which suggests a role in the establishment of oral tolerance (Li, Jin *et al.,* 2020).

1.2 Physiological adaptations of the immune system in different life stages

1.2.1 The immune system development

During early development, the immune system lacks immunological memory due to the low exposure to antigens and therefore is considered "immature". Mothers transfer IgG antibodies, among other factors, through the placenta and breastmilk to provide critical protection against infections (Mor & Cardenas 2010). The immune system develops as we age and is highly influence by components of the environment and the diet. The immune repertoire is also modulated by exposure to infections and vaccination (Childs, Calder *et al.,* 2019, Simon, Hollander *et al.,* 2015). In rodents, similar to what we see in humans, B cells are the population to be first presence in the spleen and present an immature phenotype with a low IgM expression on their surface (Perez-Cano, Franch *et al.,* 2012). The antibody responses in neonates are slower than in adults and there is less diversity of Igs. IgM and IgG are detectable in rats' serum at 5 days of age and increased gradually during the suckling period (Albers, Antoine *et al.,* 2005, Crockett 1995). IgA is the last

antibody to appear in rats' serum, with breastmilk being the major source of IgA (Butler 2005, Perez-Cano, Castellote *et al.,* 2007).

T cells are found in low proportion in spleen within the first 2 weeks of life in rodents. The first half of the suckling period is characterized by a low proportion of immature CD4+ and CD8+ cells (Perez-Cano, Castellote *et al.,* 2007, Perez-Cano, Franch *et al.,* 2012). Th responses develop rapidly during the early postnatal period, contrary to cytotoxic T cells responses which develop at a slower rate (Perez-Cano, Franch *et al.,* 2012, Prescott 2003). Neonates (rodents and humans) have diminished Th1 responses when compared to adults and they rely heavily on Th2 responses characterize by the production of IL-10, IL-4, and IL-5. Th cells also have a reduced proliferative capacity (Albers, Antoine *et al.,* 2005) and the secretion of Th1 cytokines TNF-α, and IFN-y, is significantly attenuate in neonates but gradually increase during the suckling period in rodents and the first year of life in infants (Hartel, Adam *et al.,* 2005). The inability to switch from a predominant Th2 to a Th1 responses later in life has been associated with an increased risk of atopic diseases (Prescott 2003).

1.2.2 The immune system during pregnancy and lactation

During pregnancy, the immune system is expose to major changes to protect the mother and future baby from invading pathogens, in addition to suppressing immune responses towards the fetus (Abu-Raya, Michalski *et al.,* 2020). The first trimester is characterized by an overall pro-inflammatory environment whereas the second and third trimester are characterized by an anti-inflammatory environment (Orefice 2021). The human decidua, a specialized tissue that its form in the uterus in preparation for pregnancy, contains a considerably amount of immune cells that includes NK cells, DC, macrophages, and Treg, that work collectively to maintain tolerance and

overall function of the placenta (Aluvihare, Kallikourdis *et al.,* 2004, Orefice 2021). During the first trimester of pregnancy, invasive trophoblasts are surrounded by DC, NK cells and macrophages. In fact, NK cells have shown to play a crucial role in preventing trophoblast in reaching the endometria (Shimada, Nishida *et al.,* 2006, Wicherek, Basta *et al.,* 2009). DC are needed for decidual formation and Treg are responsible for modulating immune responses and the establishment of tolerance (Abu-Raya, Michalski *et al.,* 2020). B cells are absent in the decidua, making T cells responsible for adaptive immune responses of the fetus. With humoral responses absent in the fetus, maternal IgG antibodies cross the human placenta to protect the fetus from infections (Crockett 1995, Mor & Cardenas 2010).

A tolerogenic adaptation that has been suggested during pregnancy is the switch from Th1 cytokine response to Th2. The production of progesterone, estradiol, and prostaglandin D2 promotes a Th2 environment, which is associated with a normal pregnancy (Mor & Cardenas 2010, Roth, Corry *et al.,* 1996). Th1 responses are downregulated during pregnancy, in part due to the presence of Th2 cells that drive naïve T cells into becoming Th2 cells (Sykes, MacIntyre *et al.,* 2012). These changes are accompanied by an increase in Th2 cytokine production (IL-4, IL-10). The production of Th2 cytokines, along with macrophages and Treg, maintain immune tolerance towards the fetus (Mor, Straszewski-Chavez *et al.,* 2006). Because Th1 responses are associated with the clearance of bacterial and viral infection, the skewed Th2 responses during pregnancy increased the risk of infection in pregnant women. This phenomenon is usually reversed shortly after giving birth (Abu-Raya, Michalski *et al.,* 2020).

During lactation, the infant continues to be exposed to maternal immunity through breast feeding. Breastmilk is crucial for protection against infections, allergies, and asthma, and can influence the infant's gut microbiome and the development of the immune system. Human

breastmilk contains numerous immune cells, including T and B cells, macrophages, and neutrophils, in addition to immunoglobulins (IgA, IgG and IgM) (Jarvinen, Martin *et al.,* 2019). Human breastmilk composition can be affected by the diet and immune cells concentrations will vary depending on the period of lactation. The number of immune cells and immunoglobulins are found to be higher in colostrum and early milk (Miles, Childs *et al.,* 2021).

1.2.3 Alterations in the immune system in the context of obesity

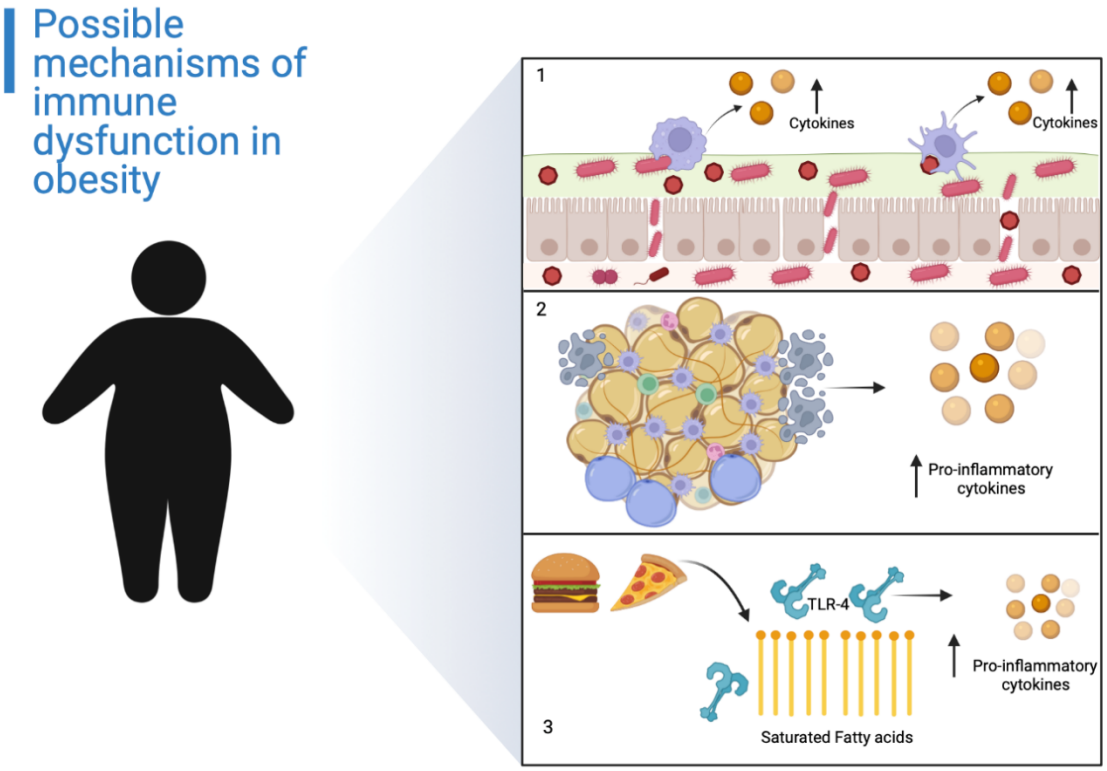
The World Health Organization defined obesity as an excessive body fat accumulation that compromise health. Evidence suggests that the association between obesity and other comorbidities such as, type 2 diabetes (T2D), cancer, cardiovascular diseases (CVD), nonalcoholic fatty liver disease (NAFLD) and, steatohepatitis, is closely related to the state of chronic low-grade systemic inflammation commonly observed in obesity (Gonzalez-Muniesa, Martinez-Gonzalez *et al.,* 2017, Reilly & Saltiel 2017). Chronic low-grade systemic inflammation is characterized by elevated levels of circulating inflammatory markers (IL-6, TNF-a, C-reactive protein (CRP)) (Calder, Ahluwalia *et al.,* 2011). Several possible causes for this increased systemic inflammation have been proposed (**Figure 1-2**) including increased gut permeability, adipocytes death/hypoxia in adipose tissue and/or poor dietary patterns (Reilly & Saltiel 2017). Obesity is associated with increased intestinal permeability and changes in the gut microbiota, which results in bacterial translocation and higher levels of circulating LPS. LPS can be recognize by the toll-like receptor 4 (TRL-4) present on immune cells and, thus, initiate an inflammatory response (Scheithauer, Rampanelli *et al.,* 2020). Adipocytes expansion can lead to hypoxia and adipocytes death which lead to the infiltration of macrophages forming crown-like structures. These macrophages are known to switch to a pro-inflammatory phenotype (M1-like macrophages) and secrete cytokines

such as IL-1β, TNF-α, IL-12 (Reilly & Saltiel 2017). Finally, components of the diet are also capable of initiating an inflammatory response. Some saturated fatty acids, such as, lauric and palmitic acid, have been shown to be natural ligands for the TLR-4 and able to trigger an immune response (Childs, Calder *et al.,* 2019).

Obesity has been associated with an elevated total number of macrophages, B and T cells in both adipose tissue and in circulation (Misumi, Starmer *et al.,* 2019). Despite this, obesity is associated with an impaired immune function and poor response to vaccination in both human and rodents (Karlsson, Sheridan *et al.,* 2010, Milner & Beck 2012, Sheridan, Paich *et al.,* 2012). Studies have shown that feeding mice with a high-fat diet (HFD) resulted in increased mortality and impaired memory T cell response upon influenza infection (Karlsson, Sheridan *et al.,* 2010). In humans, individuals with obesity and T2D showed an impaired T cell response characterized by a lower cytokine production (IL-2, TNF-a and IL-6) by peripheral blood mononuclear cells (PBMCs) after T cell stimulation, when compared to normo-glycemic individuals with obesity (Richard, Wadowski *et al.,* 2017). Similarly, in Wistar rats fed a HFD, there was a lower production of IL-2 following T cell mitogen stimulation (Lamas, Martinez *et al.,* 2002). A HFD is associated with changes in the gut microbiota that could directly impact the gut-associated immune system. Alterations in the gut composition can elicit an inflammatory response within the gut and result in increased intestinal permeability and LPS translocation (Moreira, Texeira et al., 2012, Teixeira, Collado et al., 2012). In rodents, both diet-induced, and genetically-induced obesity has been associated with alteration in intestinal permeability (as measured by plasma LPS, FITC dextran) (Gao, Wu et al., 2020, Gong, Hu et al., 2017, Ruth, Proctor et al., 2009). However, in remains controversial to what extent obesity can alter immune responses in the gut as there has been discrepancies among studies. Some studies have shown a decrease T cell responses as

measured by IL-2 production and other no change and even increased in anti-inflammatory cytokine production (Blewett, Gerdung et al., 2009, Molina, Bolin et al., 2015, Ruth, Proctor et al., 2009).

Figure 0-2 Possible mechanisms of immune dysfunction in obesity. 1) Obesity is associated with a "leaky gut" which result in bacterial translocation with higher LPS levels in circulation which can activate immune responses and increase production of pro-inflammatory cytokines. 2) Adipocytes size and number are increased in the context of obesity which leads to adipocyte hypoxia/death that favors the recruitment of M1-like pro-inflammatory macrophages secreting cytokines. 3) Components of the diet such as saturated fatty acids that are natural ligands of the TLR-4 activating immune cells to produce cytokines. Created with http:BioRender.com.



Abbreviations: LPS, lypoporysaccharide; TLR-4, toll-like receptor 4.

1.3 Choline, an overview

Choline has been officially considered as an essential dietary nutrient by the Institute of Medicine (IOM) since 1998. Choline is required for a wide range of critical processes in the body, including lipoprotein assembly and secretion, cell membrane formation and signalization, lipid transport, acetylcholine synthesis, optimal immune function, and it is also considered an important dietary source of methyl groups (Zeisel & da Costa 2009). Choline can be obtained via *de novo* synthesis, which occurs mainly in the liver, by the methylation of phosphatidylethanolamine (PE) to phosphatidylcholine (PC), a reaction catalyzed by the enzyme phosphatidylethanolamine *N*-methyltransferase (PEMT) (Vance 2014). However, the amount of choline acquired via *de novo* synthesis is not sufficient the meet the demands of the human body, and thus, must be additionally obtain from dietary sources. The OIM set an adequate intake (AI) for choline at 550 mg/day for men and 425 mg/day for women (Zeisel & da Costa 2009).

1.3.1 Choline forms and dietary sources

Choline is available in the diet in the form of free choline (FC), phosphocholine (Pcho), glycerophosphocholine (GPC), PC, lysophosphatidylcholine (Lyso-PC) and sphingomyelin (SM). Foods that are particularly high in choline includes, meat (liver, pork), eggs, soybean, wheat germ and spinach (Lewis, Subhan *et al.,* 2014, Zeisel, Mar *et al.,* 2003). Even though milk and dairy products are not the richest sources of choline they are considered a good dietary source due to the frequency of their consumption, which helps considerably in meeting daily choline recommendations. The United States Department of Agriculture (USDA) database for choline content of common food is use as a reference to determined choline and choline moieties intake across the population (Patterson, Bhagwat *et al.,* 2008). This database contains an approximate of

630 foods commonly consumed in the USA; however, the database does not include certain foods that might be of relevance for other regions. In the University of Alberta Choline Database (Alberta Database), our group has increased the number of foods providing choline content to include approximately 3,800 foods. In that regard, we have measured the choline content in a variety of meat, dairy products and pulse that are absent from the USDA database, which can be useful when estimating choline intake in different populations
(Lewis, Kosik *et al*., 2014, Lewis, Zhao *et al*., 2015, Richard, Lewis *et al*., 2016).

1.3.2 Buttermilk as source of dietary choline

Buttermilk can be obtained as a result of the churning of cream into butter. Buttermilk has many similarities when compared to skim milk; however, they differ significantly in fat composition and amount of choline derived from the presence of the milk fat globule membrane (MFGM) in buttermilk (Conway, Gauthier *et al.,* 2014). Buttermilk contains a substantially higher amount of phospholipids compare to skim milk (Conway, Gauthier *et al.,* 2014). The MFGM is rich in polar lipids, cholesterol, and proteins specific to the membrane. The protein composition represents about 25-75% of the MFGM mass. Butyrophilin, CD36, Xanthine dehydrogenase/oxidase, Lactadherin, Adipophilin, Mucin-like glycoproteins 1 are among some of the proteins found in high amounts within the MFGM (Lopez 2018). The structure and major function of the most abundant proteins in the MFGM have been described and discuss in more detail elsewhere (Affolter, Grass *et al.,* 2010, Ali 2019). The MFGM lipids mixture is composed of neutral lipids (70%) and polar lipids (26-30%). Cholesterol accounts for roughly 90% of the sterols within the MFGM. The polar lipid composition of the MFGM has been shown to be similar to the one found in human's mammary gland (Lopez 2018). Polar lipids within the MFGM can be

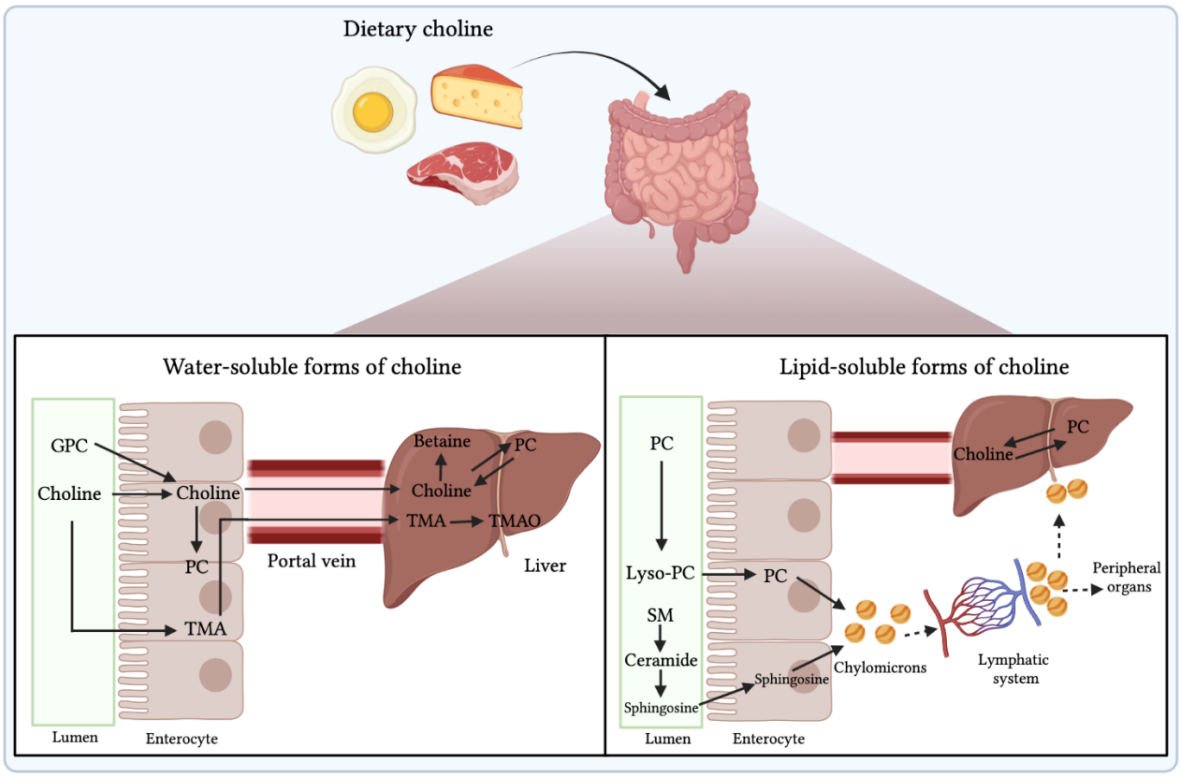
classified into two major classes, glycerophospholipids and sphingolipids. The most abundant glycerophospholipids are PE (31-42%) and PC (19-34%) and, in smaller amounts, we can also find phosphatidylserine and phosphatidylinositol. Sphingolipids compose one-third to one-half of the polar lipids in the MFGM (Conway, Gauthier *et al.,* 2014, Lopez 2018). Therefore, milk fat and, the MFGM in particular, is considered a major source of dietary sphingolipids in the human diet. SM and glycosphingolipids (i.e. gangliosides) stand for the majority of sphingolipids in the membrane. MFGM sphingolipids interact with cholesterol to form organized microdomains termed lipid rafts (Lopez 2018). Lipid rafts play important roles in cell signaling, intracellular traffic and transduction (Dykstra, Cherukuri *et al.,* 2003). MFGM phospholipids have been associated with health benefits regarding CVD, cancer, and inflammation (Conway, Couture *et al.,* 2014, Conway, Couture *et al.,* 2013). In addition, supplementation of MFGM has also been linked to improvement in brain and cognitive development, infection prevention, intestinal health and immunity, and lipid and cholesterol absorption (Ali 2019). The health benefits attributed to the MFGM from buttermilk in addition to differences in the fat composition when compared to skim milk make it a valuable source of dietary phospholipids (including choline).

1.3.3 Absorption and metabolism of the different forms of choline

Choline is present in the diet as water-soluble (FC, Pcho, GPC) and lipid-soluble (PC, SM) forms of choline, which are absorbed and metabolized distinctly in the body (Sheard, Tayek *et al.,* 1986). FC is absorbed in the jejunum and ileum and transported into the enterocyte by a sodium-dependent transporter. Once in the enterocyte, FC can either directly enter the portal circulation and be taken up by the liver or can be irreversible oxidized to betaine (Tsubaki & Komai 1987). In the liver, FC can be oxidized to betaine or utilized to synthesize PC by entering the CDP-choline

pathway. Unabsorbed FC reaches the large intestine, and it is metabolized by intestinal bacteria to produce trimethylamine (TMA). TMA is then excreted in the urine or transported to the liver and oxidized to form trimethylamine-N-oxide (TMAO) (Zeisel, Wishnok *et al.,* 1983). The lipid-soluble form PC is first hydrolyzed to Lyso-PC and a free fatty acid by the action of phospholipase A2 to be absorbed by the enterocyte. Once in the enterocyte, Lyso-PC can be either reacetylated to PC, and further incorporated into chylomicrons, or hydrolyzed to GPC. GPC is absorbed directly or further hydrolyzed to form FC (Parthasarathy, Subbaiah *et al.,* 1974). In the small intestine, alkaline sphingomyelinase hydrolyzed SM to ceramide and then to sphingosine. Sphingosine its directly absorbed by the enterocyte. A small amount its incorporated into the mucosal ceramide and/or other complex sphingolipids and the rest its converted to palmitic acid, which is further incorporated into chylomicrons (Nilsson & Duan 2006). Once incorporated into chylomicrons, the lipid-soluble forms of choline, are secreted into the lymphatic system and, therefore, can be delivered to peripheral organs first (adipose tissue, muscle) before being on their way to the liver (Lewis, Field *et al.,* 2015) (**Figure 1-3**).

Figure 0-3 Absorption and metabolism of the different forms of choline. Once absoberd by the enterocyte, FC can enter the portal circulation, taken up by the liver and/or be irreversible oxidized to betaine. Unabsorbed FC is metabolized by gut bacteria to produce TMA that can be transported to the liver and oxidized to TMAO. The lipid-soluble forms of choline are first packed into chylomicrones and can be delivered to peripheral tissues before reaching the liver. Created with http:BioRender.com.



Abbreviations: GPC, glycerophosphocholine; Lyso-PC, lysophosphocholine; PC, phosphatodylcholine; SM, sphingomyelin; TMA, trimethylamine; TMAO, trimethylamine N-oxide.

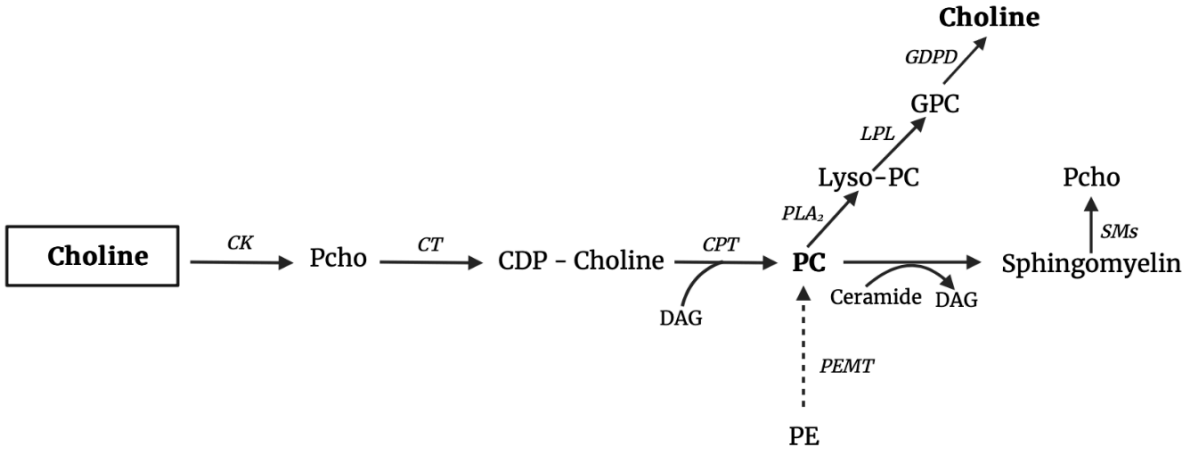
1.3.4 *De novo* synthesis of choline and derivatives

Choline can be also obtained endogenously through the synthesis of PC via the CDP-choline pathway, also termed the Kennedy pathway (van der Veen, Kennelly *et al.,* 2017). This is thought to be the primary source of PC biosynthesis in liver. Once inside the cell, choline is phosphorylated by ATP to Pcho via the action of the enzyme choline kinase, followed by the conversion of CTP and Pcho into CDP-choline, a reaction catalyzed by the enzyme CTP:phosphocholine cytidyltransferase (CT) (Vance 2014). The final step of the CDP- choline

pathway occurs in the endoplasmic reticulum (ER) and consists in the transfer of Pcho from CDP-choline to diacylglycerol (DAG), thus generating PC. This reaction is catalyzed by CDP-choline:1,2-dyacylglycerol cholinephosphotransferase (CTP) and CDP-choline:1,2-diacylglycerol choline/ethanolamine phosphotransferase (CEPT) (Li & Vance 2008, van der Veen, Kennelly *et al.,* 2017). Additionally, PC can be also obtained by three successive methylations of PE in the liver, in which S-adenosylmethionine is the methyl group donor. All three methylation reactions are catalyzed by the enzyme PEMT. PEMT is mainly found in the liver, and it is suggested that 30% of the PC pool is obtained through this pathway (Bremer J & Greenberg 1961, van der Veen, Kennelly *et al.,* 2017) (**Figure 1-4**).

SM can be synthesized from PC and ceramides by the action of cerimide:phosphatidylcholine cholinephophotransferase (sphingomyelin synthase). This reaction takes place in the Golgi apparatus and plasma membrane (Linardic & Hannun 1994, Tafesse, Ternes *et al.,* 2006). Lyso-PC is generated from the hydrolysis of PC by phospholipase A2 (Burke & Dennis 2009). Lyso-PC can then undergo hydrolysis to form GPC by the action of the enzyme 2-lysophosphatidylcholine acylhydrolase (lysophospholipase) (Parthasarathy, Subbaiah *et al.,* 1974). GPC can be further hydrolyzed to glycerol-3-phosphate and choline by GPC:choline phosphodiesterase (Fernandez-Murray & McMaster 2005, Kwon, Jung *et al.,* 1995).

Figure 0-4 Biosynthesis of choline and derivatives. Created with http:BioRender.com.



Abbreviations: CK, choline kinase; CT, CTP:phosphocholine cytidylyltransferase; CTP, choline phosphotransferase; DAG, diacylglycerol; GDPD, glycerophosphocholine phosphodiesterase; GPC, glycerophosphocholine; Lyso-PC, lysophosphatidylcholine; LPL, lysophospholipase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEMT, phosphatidylcholine-N-methyltransferase; Pcho, phosphocholine; PLA_2, phospholipase A2; SMs, sphingomyelinase.

1.4 Physiological relevance of choline

1.4.1 Structural integrity of cell membranes and signaling

PC is the most abundant phospholipids in cell membranes, accounting for approximately 50% of membrane phospholipids. In addition, PC is the precursor of SM and Lyso-PC, both being important components of membrane phospholipids (van Meer, Voelker *et al.,* 2008). SM represents roughly 85% of all sphingolipids and it is mainly found in neurons (Quinville, Deschenes *et al.,* 2021). PC and SM, as part of the cell membrane, play a key role in both intracellular and extracellular signals, modulating important processes such as apoptosis, phagocytosis, cell differentiation, cell growth, enzymatic activity, among others. The products of PC and SM hydrolysis also have been thought to be crucial for cell signaling (Chakraborty & Jiang 2013, Exton 1994, MerrillJr., Liotta *et al.,* 1996). Lyso-PC is needed for T cell activation, cell lysis and chemotaxis (Nishizuka 1992). Ceramides, the result of SM hydrolysis, is now considered an important secondary messenger involve in cell differentiation and proliferation, apoptosis, and

modulation of inflammatory immune responses (Avota, de Lira *et al.,* 2019, Ohanian & Ohanian 2001).

1.4.3 Lipoprotein assembly and secretion

Phospholipids are essential for the formation and secretion of lipoproteins, which are responsible for delivering lipids from the enterocyte and the liver to peripheral tissues. It has been demonstrated that PC is required for the synthesis and secretion of very low-density lipoprotein (VLDL) and chylomicrons (Yao & Vance 1988, Yao & Vance 1989). Previous studies have shown that a diet deficient in choline resulted in lower VLDL secretion and hepatic steatosis in both humans and rodents. Moreover, mice lacking CTa and PEMT have an impaired VLDL secretion in addition to lipid accumulation in hepatocytes, thus highlighting the importance of PC in VLDL formation and secretion (Jacobs, Devlin *et al.,* 2004, Jacobs, Lingrell *et al.,* 2008, Noga, Zhao *et al.,* 2002).

1.4.4 Methyl group donation and methylation reactions

The action of the enzyme choline dehydrogenase irreversibly converts choline to betaine. This reaction can occur in the liver or kidney, where betaine function as an osmolyte. Betaine is an important coenzyme in the remethylation of homocysteine and provides its methyl group to form methionine in the methionine cycle, a reaction catalyzed by betaine homocysteine methyltransferase (Day & Kempson 2016, Hollenbeck 2012). Folate is also required in the methylation of homocysteine, thus closely interlinking the three different pathways. Methionine adenosyl transferase converts methionine to S-adenosylmethionine (SAM), the active methyl donor in the methionine cycle and, it is also involved in many other methylation reactions,

including the methylation of PE to PC and the methylation of DNA and histones (Hollenbeck 2012, Watkins, Zhu *et al.,* 2003, Zeisel & da Costa 2009).

1.4.5 Neurotransmission

Small amounts of choline are acetylated to form acetylcholine in a reaction catalyzed by choline acetyltransferase (Leventer, Rowell *et al.,* 1982). Acetylcholine is a neurotransmitter that acts at the neuromuscular junction and the ganglia as well as other non-neural tissues, including the placenta (Wessler & Kirkpatrick 2008). Acetylcholine influences synaptic transmission, myelination, neurogenesis, muscle function, breathing, heart rate, in addition to memory, sleep and learning (Hasselmo 2006). The synthesis of acetylcholine is affected by choline availability, therefore, suggesting that dietary choline may directly influence cholinergic stimulation. The role of acetylcholine as neurotransmitter has been reviewed in more detail by (Picciotto, Higley *et al.,* 2012).

1.5 Choline requirements during different life stages

1.5.1 Choline during the prenatal period

During pregnancy, fetal organ development requires high amounts of choline for membrane biosynthesis. Expression of PEMT is nearly absent in fetal liver and placenta tissue, relying solely on maternal supply. Choline is delivered to the placenta through a choline transported system to ensure the availability of choline to tissues (Caudill 2010). In fact, choline is store in large amounts as acetylcholine in the placenta, one of the few non-neural tissues capable of doing it. In neonates and fetus, plasma concentration of choline is three times higher than they are in adults. Studies suggest that the choline levels in placenta are 50 times higher when compared

to maternal choline plasma levels (Zeisel 2006). The increased in estrogen production directly affect the endogenous synthesis of choline, which is increased during gestation. Estrogen responds elements are located in the promoter region of the PEMT gene, therefore, increasing PEMT expression during gestation, resulting in increased PC synthesis (Fischer, daCosta *et al.,* 2007, Zeisel 2006).

In North America, it is estimated that the daily choline intake of pregnant women is below the AI. In fact, epidemiological data from the Alberta Pregnancy Outcomes and Nutrition (APrON) study, suggests that only 23% of pregnant women in Alberta are meeting the daily choline recommendations (Lewis, Subhan *et al.,* 2014). Women that were consuming an average of 500 mL of milk or more and/or 1 egg per day were more likely to meet daily choline intake recommendations. In the same study, it was shown that PC was the form of choline mostly consume by women (about 50%), followed by GPC and FC (about 25% each) (Lewis, Subhan *et al.,* 2014). A dietary intake of choline below the AI has been associated with an increased risk of neural tube defects. In contrast, choline-supplemented studies demonstrated that a supplementation of choline above the AI was associated with a lower risk of preeclampsia and a higher choline content in breastmilk (Caudill 2010, Davenport, Yan *et al*., 2015, Jiang, Bar *et al*., 2013). Currently, the majority of prenatal multivitamins available do not contain choline, which highlights the importance of dietary choline in pregnant and lactating women.

1.5.2 Choline during the postnatal period

The demands of choline continue to be high during the postnatal period where it is up taken by the infants' brain and other tissues (Caudill 2010). Choline is delivered to infants via breastmilk or infant formula (Zeisel 2006). To ensure an adequate supply to the mammary gland, serum

choline concentrations are 50-100% higher in breastfeeding women which increased the demands of choline during the lactation period or storage of choline are also depleted, similarly to the pregnancy period (Caudill 2010). The endogenous synthesis of choline is not sufficient to meet the demands of both the mother and the infant, highlighting the importance of an adequate dietary choline intake. Data from the APrON study showed that only 10% of lactating women were meeting the daily choline intake requirements. Interestingly, the average daily choline intake has been shown to be fairly consistent throughout pregnancy and lactation periods (Lewis, Subhan *et al.,* 2014). Yet, the AI increase considerably during lactation compared to the pregnancy period.

Previous studies in lactating rats demonstrated that the choline content in the breastmilk was correlated with the choline content of the maternal diet (choline-deficient diet or choline-supplemented diet) (Dellschaft, Ruth *et al.,* 2015). Typically, human milk is composed mainly by water-soluble forms, with Pcho being the most abundant form of choline in breastmilk followed by GPC and both represent close to 70% of the total choline composition in breastmilk (Holmes, Snodgrass *et al.,* 2000, Holmes-McNary, Cheng *et al.,* 1996). In contrast, the lipid soluble forms of choline represent a relatively small proportion of the human milk content (Wiedeman, Barr *et al.,* 2018). Supplementation of choline during lactation have shown to increase both the total amount of choline and the proportion of FC, Pcho and GPC in human milk (Davenport, Yan *et al.,* 2015, Fischer, daCosta *et al.,* 2007). In animal studies, it has been demonstrated that the form of choline in the maternal diet can modulate the breastmilk composition (Dellschaft, Richard *et al.,* 2017, Lewis, Richard *et al.,* 2016, Richard, Lewis *et al.,* 2017). Infant formula is usually the chosen option when breastfeeding it is not possible. The nutrient composition of breastmilk is often followed as a guideline for the nutrient formulation in infant formula. The proportion of choline forms usually found in infant formula varies considerably and, even though they are mainly

composed of water-soluble forms (Pcho, GPC), they mostly differ from breastmilk significantly in terms of total amount of choline and the choline moieties composition (Wiedeman, Barr *et al.,* 2018).

1.5.3 Choline in obesity

Choline is critical for the formation of all biological membranes, dietary lipid absorption and lipoprotein secretion (van der Veen, Kennelly *et al.,* 2017). In the context of obesity and high-fat diet (HFD) feeding, choline requirements increased due to increase VLDL assembly and secretion and tissue expansion. Studies have demonstrated that mice on a choline deficient-HFD had impaired PC synthesis that led to an impaired VLDL and triglyceride secretion that resulted in an accumulation of fat in liver (Jacobs, Devlin *et al.,* 2004). In addition, mice lacking CTα shown impaired lipid uptake in the intestine which was associated with limited free fatty acids and cholesterol passage from the intestinal lumen and lower plasma triglycerides levels (Kennelly, van der Veen *et al.,* 2018). In rodents, a standard diet will provide 1g/kg of diet of choline in the form of FC whereas in the context of a HFD, rodent diets typically contain between 1.5 to 2 g/kg of choline (Hariri & Thibault 2010, Reeves 1997). In the context of obesity, the total amount of choline in the diet is important for lipoprotein formation; however, little is known about the impact of the different forms of choline in obesity.

1.6 Choline and the immune system

1.6.1 The form of choline in the diet modulates the immune system development

In recent years, research has been focused on determining the impact of feeding different forms of choline on the immune system development, which are presented in **Figure 1-5**. As

mentioned before, choline can be divided into water and lipid-soluble form. Each of these forms are absorbed and metabolized distinctly which suggests different functions in the body and possibly the immune system.

In a study conducted by Lewis, *et al*., (2016), the impact of feeding different forms of choline on the development of the immune system was examined. Two experimental diets were use in this study. The control diet was composed only by FC (1g choline bitartrate/kg of diet). The second diet contained only PC as the main source of choline in the diet (1 g choline from PC/kg of diet) (Lewis, Richard *et al.,* 2016). Feeding a maternal diet containing only PC had little impact on the proportion of the major T cell populations in splenocytes (CD3+, CD3+CD4, CD3+CD8); however, PC increased the proportion of B cells (OX6+) when compared to the control diet in offspring. In addition, splenocytes from the PC group had an increased IL-2, IL-6, IFN-γ production after T cell mitogen stimulation (concanavalin A (ConA)) and TNF-α and IL-6 after stimulation with an antigen-presenting cell mitogen (LPS) when compared to the control diet. Collectively, results from this study showed that feeding PC improve Th1 responses in offspring when compared to a maternal diet containing only FC. These results suggest that the lipid-soluble forms of choline are of more benefit for the maturation of the immune system in offspring. Although these studies show that feeding a diet that contained only PC prove to have a greater impact on immune responses, in reality, no human diet will contain only one form of choline but instead will be composed of a mixture of choline forms.

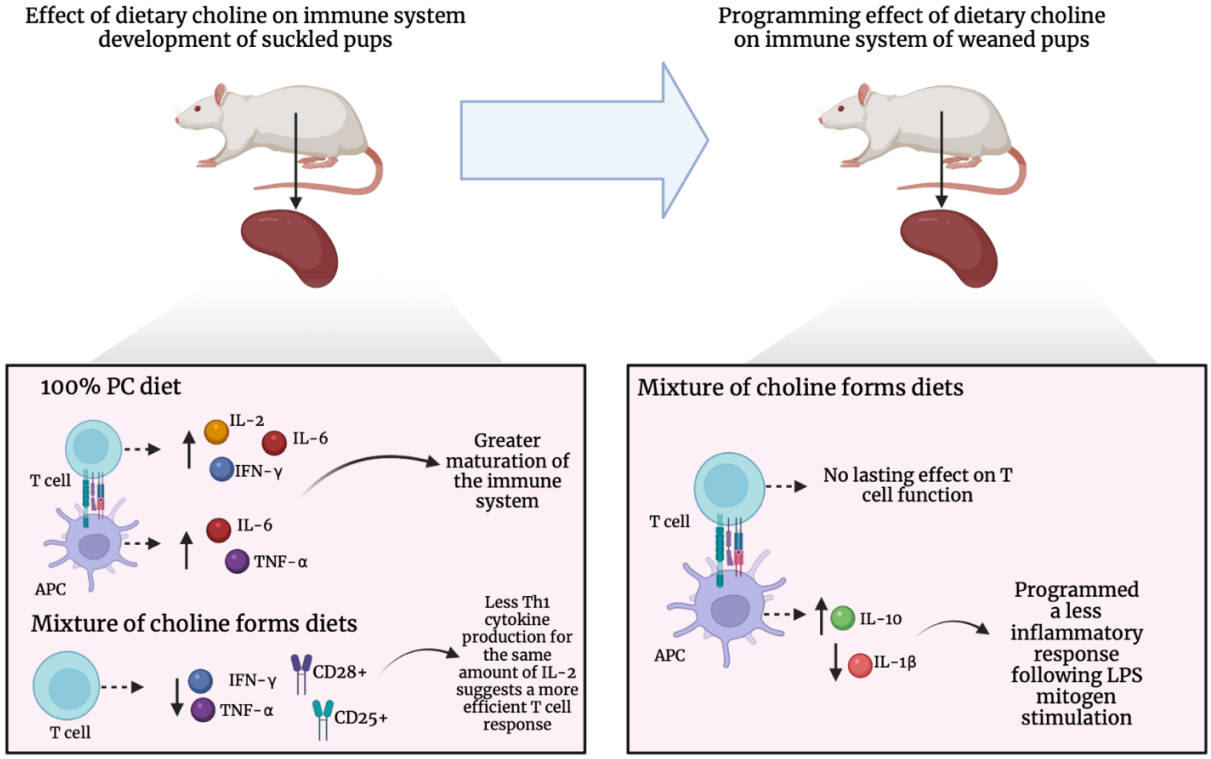
In a study conducted by Richard, *et al.*, (2017), diets containing a mixture of choline forms were fed to lactating dams and the programming effect on the immune system was assessed in their offspring when compared to a diet containing only FC (Richard, Lewis *et al.,* 2017). Three different diets were use in this experiment: The mixed choline (MC) diet was composed of 50%

PC, 25% FC, 25% GPC; the Control diet contained only FC, and the high GPC diet (HGPC) contained 75% GPC, 12.5% PC, and 12.5% FC. Diets were matched in total choline content (1 g/kg of diet) and fatty acids composition. The MC diet was based on epidemiological data from the APrON study that showed that PC, FC and GPC were the forms of choline mainly consumed by pregnant and lactating women in North America. Diets were fed to lactating dams (MC, HGPC and Control diets) through the lactation period and at weaning, pups were assigned to consume the control diet to examine the programming effect of dietary choline forms on immune system. At 3-weeks, splenocytes of pups from the MC and HGPC groups were producing less IFN-y and TNF-α, when compared to the control diet; however, no changes were found in IL-2 production among groups, meaning that pups from the MC and HGPC groups do not need to produce a high amount of Th1 cytokines in order to maintain a proliferative response. Following LPS stimulation, there was a lower production of IL-10 in the MC and HGPC groups when compared to the control diet. There was also a higher proportion of helper T cells expressing CD25+ and CD28+ markers. At 10 weeks, there was no changes in body weight nor organs' weight among dietary groups. Splenocytes from the MC and HGPC had a higher production of IL-10 and a lower production of IL-1β following LPS stimulation when compared to the control diet. In addition, there was a lower proportion of macrophages and dendritic cells in splenocytes from both the MC and HGPC diets. Altogether, these results suggest that feeding a mixture of choline forms during early developmental period led to a more efficient T cells response in suckled pups and that there was a programming effect of the choline forms later in life which led to a lower inflammatory response upon bacterial challenge.

In summary, the studies reviewed provide evidence that the form of choline in the diet differently modulate the immune system development in offspring. Of the reviewed studies, only

one determined the programming effect on the immune system later in life of feeding different choline forms during the suckling period. Indeed, offspring were not fed with the experimental diets at weaning, instead they were fed the control diet containing only FC to assess the programming effects of the MC and HGPC diets at 10 weeks of age. It will be interesting to examine the impact of feeding a mixture of choline forms during both the lactation and weaning periods, since the immune system continues to develop during the weaning period. In addition, SM is another lipid-soluble form of choline that has not been studied for its impact on immune responses and immune system development. SM can be found as part of the cell membrane and lipid rafts and some metabolites from its metabolism (ceramides, sphigonsine-1-phosphate) have shown to modulate immune responses. Therefore, future studies should aim to determine the impact of dietary SM on immune responses and immune system development. Furthermore, the gut-associated immune system also develops as we age and is modulated primarily by the diet. No study to date has investigated the impact of dietary choline on the development of the gut-associated immune system and, therefore, it is unknow if dietary choline will modulate the maturation of immune responses in the gut to the same extent as they modulate peripheral immune function. As of now, no studies have examined the impact of dietary choline on the establishment of oral tolerance, an important process that occur early in life, and that is involved in the prevention of atopic and allergic diseases. Thus, the effect of feeding a mixture of choline forms that contain SM on the development of both local and systemic immune responses and the establishment of oral tolerance warrant further investigation. Finally, other key points we could take from these studies is that the diets used in all of them were closely match for choline content and fatty acid composition. Fatty acids are known to influence immune responses and therefore is important to take them into account in order to attribute the benefits only to the form of choline.

Figure 0-5 Summary of the effects of feeding different forms of choline during the lactation period and their programming effect later in life (Lewis, Richard *et al*., 2016, Richard, Lewis *et al*., 2017). Created with http:BioRender.com.



Abbreviations: CD, cluster of differentiation; IFN-y, interferon gamma; IL, interleukin; LPS, lipopolysaccharide; TNF-α, tumor-necrosis alpha.

1.6.2 The form of choline in the diet modulates maternal immune function

It has been previously demonstrated, in rodents, that choline is needed for optimal maternal immune function and that feeding a diet devoid in choline (FC) has detrimental effects on immune responses in lactating dams (Dellschaft, Ruth *et al.,* 2015). In recent years, studies have been focused on examining the effect of feeding different amount and forms of choline on maternal immune responses and are summarize in **Figure 1-6**.

Dellschaft, *et al*., (2017) assessed the impact of feeding lactating dams a diet containing either 1 g of PC (1PC diet) or 2.5 g of PC (2.5PC diet) per kg/diet versus a control diet containing

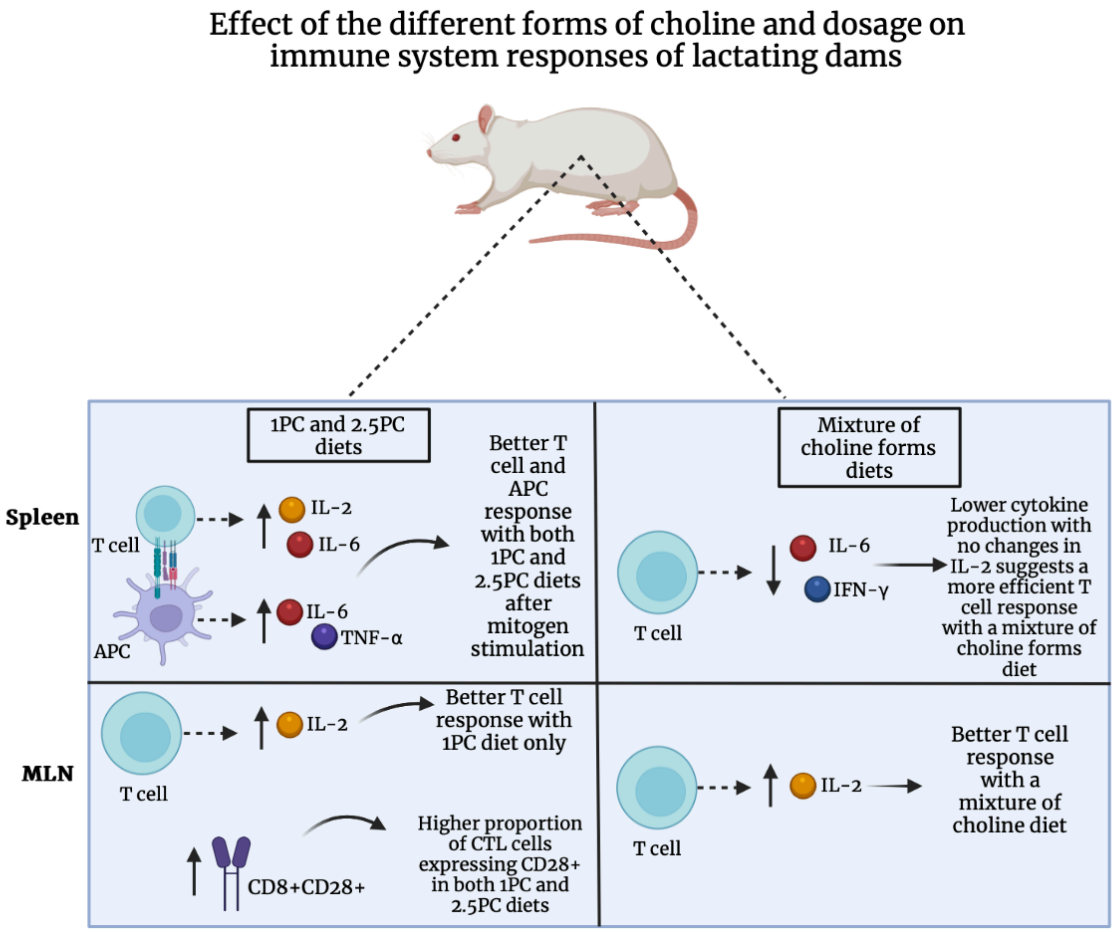
1 g of FC per kg/diet (control diet), the form of choline mainly found in a standard rodent diet (Dellschaft, Richard *et al.,* 2017). Both PC diets led to a higher final body weight when compared to dams fed the control diet. Feeding 1PC or 2.5PC diets improved APC cell response, in spleen, by increasing IL-6 production after LPS stimulation, in addition to an increased proportion of DC and macrophage populations. T cell responses were enhanced when 2.5PC was fed to lactating dams by increasing IL-2 production, in spleen, following ConA stimulation compared to both PC1 and control diets. There was also a higher proportion of cytotoxic T cells expressing co-stimulatory molecule (CD28+) in both 1PC and 2.5PC versus the control diet. In this study, immune responses in MLN were also measured. Feeding 1PC diet led to a higher production of IL-2 by MLN after ConA stimulation. Both 1PC and 2.5PC diet increased the proportion of T cells and cytotoxic T cells expressing CD28+ in MLN. The results from this study suggest that feeding 2.5g of PC per kg/g improves T cells responses in spleen and 1PC diet enhanced T cells responses in MLN of lactating dams when compared to a diet containing only water-soluble forms of choline (FC).

Lewis, et al., (2017) determined the impact of feeding a mixture of forms of choline to lactating dams on immune responses. Dams were fed one of three experimental diets containing the same amount of choline but varying in the form of choline (Lewis, Richard *et al.,* 2017). As described above, the mixed choline (MC) diet was composed of 50% PC, 25% FC, 25% GPC and its composition was based on data from the APrON study; the control diet contained only FC, and the high GPC diet (HGPC) contained 75% GPC, 12.5% PC, and 12.5% FC. Diets had little impact on dam's body weight. Feeding MC and HGPC diets lower the production of IFN-y and IL-6 in splenocytes following stimulation with a T cell mitogen (ConA); however, there were no changes in IL-2 production, suggesting that the proliferative response remained unaffected by the choline form in the diet. Moreover, there was a higher proportion of helper T cells, DC and helper T cells

expressing CD28 (MC group only) in spleen. Following LPS stimulation, splenocytes from MC and HGPC groups produced less IL-1β and IL-6 when compared to the control group. In MLN, there was a higher production of IL-2 and IFN-y in both the MC and HGPC groups when compared to the control diet after ConA stimulation. It appears that, based on the results of this study, feeding a mixture of choline forms during pregnancy and lactation improve maternal immune responses to mitogen stimulation.

Collectively, the studies presented above suggest that the choline form in the diet distinctly affect immune responses in lactating dams. It appears that a diets with a higher content of lipid-soluble forms of choline have a greater beneficial impact on immune responses of lactating dams. Feeding a mixture of choline forms during lactation enhanced T cells responses in lactating dams and also positively modulated immune responses in the gut. Similarly, to the offspring, no study has assessed the impact of a diet containing SM on immune responses of lactating dams. SM is an important component of cells membrane and another lipid-soluble forms of choline. As it appears from the studies above, the lipid-soluble forms of choline have a greater impact on immune responses when compared to the water-soluble forms of choline and therefore, the immunomodulatory effect of SM in the maternal diet on the dams' immune system should be tested.

Figure 0-6 Summary of the effects of the different forms of choline during pregnancy and lactation periods on immune responses in rat dams (Dellschaft, Richard *et al*., 2017, Lewis, Richard *et al*., 2017). Created with http:BioRender.com.



Abbreviations: APC, antigen presenting cells; CD, cluster of differentiation; CTL, cytotoxic T cell; IFN-y, interferon gamma; IL, interleukin; MLN, mesenteric lymph node; PC, phosphatidylcholine; TNF-α, tumor-necrosis alpha.

1.6.3 The form of choline in the diet modulates immune function in the context of obesity

Obesity is associated with an impaired T cell responses in both, humans and rodents, characterize by lower IL-2 production (Lamas, Martinez *et al.,* 2002, Richard, Wadowski *et al.,* 2017). Interestingly, dietary PC has consistently been associated with an overall more efficient immune response and a higher IL-2 production early in life in offspring but also in lactating dams.

Therefore, one could speculate that the lipid-soluble forms of choline, especially PC, could attenuate the immune dysfunction typically observed in obesity (Lewis, Richard *et al.,* 2016, Richard, Lewis *et al.,* 2017). Only few studies have assessed the role of dietary choline in the context of obesity or HFD feeding and are summarized **in Figure 1-7**.

In a study conducted by Aldana-Hernandez, *et al*., (2021) $LDL^{-/-}$ male mice were randomized to one of three experimental HFD (40% calories from fat, with 0.5% w/w of cholesterol): 1) control diet (0.1% w/w FC wt/wt); 2) choline-supplemented (CS, 0.4% FC wt/wt); and 3) PC supplemented (PCS, 0.3% choline from PC wt/wt) (Aldana-Hernandez, Azarcoya-Barrera *et al.,* 2021). Immune cell phenotypes of splenocytes and peripheral blood cells were measured by flow cytometry as well as plasma chemokines and cytokines levels. There was no significant difference among dietary groups on immune cell phenotypes of splenocytes nor peripheral blood cells. In addition, there was no significant difference on circulating levels of IFN γ, TNF α, IL-1 β, IL-6, IL-2, and IL-10. To point out, this study aimed to determine the role of dietary PC in an atherogenic mouse model and did not intend to investigate the effect on immune dysfunction as a result of a HFD feeding.

Gao, *et al*., (2021) investigated the effect of PC from different biological sources in a HFD-insulin resistant mice model. Male C57BL/6J mice were randomly assigned to one of six experimental diets: 1) control group (Con), 2) high fat diet-fed group (HF), 3) eicosapentaenoic acid (EPA)/ docosahexaenoic acid (DHA; EPA/DHA-PC) group extracted from *Sthenototeuthis oualaniensis* (squid), 4) soybean PC group (Soy-PC), 5) PC extracted from egg yolk group (Egg-PC) and 6) EPA/DHA-TG group (Gao, Du *et al.,* 2021). The PC groups were fed a modified HFD based on AIN-93G rodent diet mixed with 2% EPA/DHA-PC, Soy-PC or Egg-PC. Body weight gain was higher in the HF groups when compared to the control group. The EPA/DHA-PC group

had lower body weight gain when compared to the HF group. Feeding mice with EPA/DHA-PC was associated with a significant decreased in mRNA relative expression of TLR-4, C-junk terminal kinase (JNK), Nuclear factor kappa B (NF-κB), NLR family pyrin domain containing 3 (NLRP3), IL-1β, and TNF-α and an increased expression of IL-10 in white adipose tissue when compared to the HF group. Protein levels of phosphorylated-JNK/total-JNK and TLR-4 were also lower in the EPA/DHA-PC group when compared to the HF group. Serum levels of TNF-α and IL-1β where lower in mice fed the EPA/DHA-PC diet while IL-6 was lower in both EPA/DHA-PC and Soy-PC groups when compared to the HF group. IL-10 serum levels were higher in mice fed with both the EPA/DHA-PC and Soy-PC diets when compared to the HF group. Egg-PC diet had no effect on plasma cytokines levels nor mRNA cytokines expression. The results of this study suggest a possible effect of dietary PC combined with EPA/DHA in downregulating inflammatory pathways typically activated in the state of obesity and insulin resistance (NF-κB, JNK). EPA/DHA-PC also showed potential anti-inflammatory properties by downregulating inflammatory cytokines expression (IL-1β, TNF-α) and upregulating IL-10, an important immunomodulatory cytokine. These results were also confirmed in circulation where lower concentrations of cytokines were observed after the EPA/DHA-PC consumption.

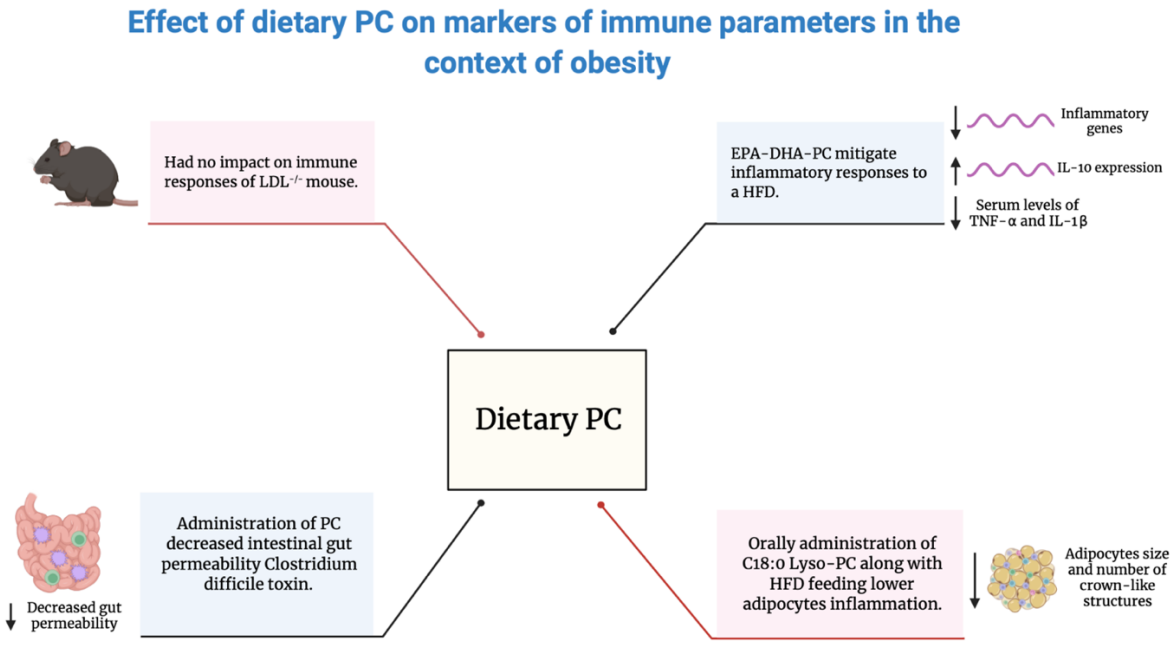
In a study conducted by Han, *et al*., (2021) male C57BL/6 mice were randomly assigned to one of four different groups (n=6): 1) a control group fed a low-fat commercial diet (10% of calories from fat) and 0.05% of polysorbate (Tween-20) in PBS via oral gavage; 2) the HFD group fed a commercial high-fat diet (45% calories from fat) and 0.05% of polysorbate in PBS via oral gavage; 3) the treatment group fed a HFD (45% fat) and administrated 100 mg/kg (body weight) of 18:0 Lyso-PC extracted from lentils and dissolved in PBS via oral gavage; 4) the positive control group fed a HFD and administrated 100 mg/kg (body weight) of metformin dissolved in PBS via

oral gavage (Han, Park *et al.,* 2021). No changes in body weight where observe among groups. Feeding mice with a HFD and 18:0 Lyso-PC led to a decrease in adipocytes size and the number of crown-like structures in adipose tissue when compared to the HFD group, suggesting a protective role of Lyso-PC against the obesity-related adipose tissue inflammation. In intestine, Olson *et al*., (2014), demonstrated that intestinal epithelial cells treated with *Clostridium difficile,* as a model of colitis, improve intestinal barrier in the presence of PC, suggesting that PC can improve intestinal permeability (Olson, Diebel *et al.,* 2014).

In summary, the studies reviewed above, demonstrated that dietary choline (lipid-soluble forms in particular) could be used to counteract the immune dysfunction observed in obesity and/or during HFD feeding. However, in the studies conducted by Gao, *et al*., (2021) and Han, *et al*., (2021), the diets fed to the mice were commercially purchased and the fatty acid composition of the diets was not considered when designing the experiments. In fact, Gao, *et al*., (2021) mentioned that the purpose of their study was to determine if there were any differences between the types of PC due to their differences in their fatty acid composition. Since fatty acids exert immunomodulatory properties, it is important to design diets that are closely match in fatty acid composition to determine if dietary choline is responsible for the changes observe on immune responses. In addition, studies should also aim to determine the impact of the lipid-soluble forms of choline on immune cell phenotypes as well as the function of immune cells as measure by *ex vivo* cytokine production. The three animal studies presented above were all conducted in male mice, and little is known about the sex-differences in these models. Moreover, *in vitro* studies showed that PC can improve intestinal barrier and, as obesity is associated with a "leaky gut", studies should aim to determine if dietary PC can improve to some extent the increase in intestinal permeability often observed in obesity. Finally, Wistar rats have shown to develop an obesity

phenotype and immune dysfunction similar to the one observed in humans (lower IL-2 production) and, therefore, it would be an appropriate model to understand some of the mechanisms behind the impaired T cell responses observed in obesity and how the lipid-soluble forms of choline (PC) can possibly ameliorate the T cell dysfunction observed in obesity.

Figure 0-7 Summary of choline studies and the effect in obesity-related immune dysfunction parameters (Aldana-Hernandez, Azarcoya-Barrera *et al*., 2021, Gao, Du *et al*., 2021, Han, Park *et al*., 2021, Olson, Diebel *et al*., 2014). Created with http:BioRender.com.



Abbreviations: DHA, docosahexaenoic acid, EPA, eicosapentaenoic acid; HFD, high-fat diet; IL, interleukin; Lyso-PC, lysophosphatidylcholine; LDL, low-density lipoprotein; PC, phosphatidylcholine; TNF-α, tumor-necrosis alpha.

1.7 Sphingomyelin and the immune system

1.7.1 Cell culture studies

A summary of all cell culture studies examining the role of SM on immune system parameters are presented in **Table 1.1**. Sakamoto, *et al*., (2017) examined the pro-inflammatory properties of SM in thioglycolate-elicited peritoneal macrophages that were isolated from SMS2

(sphingomyelin synthase 2) knockout mice ($SMS2^{-/-}$) (Sakamoto, Yoshida *et al.,* 2017). Plasma sphingomyelin is synthesized, in majority, by the action of SMS2 in the liver and SMS2 deficiency has been related to a decreased in SM plasma and liver levels. When treated exogenously with bovine SM, macrophages from $SMS2^{-/-}$ mice increased inducible nitric oxide synthase (iNOS) and intercellular adhesion molecule 1 (ICAM1) expression. Moreover, it was shown that C24:0 SM but not C16:0 SM nor C6:00 SM was able to increase iNOS and ICAM1 expression. Suggesting a role of SM in macrophage activation and, more specifically, from plasma long chain SM.

Norris, *et al.*, (2017) investigated the effects of milk SM on chronic inflammation. RAW264.7 macrophages were incubated in the presence or absence of LPS and two different concentrations of milk SM (0.8 or 8 μg) (G. Norris, C. Porter *et al.,* 2017). Macrophage cell viability was unaffected by milk SM. However, both concentration of milk SM were able to ameliorate the pro-inflammatory effects of LPS by decreasing the relative expression of TNF-α and CCL2. This was mitigated by the presence of imipramine, which causes the degradation of acid sphingomyelinase, an enzyme responsible for the hydrolysis of SM to ceramide and phosphorylcholine. These results suggest that the products of SM hydrolysis play a key role in the anti-inflammatory properties of SM.

Milard, *et al*., (2019) assessed the impact of milk SM on local immune responses by using Caco-2/TC7 human intestinal cells. Micelles were mix with different doses of SM (0.2, 0.4 and 0.6 mM milk SM) and added into the cell culture (Milard, Penhoat *et al.,* 2019). Cells where then incubated for 24 hours, and mRNA relative expression of IL-8 was measured. The results from this study showed that 0.4 mM of milk SM was able to increase IL-8 expression in intestinal cells vs. control. No changes were found in the expression of other cytokines (TNF-α, MCP-1, IL-1β) among groups. IL-8 has been shown to promote cell differentiation, proliferation, and migration

in Caco-2 cells. It is worth to mention, that this study was performed in order to determine the impact of milk SM on the expression of tight junction proteins in the gut. The upregulation in IL-8 expression could be the potential mechanism by which SM upregulate tight junction proteins expression.

To date, studies assessing the role of SM on immune system parameters *in vitro* are scarce. The three studies above demonstrated that SM may be involved in macrophages activation and, also, in the suppression of immune responses in the gut. Two studies showed that SM can be used to ameliorate chronic inflammatory responses both systemic and locally. All these studies used milk SM as their source of SM; however, a different range of doses was use, and it is unclear what is the optimal dose of SM and if SM would exert the same immunomodulatory properties *in vivo*. Furthermore, Sakamoto, *et al*., (2017) demonstrated that the type and composition of SM use matters. Hence, a study comparing different types of SM coming from different sources (egg, milk) warrants future studies. In addition, SM have shown promising results in modulating the activation of immune responses; however, to date, this has not been fully investigated. It will be interesting to examined if immune cells can possibly modulate and mount a better or more efficient immune responses upon mitogen stimulation in the presence of SM. Other markers of inflammation should also be considered, such as, IL-2 (marker of proliferation), IFN-y and IL-6.

Table 0-1 Summary of cell culture studies examining the role of SM on immune system parameters.

Cell type	SM concentration use	Length of incubation	Immune parameters measured	Results	Reference
Thioglycolate-elicited peritoneal macrophages isolated from SMS2-deficient mice.	100 µM bovine SM 100 µM SM (C24:0) 100 µM SM (C16:0) 100 µM SM (C6:0)	24 h	iNOS and ICAM-1 mRNA relative expression after incubation with bovine SM	↑ relative expression of iNOS and ICAM-1 after incubation of macrophages with bovine SM	Sakamoto, *et al*, 2017.
RAW264.7 macrophages	0.8-8 µg/mL of milk SM	1 h following by a 4 h co-incubation in the presence or absence of LPS and a 2-hour incubation with Imipramine (100 µg) only with milk SM	mRNA relative expression of TNF-α, CCL2 and macrophage viability	↓TNF-α and CCL2 relative expression after LPS stimulation in both 0.8 and 8 MSM. ↔ in macrophage viability. ↔ TNF-α relative expression in the presence of LPS and Imipramine.	Norris, *et al*, 2017.
Caco-2/TC7-Human epithelial intestinal cells	mixed micelles + 0.2, 0.4 or 0.6 mM of Milk SM	24 h	IL-8	↑ IL-8 relative expression with 0.4 mM MSM	Milard, *et al.*, 2019.

Abbreviations: CCL2, chemokine (C-C motif) ligand 2; ICAM-1, Intercellular Adhesion Molecule 1; IL-8, interleukin-8; iNOS, inducible nitric oxide synthase; LPS lipopolysaccharide; MCP-1, monocyte chemotactic protein; mRNA, messenger RNA; SMS2, sphingomyelin synthase 2; TNF-α, tumor necrosis factor-alpha. ↑ indicates an increase; ↓ indicates a decrease; ↔ indicates no change.

1.7.2 Animal studies

There have been some studies assessing the impact of SM *in vivo* on immune system parameters. These studies have been focused mostly on the effect of SM in the context of chronic inflammation and are summarize in **Table 1.2**. Mazzei, *et al*., (2011) investigated the impact of dietary choline on intestinal inflammation in a colonic cancer animal model (Mazzei, Zhou *et al.,* 2011). Mice were fed a semi-purified diet (AIN76A diet) with no sphingolipids added and with or without SM. After 7 days of consuming the experimental diets, mice were injected with azoxymethane to induce colon cancer. A week later, 2.0% dextran sodium sulfate (DSS) was added to the drinking water to induce inflammation. Mice were fed regular water after one week on DSS and for the duration of the study (68 days total). MLN were collected at the end of the study. Mice fed the SM diet had a decreased F4/80+ macrophages population as measured by flow cytometry. Colonic mRNA markers of inflammation were also measured; however, the sample size for this experiment was n=3 and, therefore, no statistical analysis was performed. Nonetheless, genes involved in cell trafficking to the gut (CCL19, CCL11, CCL20, CXCLl9, CXCL11) and CD4+ T cell differentiation and fate (IFN-γ, IL-17, IL-4, IL-13, IL-13R, Treg) were upregulated in colon in the SM group. The pattern of genes upregulated, showed that both pro- and anti-inflammatory pathways are modulated by dietary SM.

To date, there have been three studies examining the impact of dietary SM in mice fed a HFD. HFD are known to increase intestinal permeability, increase circulating LPS and pro-inflammatory circulating cytokines levels. Norris, *et al*., (2016) investigated the impact of feeding a HFD with either SM coming from milk or from egg (Norris, Jiang *et al.,* 2016). Mice were fed for 4 weeks with one of three experimental diets. The three diets were all HFD (46% calories from fat) and were also adjust for other macronutrients composition (carbohydrates and protein). The

control diet did not contain SM and the other two experimental diets contained either milk SM or egg SM (2.5 g/kg of diet). In liver, there was no change among groups in mRNA relative expression of monocyte chemoattractant protein-1 (MCP-1). Milk SM decreased serum LPS levels when compared to the control HFD (no SM). In addition, milk SM decreased IL-6 mRNA relative expression in skeletal muscle when compared to the control diet only. Similarly, Norris, *et al*., (2017) also investigated the impact of HFD feeding in mice and the impact of dietary milk and egg SM (1g/kg of diet) (G. Norris, C. Porter *et al.,* 2017). In this study, mice were fed with the HFD for 10 weeks and 60% of the total energy intake was coming from fat. Macrophage infiltration, as measured by the number of crow-like structures, was decreased in the egg SM group when compared to the control HFD. Serum CCL2 was decreased in both milk and egg SM groups when compared to the control HFD. In addition, the relative expression of F4/80 was decreased in both milk and egg SM groups in adipose tissue. Egg SM decreased the relative expression of CD11c and CD68 in adipose tissue when compared to the control HFD only. Norris, *et al*., (2017) determined the impact of dietary SM on serum inflammatory markers (G. H. Norris, C. M. Porter *et al.,* 2017). Mice were once again fed a control HFD (without SM) and a HFD containing milk SM. Milk SM decreased serum concentrations of IFN-γ, TNF-α, IL-6 and macrophage inflammatory protein-1beta (MIP-1β) when compared to the control HFD. From the three studies previously discussed, we can conclude that SM in the diet can counteract some of the known negative impact of obesity on immune responses. It appears that SM coming from milk provides more beneficial effects when compared to SM from eggs. However, the authors highlight that there are differences in the fatty acid composition between the milk and egg SM and that the fatty acid composition of the diets was not considered when diets were designed. Therefore, it is not possible

to attribute entirely the benefits seen to SM because fatty acids are also known to directly impact immune responses and, hence this is an important limitation from these studies.

In summary, studies have shown that dietary SM exert anti-inflammatory properties by decreasing pro-inflammatory markers and serum LPS in the context of chronic inflammation (either HDF induced inflammation or colonic cancer model). However, inflammatory markers have been determined only by relative expression on different tissues (intestine) and there is no evidence of the impact of dietary SM on *ex vivo* cytokine production by immune cells stimulated with different mitogen, such as ConA or LPS. It is known that SM is a component of cell membranes and is needed for T cell activation. However, the effect of SM on immune function and immune cell phenotypes remain largely unknown. In addition, studies have mostly focus on dietary SM in the context of obesity and, it would be interesting understand the impact of SM on the development of the immune system early in life and immune reponses during pregnancy and lactation.

Table 0-2 Summary of animal studies examining the role of SM on immune system parameters.

Animal model	Sex and age	Dietary intervention	Dose and type of SM	Length of feeding	Immune parameters measured	Results	Reference
PPAR-γ−/− mice	Female and male	Mice were fed a semi-purified sphingolipid-free AIN76A diet (5% w/w corn oil) with or without SM through the whole experiment (n=10).	0.1% (w/w) of milk SM	Colon: One week on diet + 7 days with 2% DSS in the water (n=3). MLN: 68 days	Colonic mRNA relative expression of cytokines. MLN immune cells phenotype	↓ F4/80+ macrophage population in MLN with SM. Colon: ↑ CCL19, CCL11, CCL20, CXCLl9, CXCL11, IFN-γ, IL-17, IL-4, IL-13, IL-13R, Treg (IL-10R) mRNA relative expression with SM.	(Mazzei, *et al*., 2011)
C57BL/6J mice	Male 8-weeks-old	Mice were randomly assigned to consume one of three experimental diets: 1) High-fat diet control (CTL; 46% kcal as fat, 36% cho, 18% prot; n=10). 2) High-fat diet with milk SM (MSM; 46% kcal as fat, 36% CHO, 18% prot; n=10). 3)High-fat diet with egg SM (ESM; 46% kcal as fat, 36% CHO, 18% prot; n=10).	2.5 g/kg of diet of milk SM or 2.5 g/kg of egg SM	4 weeks	mRNA relative expression of inflammatory markers in liver Serum LPS mRNA relative expression of inflammatory markers in skeletal muscle	↔ MCP-1 in liver. ↓ serum LPS in MSM vs CTL. ↓ IL-6 relative expression in skeletal muscle in MSM vs. CTL.	(Norris, *et al.,* 2016)
C57BL/6J	Male 6-weeks-old	Mice were randomly assigned to consume one of four experimental diets: 1) Low-fat diet (LFD: 10% fat, 65% CHO, 25% prot; n=10). 2)High-fat diet (HFD, 60% fat, 20% CHO, 20% Prot; n=14).	1 g/kg of diet of milk SM or 1 g/kg of diet of egg SM	10 weeks	mRNA expression of inflammatory markers and macrophage infiltration in adipose tissue and serum CCL2 concentrations	↓ macrophage infiltration (# of crown-like structure) in adipose tissue in ESM vs HFD. ↓ serum CCL2 in MSM & ESM vs. HFD. ↓ F4/80 adipose tissue relative expression in MSM &ESM vs. HFD.	(Norris, *et al.*, 2017)

		3) High-fat diet with milk SM (MSM; 60% fat, 0.15% chol; n=10). 4) High-fat diet with egg SM (ESM; 60% fat; 0.15% chol; n=10).				↓ CD68 and CD11c adipose tissue expression in ESM vs. HFD.	
C57BL /6J mice	Male 6-weeks-old	Mice were randomly assigned to consume one of two experimental diets: 1) High-fat diet (HFD, 60% fat, 20% CHO, 20% Prot; n=14). 2) High-fat diet with milk SM (MSM, 60% fat, 20% CHO, 20% Prot; n=14).	1 g/kg of diet of milk SM (bovine, >99% purity)	10 weeks	Serum inflammatory markers mRNA relative expression in intestine, colon and adipose tissue	↓ in serum IFN-γ, TNF-α, IL-6 and MIP-1β in MSM vs HFD. ↓ serum LPS in MSM vs HFD. ↔ mRNA relative expression of inflammatory markers in small intestine. ↑ relative expression of CCL2 in colon in MSM vs. HFD. ↔ mRNA relative expression of inflammatory markers in mesenteric adipose tissue.	(Norris, *et al.*, 2017)

Abbreviations: CCL2, chemokine (C-C motif) ligand 2; CD, cluster of differentiation; CHO, carbohydrates; Chol, cholesterol; HFD, high-fat diet; Il, interleukin; LPS lipopolysaccharide; MCP-1, monocyte chemotactic protein; mRNA, messenger RNA; NaCl, sodium chloride; TNF-α, tumor necrosis factor-alpha; SM, sphingomyelin.
↑ indicates an increase; ↓ indicates a decrease; ↔ indicates no change.

1.7.3 Human studies

No studies in humans have yet focused on determining the impact of dietary SM, specifically, on immune system. The intake of dietary SM in humans could only be assess by the pattern of food they consume and if they are known to be high in SM as it would be difficult to have a diet containing only SM as source of fat due to the complexity of human diets. The MFGM, as mentioned before, it is found in dairy products and the membrane is especially high in phospholipid content, including SM. There have been 2 studies so far examining the effect of MFGM on the immune system in humans in the context of obesity and are summarize in **Table 1.3**.

Demmer, *et al*, (2016) examined the impact of a high saturated-fat test meal, with or without MFGM, on plasma inflammatory markers in obese or overweight adults (Demmer, Van Loan *et al.,* 2016). Individuals were randomly assigned to one of the two test meals, 1) Palm oil (PO); 2) PO + MFGM (14% of total fat was phospholipids) in smoothies, in addition to a bagel with strawberry preserves. Individuals consumed both test meals in random order on different days separated by a washout phase of a minimal of 1 week and maximum of 2 weeks to avoid any carry-over effects. Blood samples were collected at time 0 (baseline) and, 1, 3 and 6 hours post meal. The addition of MFGM to the meals increased serum levels of IL-10 and decreased serum levels of sICAM (soluble intracellular adhesion molecule). In addition, there was a time and treatment interaction effect for IL-8, IL-10 and sICAM, which overall suggest an anti-inflammatory effect of the MFGM.

Similarly, Rogers, *et al*., (2017) examined the effect of four different meal test in obese and overweight adults with or without MFGM (Rogers, Demmer *et al.,* 2017). The test meals consisted of a bagel with strawberry preserves and a smoothie, high in saturated fat, containing PO

or whipping cream with or without MFGM. The participants were consuming the meals randomly, and they all consumed the four meals in different visits separated by 1-2 weeks washout period. Blood was collected at time 0 (baseline) and, 1, 3 and 6 hours after consuming the meal. The addition to the MFGM to the meals had no effect on plasma postprandial levels of IL-6, IL-10, TNF-α, CRP, and IL-18. There was only a significant time effect for IL-6, IL-10, TNF-α, CRP, and IL-18 levels.

In summary, only one study so far showed a potential anti-inflammatory effect of MFGM in the context of a high-saturated meal. The study conducted by Rogers, *et al*, (2017) showed no effect of the MFGM in the postprandial state on plasma inflammatory markers. Importantly, these were exploratory studies that only offered a test meal and longer intervention periods may be required to observe a consistent anti-inflammatory effect of MFGM. Yet, the addition of the MFGM showed some beneficial effect by increasing IL-10 concentrations in plasma. MFGM contains other major components, besides SM, such as PC and bioactive peptides that have also been shown to exert anti-inflammatory properties and, therefore, we cannot attribute the benefits observed from MFGM solely to SM. Future studies should aim to better understand the effect of SM *per se* on the immune system in the context of obesity.

Table 0-3 Summary of human studies examining the role of SM on immune system parameters.

Study design	Population of study	Dietary intervention	Immune parameters measured	Results	Reference
Randomized, double-blinded, two-way, cross-over trial	Overweight and obese men and women (18-65, n=36)	Subjects were randomized to consumed 1 of 2 test meals in two different occasions. 1) Palm oil (PO) 2) palm oil plus milk fat globule membrane (PO + MFGM).	Serum and plasma inflammatory markers at baseline, 1, 3 and 6 h postprandial.	↑serum IL-10 and ↓sICAM in PO + MFGM. Time x treatment effect for IL-10, IL-8, and sICAM.	Demmer, *et al*., 2016.
Randomized, double-blinded, cross-over trial	Overweight and obese men and women (18-65 y, n=36)	Subjects were randomized to consumed 1 of 4 test meals in four different occasions. 1) Palm oil (PO) 2) palm oil plus milk fat globule membrane (PO + MFGM). 3) Whipping cream (WC). 4) Whipping cream plus milk fat globule membrane (WC + MFGM).	Serum and plasma inflammatory markers at baseline, 1, 3 and 6 h postprandial.	↔ IL-6, IL-10, TNF-α, CRP, IL-18. Significant time effect in IL-6, IL-18, TNF-α, CRP.	Rogers, *et al*., 2017.

Abbreviations: CRP, C-reactive protein; IL, interleukin; MFGM, milk-fat globule membrane; PO; palm oil; sICAM, soluble intracellular adhesion molecule; WC, whipping cream.
↑ indicates an increase; ↓ indicates a decrease; ↔ indicates no change.

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Chapter 2: Research plan

2.1 Rationale

Choline is an essential nutrient for different critical processes in the body (Zeisel & da Costa 2009). During pregnancy and lactation, the requirements of choline increased significantly to meet the demands of the fetus and the infant, yet epidemiological data suggest that pregnant and lactating women in North America are not meeting the daily requirements for choline intake (Caudill 2010, Lewis, Subhan *et al.,* 2014). Choline exists in the diet as water-soluble forms (free choline (FC), glycerophosphocholine (GPC), phosphocholine) and lipid-soluble forms (phosphatidylcholine (PC), sphingomyelin (SM). Previously, it has been demonstrated that choline is necessary for optimal maternal immune function and that the choline forms present in the diet modulate distinctly immune responses in lactating dams and offspring (Dellschaft, Ruth *et al.,* 2015, Lewis, Richard *et al.,* 2016, Lewis, Richard *et al.,* 2017). However, the effect of SM on immune responses during pregnancy and lactation and early developmental periods has yet to be investigated. Buttermilk is the result of the churning of cream into butter. It significantly differed in fat content compared to skim milk due to the presence of a milk fat globule membrane (MFGM) (Conway, Gauthier *et al.,* 2014). MFGM is composed mostly by neutral lipids (70%) and phospholipids (30%, including PC (19.2-34.5%) and SM (17.9- 34.5%)) (Lopez 2018). Therefore, buttermilk could be a valuable source of dietary choline and specific forms of choline (high in SM) during pregnancy, lactation and later in life. Moreover, most of the research on dietary choline has been focused at determining its impact on peripheral immune responses. Little is known about how dietary choline and the different forms of choline will affect the development of the gut-associated immune system, which is highly affected by the diet especially early in life when it is still maturating. PC is the most abundant phospholipid in cells membrane, with eggs, liver, soy

products and beef being the major sources of dietary PC (Vance 2015). In addition, it has been shown that PC can improve T cells responses by increasing IL-2 production (Lewis, Richard *et al.,* 2016). In the state of obesity, immune responses have been shown to be impaired. Wistar rats on a diet-induced obesity (cafeteria diet) had a lower helper T cell population and a lower production IL-2 after phytohemagglutinin stimulation (Lamas, Martinez *et al.,* 2002). In humans, individual with type 2 diabetes and obesity showed a decreased IL-2, IL-6 and TNF-a following T cell mitogen stimulation when compared to obese normoglycemic individuals (Richard, Wadowski *et al.,* 2017). So far, no study has investigated the impact of dietary PC on immune responses in the context of obesity.

2.2 Objectives and hypotheses

The overall objective of this research is to determine the impact of feeding different forms of choline on immune system through different life stages in life (i.e. early in life and obesity). To address this overall objective, three different objectives and their hypotheses were established:

1. The first objective of this research was to determine the effect of feeding buttermilk-derived choline forms during pregnancy and lactation on immune function of lactating dams and the development of the immune system in their offspring. Two specific objectives were proposed with their respective hypotheses:
    a) Determine the effect of feeding a diet containing buttermilk-derived choline forms on the immune system during pregnancy and lactation in rat dams and the distribution of choline forms in breastmilk. We hypothesized that feeding whole buttermilk to dams during pregnancy and lactation 1) will have a greater beneficial effect on immune

responses of lactating dams compared to our placebo and control diets; 2) will modulate the choline forms distribution in breastmilk reflective of the maternal diet.

b) Determine the impact of feeding buttermilk-derived choline forms during the lactation and weaning periods on the immune system development in rat offspring. We hypothesized that feeding whole buttermilk during the lactation and weaning periods will have a beneficial effect on the immune system development in rat pups when compared to our control and placebo diets.

2. The second objective of this research was to determine the effect of feeding different forms of choline during suckling and weaning periods on the development of the gut-associated lymphoid tissue (GALT). We hypothesized that 1) the choline forms in the diet will affect differently the development of the GALT, and 2) a diet containing higher proportion of lipid-soluble forms of choline will beneficially impact, to a greater extend, the development of the GALT compared to diets higher in water-soluble forms.
3. The third objective of this research was to determine the effect of providing egg-PC as part of a high-fat diet (HFD) on immune responses in Wistar rats. We hypothesized that 1) feeding a HFD will impaired immune responses (especially T cell) in Wistar rats; 2) providing egg-PC as part of a HFD will attenuate the obesity-related immune dysfunction.

2.3 Chapter format

The objectives and hypotheses stated above were tested in a series of different studies. These studies were organized into thesis chapters and have been accepted and/or submitted for publication as individual manuscripts.

Chapter 3 examines the impact of buttermilk-derived choline forms in the maternal diet on immune responses of lactating dams. The impact of buttermilk on breastmilk composition was also determined. Objective 1(a) was addressed in this chapter. We found that buttermilk-derived choline forms had a greater impact on T cells responses in lactating dams and that the choline form present in the maternal diet modulated breast milk composition in choline.

Chapter 4 examines the impact of buttermilk-derived choline forms in the maternal and weaning diets on immune system development in rat offspring. This was the first choline study examining the effect of feeding choline forms in both the lactation and weaning periods on immune system development and, in addition, the first examining the effect of dietary choline on immune responses to a dietary antigen (ovalbumin). In summary, diets containing a higher content of lipid-soluble forms of choline favored, to a greater extend, the maturation of peripheral immune responses. Objective 1(b) was addressed in this chapter

Chapter 5 examines the impact of feeding dietary choline early in life on the development of local immune responses in the GALT. Three previous studies from our group were included in this study. Two of the three studies examined the programming effect of dietary choline on immune responses in the GALT. The other study examined the impact of dietary choline when fed during both the lactation and weaning periods. Overall, the diets that contained a mixture of choline forms mostly composed of lipid-soluble forms had a greater impact on the maturation of the GALT, especially when fed in both the lactation and weaning periods. Objective 2 was addressed in this chapter.

Chapter 6 examines the impact of feeding a HFD with or without egg-PC on immune responses in Wistar rats. Objective 3 was addressed in this chapter. In summary, feeding rats with a HFD

impaired T cells responses by decreasing IL-2 production, and this was counteracted by the addition of egg-PC to the HFD.

Chapter 7 provides an overall discussion that includes the results from each objective and hypotheses, in addition to, directions for future research.

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Chapter 3: Feeding buttermilk-derived choline forms during gestation and lactation modulates *ex vivo* T cell response in rat dams.

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3.1 Introduction

During pregnancy mothers undergo physiological and metabolic changes required for a successful pregnancy. These changes affect every system in the mother's body (Lockitch 1997). During this period, the immune system, in particular, needs to maintain a balance between pro- and anti-inflammatory immune responses but also maintain efficient immune responses to viral and bacterial infections to protect not only the mother but also the fetus (Mor & Cardenas 2010, Racicot, Kwon *et al.,* 2014). Innate immunity is the first line defense and consists of physical barriers such as skin and mucus but also immune cells, which includes granulocytes (i.e. neutrophils) and monocytes/macrophages. Acquired immunity, which includes T (T cells) and B (B cells) lymphocytes, possesses the ability to develop immunological memory which is the recognition of antigens previously encountered (Yaqoob & Calder 2011).

Choline is an essential nutrient needed for critical processes during pregnancy and lactation including, organ and tissue development, offspring growth, brain development and optimal maternal and offspring immune function (Dellschaft, Ruth *et al.,* 2015, Lewis, Goruk *et al.,* 2016, Zeisel 2006a, Zeisel 2006b). Choline can be obtained via *de novo* biosynthesis; however, this is not sufficient to meet the daily requirements and therefore a dietary source of choline is crucial for humans. Choline can be found in the diet both in primarily water-soluble forms (free choline (FC), phosphocholine (Pcho), glycerophosphocholine (GPC)) and lipid-soluble forms (phosphatidylcholine (PC, sphingomyelin (SM)) (Zeisel, Mar *et al.,* 2003). During pregnancy and lactation, the recommendations for choline increase significantly from 425 mg/day for non-pregnant women to 450 mg/day and 550 mg/day for pregnant and lactating women, respectively (Institute of Medicine, 1998). Epidemiological data from the Alberta Pregnancy Outcomes and Nutrition (APrON) study reported that only 23% of pregnant and 10% of lactating women are meeting the daily choline recommendations (Lewis, Subhan *et al.,* 2014). Recently we demonstrated that the form of choline in the maternal diet during lactation modulates immune function in both lactating dam rats and their offspring. Feeding a maternal diet containing PC instead of FC (the form of choline found in the standard rodent diet) improved immune function in lactating dams (Lewis, Richard *et al.,* 2016). In addition, providing a mixture of choline forms representative of the human consumption (50% PC, 25% GPC, 25% FC) to lactating dams led to a more efficient immune response to a T cell mitogen (Lewis, Richard *et al.,* 2017).

Buttermilk, the result of the churning of cream into butter, is a unique by-product that is similar in nutrient composition to skim milk but differs considerably in total fat content due to the presence of milk fat globule membrane (MFGM). MFGM is a three layer membrane structure composed of proteins and non-polar (62% triglycerides, 9% diglycerides) and polar lipids (26-

31% phospholipids) (Conway, Gauthier *et al.,* 2014, Doreen 2011). The three layers within the MFGM are: a monolayer, located in the interior and made mostly by polar lipids such as phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS), a protein layer and an outer bilayer made of phospholipids, protein, polar and non-polar lipids and enzymes. SM and PC are located in the external bilayer of the MFGM (Lopez 2018, Smoczyński 2012). Emerging research has attributed health benefits to some components of buttermilk, including sphingolipids (i.e. SM and gangliosides). It has been demonstrated that certain SM exert anti-inflammatory properties in the context of high-fat diet induced inflammation models (G. Norris, C. Porter *et al.,* 2017, G. H. Norris, C. M. Porter *et al.,* 2017). In addition, bioactive peptides found in buttermilk have been associated with a reduction in blood pressure in humans (Conway, Couture *et al.,* 2014). Buttermilk contains twenty times more phospholipids when compared to skim milk and the main phospholipids present in buttermilk are PE (31.1-42.0%), PC (19.2-34.5%) and SM (17.9-34.5%) (Conway, Gauthier *et al.,* 2014).

The objective of the study was to assess the effect of feeding whole buttermilk on the immune system of lactating dams and the distribution of choline forms in breastmilk. In order to assess the unique immune benefits attributed to the buttermilk-derived choline forms, we developed a "placebo" diet that was similar in macronutrient, lactose and fatty acid composition but differed in the form of choline. We hypothesized that due to its high content in MFGM (containing PC and SM) buttermilk would have a greater beneficial effect on the immune system of lactating dams compared to both a typical rodent control diet and the placebo diet. Based on our previous studies discussed above, we also hypothesized that the distribution of choline forms in breastmilk would be representative of the forms of choline in the maternal diet.

3.2 Materials and Methods

3.2.1 Animal and Study design

All animal care and experimental protocols were conducted in accordance with the Canadian Council on Animal Care guidelines and approved by the University of Alberta Animal Ethics Committee. Pregnant primiparous female Sprague-Dawley rats (n=24) were obtained from Charles River Laboratories (Montreal, Quebec, Canada) on day 7-10 days of gestation and were individually housed in a temperature and humidity-controlled environment with a 12/12-h reversed light cycle. Dams were fed standard rat chow (Lab diet 5001; PMI Nutrition International, Brentwood, MO, USA) prior to the start of the study i.e. until midway during the pregnancy period. At an estimated 10 days of gestation, dams were randomized to one of three experimental diets all containing 1.7g of choline/kg of diet: Control diet (100% FC; n=8), Buttermilk diet (34% SM, 34% PC, 17% GPC, 7% FC, 5% Pcho, 3% Lyso-PC); n=8) and Placebo diet (50% PC, 25% FC, 25% GPC; n=8). At birth, the litters were culled to ten pups per dam (5 males and 5 females when possible). Diets were fed *ad libitum* throughout pregnancy and lactation periods. At the end of the pregnancy period two dams (one from the control and one from the placebo diets) died from causes unrelated to our feeding experiment. At the end of the lactation period, two pups from the same dam (1 male and 1 female) were terminated and pooled to represent a measure of the dam since the experimental unit in this study are the dams.

The three experimental diets all contained the same amount of total choline, were isocaloric and isonitrogenous and matched for the macronutrient content (40% fat, 33% carbohydrates and 27% protein) differing primarily in the forms of choline provided. The nutrient composition of the experimental diets is presented in **Table 3-1**. As PC and SM molecules provide fatty acids, the fatty acids composition of the three experimental diets were matched closely using a mixture of

oils (**Table 3-2**). The fat mixture added to the rodent diet was composed of sunflower oil, lard, vegetable oil (soybean oil), corn oil, a high arachidonic acid (AA) oil and a high docosahexaenoic acid (DHA) oil (both AA and DHA oils were provided by DSM (Nutritional Products, Columbia, Maryland, US)). All diets met the essential fatty acid requirements of the rodent and had similar polyunsaturated fatty acid (PUFA)/saturated fatty acid (SFA) and n-6/n-3 ratios. Diets were prepared weekly and stored at 4°C until fed. Feed cups were weighted and replaced every 2-3 days to prevent oxidation. Dietary intake and body weights were monitored regularly throughout the intervention. Offspring were kept with their mothers until termination at three weeks of age (i.e. the end of the suckling/lactation period).

Table 3-1 Composition of experimental diets fed to Sprague-Dawley rat dams during pregnancy and lactation.

Ingredient (g/Kg diet)[1]	Control diet	Placebo diet	Buttermilk diet
Buttermilk powder	0	0	394
Calcium caseinate	0	131	0
Permeate of lactoserum	0	231	0
BiPRO™	0	14	0
Casein	0	135	135
Soy protein	268	0	0
Corn starch	171	171	171
Sucrose	195	0	0
Vitamin Mix (AIN-93-Vx)[2]	19	19	19
Mineral Mix[3]	50	50	50
Calcium Phosphate Dibasic	3.4	3.4	3.4
Inositol	6.3	6.3	6.3
Cellulose	80	80	80
L-cystine	1.8	1.8	1.8
Methionine	2	0	0
Fat Mixture			
Butter oil	0	17	0
Sunflower oil	0	12	0
Vegetable oil (soybean oil)	32	21.3	30
Corn oil	10	0	20
Lard	155	126	107
DHAsco	1.5	1.5	1.5
ARAsco	1.5	1.5	1.5
Choline mixture (providing 1.7g of total choline per kg of diet)			
PL700 mix	0	0	26.6
Soy Lecithin	0	31	0
Choline Bitartrate	4.2	0.6	0
GPC	0	0.6	0

[1]All ingredients were purchased from Harlan Teklad (Indianapolis, IN, USA), with the exception of the dietary oils that were all purchased from Safeway (Edmonton, AB, Canada). The hydrogenated canola oil was donated by Richardson Oilseed Limited (Lethbridge, AB, Canada), ARAsco and DHAsco were provided by DSM (Nutritional Products, Columbia, MD, USA) and PL700 mix was provided by Fonterra (Auckland, New Zeland); [2]AIN-93-VX Vitamin mix (Reeves 1997); [3]Bernhart–Tomarelli salt mixture (Bernhart & Tomarelli 1966). GPC, glycerophosphocholine.

Table 3-2 Fatty acid composition of experimental diets fed to Sprague-Dawley rat dams during pregnancy and lactation.

Fatty Acid[1]	Control Diet	Placebo Diet	Buttermilk Diet
	(% of total fatty acids)		
14:0	1.23 ± 0.03	2.02 ± 0.07	1.37 ± 0.07
16:0	23.1 ± 0.09	23.0 ± 0.49	21.5 ± 0.27
16:1	1.73 ± 0.04	1.69 ± 0.1	1.59 ± 0.02
17:0	0.27 ± 0.01	0.28 ± 0.0	0.24 ± 0.01
18:0	12.9 ± 0.03	11.6 ± 0.68	12.1 ± 0.93
18:1	36.5 ± 0.57	35.4 ± 1.12	34.9 ± 1.03
18:2n-6	22.7 ± 0.46	21.9 ± 0.12	25.8 ± 0.23
18:3n-3 (ALA)	1.65 ± 0.03	1.62 ± 0.02	1.87 ± 0.04
20:4n-6 (AA)	0.35 ± 0.02	0.35 ± 0.02	0.40 ± 0.02
22:6n-3 (DHA)	0.23 ± 0.01	0.27 ± 0.01	0.25 ± 0.03
Total SFA	39.3 ± 0.21	38.6 ± 1.31	36.8 ± 1.29
Total MUFA	38.2 ± 0.61	37.1 ± 1.37	36.5 ± 1.05
Total PUFA	24.9 ± 0.51	24.22 ± 0.15	28.31 ± 0.29
Total n-3	1.89 ± 0.03	1.89 ± 0.03	2.12 ± 0.07
Total n-6	23.0 ± 0.48	22.3 ± 0.12	26.2 ± 0.23
n-6/n-3 ratio	12.2	11.8	12.4
PUFA/SFA ratio	0.63	0.63	0.77

[1]Analysis of the fatty acid composition of the three experimental diets collected weekly (n=3 batches) and pooled; AA, arachidonic acid; ALA, α-linolenic acid; DHA, docosahexaenoic acid; MUFA, monounsaturated fatty acids; n, omega; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

3.2.2 Experimental diets and Placebo formulation

Buttermilk is comprised mainly of casein, whey protein, calcium, lactose and dairy fat containing shorter chain fatty acids. However, buttermilk also contains a significant amount of MFGM rich in choline (especially PC and SM) but also in other phospholipids such as PE (approximatively 30%). In order to study the unique effect of choline forms derived from buttermilk: 1- PL700 mix was used to complete the forms of choline in the buttermilk diet; and 2- a placebo diet was developed to match the nutritional composition of the buttermilk powder (Gay

Lea Foods Co-Operative, Mississauga, ON) in terms of fat, protein and carbohydrates in order to attribute the results obtained to the choline forms in the diet and no other macronutrients.

To meet the AIN-93M requirements for a rodent diet, the maximum amount of buttermilk powder in the buttermilk diet that could be added was 40% wt/wt which was not enough to provide 1.7 g/kg of choline. Therefore, PL700 mix (kindly donated by Fonterra Co-operative Group Limited, Auckland, New Zealand), a phospholipid extract from dairy containing 31.1% PC, 28.5% SM, 1.6% Lyso-PC and 28% PE, was used to match and complete the total amount of choline in the buttermilk diet. To match the nutritional composition of the buttermilk powder, calcium caseinate 380 for casein and calcium (Fonterra, New Zealand), whey permeate for lactose and milk salts (Agropur, Granby, Qc), BiPRO™ for whey proteins (Davisco Foods International Inc, MN, USA) and butter oil (Gay Lea Foods Co-Operative, Mississauga, ON) for short and medium chain fatty acids were used in the formulation of the placebo diet as presented in **Table 3-3**.

The proportion of choline forms in the placebo diet was based on what pregnant women reported consuming as previously described (Lewis, Richard *et al.,* 2017, Lewis, Subhan *et al.,* 2014). Soy lecithin (MJS Biolynx, Brockville, ON), GPC (Santa Cruz Biotechnology, TX, USA), and choline bitartrate (ENVIGO, IN, USA) were used in the placebo diet to provide the PC, GPC and FC choline forms, respectively. The placebo diet did not contain MFGM and other minor lipids coming from milk. The control diet differed from the buttermilk and the placebo diet by containing only FC which is the standard form of choline in rodent diet and vegetable protein (soy protein supplemented with methionine). All three diets contained the same amount of total fat, protein and carbohydrate.

Table 3-3 Nutritional composition of the ingredients used in the formulation of the placebo diet fed to Sprague-Dawley rat dams during pregnancy and lactation[1].

	Calcium caseinate	Whey permeate	BiPRO™	Butter oil	Buttermilk powder
			g/100g of product		
Total Protein	90.3	2.1	94.0	0.0	34.3
Casein	90.3	0.1	0.0	0.0	29.9
Serum protein	0.0	1.9	94.0	0.0	4.2
Lactose	0.1	84.9	0.02	0.0	49.6
Lipid	0.6	0.7	0.5	99.3	4.3
Ash	4.0	6.1	1.6	0.0	8.4
Humidity	3.6	1.4	6.6	0.0	2.7

[1]To match 100g of buttermilk powder, the "placebo" mix contained 33.1g of Calcium caseinate, 58.4g of Permeate of Lactoserum, 3.4g of BiPRO™ and 3.7g of butter oil. Ingredients were purchased as follow: Calcium caseinate 380 (Fonterra, New Zealand), Whey permeate (Agropur, Granby, Qc), Bipro (Davisco Foods International Inc, MN, USA) and butter oil (Gay Lea Foods Co-Operative, Mississauga, ON).

3.2.3 Choline metabolite analyses of offspring stomach content and diet

Dams' experimental diets and stomach contents of pups were analyzed to reflect the choline concentration in the mom's and pup's diet (mainly representative of breast milk) during lactation. Diets and frozen stomach content were extracted using a modified Bligh and Dyer method that has been previously described in (Xiong, Zhao *et al.,* 2012, Zhao, Xiong *et al.,* 2011). Extracts were quantified for all significant choline-containing metabolites and total choline content by hydrophilic interaction chromatography (HILIC) liquid chromatography-tandem mass spectrometry (LC-MS/MS) as previously described (Xiong, Zhao *et al.,* 2012, Zhao, Xiong *et al.,* 2011).

3.2.4 Tissue collection and immune cell isolation

Twenty-one days after parturition, dams and four offspring per dam were weighed and euthanized by CO_2 asphyxiation in the morning hours. From the dams, spleens were collected aseptically, weighed, and immune cells were isolated for further processing. Immune cells were isolated from spleen as previously described (Field, Wu *et al.,* 1990). Prior to *ex vivo* analyses, a haemocytometer was used to count live cells using trypan blue dye exclusion (Sigma-Aldrich) to assess cell viability and was >90% for all treatment groups. All cell suspensions were then diluted to 1.25 x 10^6 cells/ml.

3.2.5 Immune cell phenotype analysis

Immune cell subsets present in freshly isolated splenocytes were identified by direct immunofluorescence assay, as previously described (Field, Thomson *et al.,* 2000). The use of four-color flow cytometry allowed identification of the following combinations of surface molecules in splenocytes: CD28/CD3/CD8/CD4, CD25/CD152/CD8/CD4, CD25/CD127/CD8/CD4, CD27/CD8/CD4, CD27/OX12/OX6/CD45ra, CD71/CD8/CD4, OX12/OX6/CD80, CD86/CD80/CD45RA, CD68/CD284/CD11b/c, OX62/CD25/OX6, CD161/OX62/CD3, IgG/IgM, IgA. All antibodies, with the exception of IgG, IgM and OX6 (BD Biosciences, Mississauga, ON, Canada), were purchased from Cedarlane Laboratories, (Burlington, ON, Canada). After incubation, cells were washed and fixed in paraformaldehyde (10 g/l; Thermo Fisher Scientific) in phosphate-buffered saline. All of the samples were acquired within 72 h of preparation by flow cytometry (FACSCalibur; Becton Dickinson, San Jose, CA, USA) according to the relative fluorescence intensity determined using Kaluza Software (Beckman Coulter, Mississauga, ON, Canada).

3.2.6 *Ex vivo* cytokine production by mitogen-stimulated cells

The measurement of the production of cytokines by mitogen-stimulated cells in spleen has been previously described (Blewett, Gerdung *et al.,* 2009). Briefly, immune cells (1.25 x 10^6 cells/ml) were cultured without mitogen (unstimulated) or with mitogen concanavalin (ConA) (5 µg/ml; MP Biomedicals, Montreal, QC, Canada), a T cell mitogen, lipopolysaccharide (LPS), 10 µg/ml; Sigma-Aldrich, as above), a bacterial component that activates antigen presenting cell populations, ovalbumin (OVA), a food antigen. Concentrations of cytokines interleukin (IL)-1β, IL-2, IL-6, IL-10, tumor-necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) were measured by commercial ELISA kits (R&D Systems, Minneapolis, MN, USA) according to manufacturer's instructions and as previously described (Blewett, Gerdung *et al.,* 2009). The detection limits for all cytokines were 15.6-4000 pg/ml. Cytokine concentrations were quantified using a microplate reader (SpectraMax 190; Molecular Devices, Sunnyvale, CA, USA) and all measurements were conducted in duplicates, with CV <10%.

3.2.7 Statistical analyses

Data are reported as mean ± standard error of the mean (SEM) unless indicated otherwise. The study was powered to assess significant differences in immune function (i.e. *ex vivo* cytokine production as the primary outcome). Data were analyzed using one-way ANOVA in SAS (v9.4, Cary, NC) with diet as the main effect. In cases where a significant main effect of diet was found, post hoc analysis was performed using the DUNCAN adjustment to determine differences between diet groups. Variables that were not normally distributed were log-10 transformed prior to statistical analysis. Differences at $P \leq 0.05$ (two-sided) were considered significant.

3.3 Results

3.3.1 Anthropometric characteristics and daily food intake

Body and organ weights, including those of liver and spleen, did not differ among diet groups (**Table 3-4**). Dams fed the placebo diet had a longer intestine compared to dams fed the control diet. The number of splenocytes was similar among diet groups. There was no difference in food intake among diet groups throughout gestation and lactation.

Table 3-4 Anthropometric data of Sprague-Dawley rat dams fed the three experimental diets.

Variable[1]	Control diet (n=7)	Placebo diet (n=7)	Buttermilk diet (n=8)
Body weight (g)	355 ± 8.75	364 ± 8.97	354 ± 8.21
Spleen weight (g)	0.7 ± 0.02	0.8 ± 0.07	0.9 ± 0.08
Splenocytes, n x 10-^{6}/100 g tissue	13.9 ± 1.93	17.3 ± 2.03	18.9 ± 2.65
Liver weight (g)	18.1 ± 0.99	16.9 ± 0.52	17.8 ± 0.66
Intestine length (cm)	151 ± 5.14^{b}	168 ± 3.28^{a}	162 ± 3.77^{ab}
Food intake (g/day)	42.7 ± 0.95	41.8 ± 0.95	43.3 ± 0.28
Pup stomach content choline, mg/100 g	25.2 ± 1.71	27.9 ± 5.97	27.2 ± 2.24
Total			
Water-soluble[2]	18.8 ± 1.25^{a}	12.7 ± 2.48^{b}	9.56 ± 0.71^{b}
Lipid-soluble[3]	6.38 ± 0.87^{b}	15.2 ± 4.75^{a}	17.7 ± 2.01^{a}

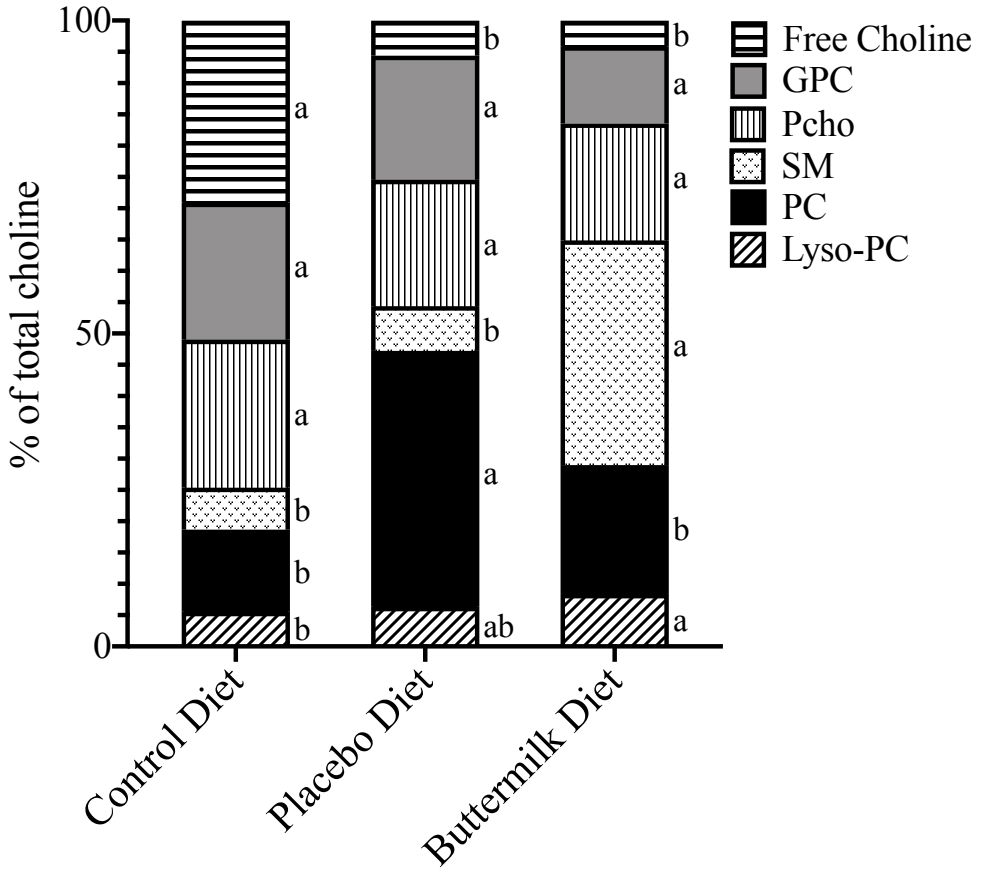
Values are presented as mean ± SEM. Labeled means in a row without a common letter differ, P<0.05.
[2]Free choline, glycerophosphocholine, and phosphocholine.
[3] Lyso-phosphatidylcholine, phosphatidylcholine and sphingomyelin.

3.3.2 Choline metabolites in pups' stomach content

Figure 3-1 represents the distribution of choline forms found in the pups' stomach content (reflective mainly of breast milk). No differences were observed in total choline concentrations in pups' stomach content among diet groups (Table 3-4). Consumption of the buttermilk and the placebo diets led to a lower proportion of FC compared to the control diet in pup's stomach content ($P < 0.01$). Pups from dams that received the buttermilk diet had a higher proportion of SM

compared to both the control and the placebo diets and a higher proportion of Lyso-PC compared to the control diet (both $P < 0.05$). Pups from dams that received the placebo diet had a higher proportion of PC compared to the control diet ($P < 0.05$). In addition, the water-soluble forms of choline (FC, GPC and Pcho) in stomach content were lower in the buttermilk and placebo groups whereas the lipid soluble forms of choline (PC, Lyso-PC and SM) were higher in the buttermilk and placebo groups compared to the control diet (both $p < 0.05$) (Table 3-4).

Figure 3-1 Percent contribution of choline-containing metabolites to total choline in stomach contents of 3-week-old Sprague-Dawley pups of dams fed control, placebo or buttermilk diet during pregnancy and lactation. Values are presented mean ± SEM. N=7-8 pups/group. Labeled means without a common letter differ, $p < 0.05$.



Abbreviations: GPC, glycerophosphocholine; Lyso-PC, lysophosphatidylcholine; PC, phosphatidylcholine; Pcho, phosphocholine; SM, sphingomyelin.

3.3.3 Ex vivo cytokine production by splenocytes after stimulation

Following ConA stimulation (a T cell mitogen), splenocytes from dams fed the buttermilk diet produced significantly more IL-2 and IFN-γ compared to both the control and placebo diets, and more TNF-α compared to the control diet (all $p < 0.05$; **Table 3-5**). There were no significant differences between the control and the placebo diets. After LPS stimulation (a bacterial challenge), splenocytes from dams fed the buttermilk and the placebo diets had a higher production

of IL-10 compared to the control diet ($p < 0.05$). The buttermilk diet also led to a higher production of IFN-γ by LPS-stimulated splenocytes compared to splenoctyes from the control diet ($p < 0.05$). There were no significant differences in IL-1β, TNF-α and IL-6 production among diets after LPS stimulation. Following OVA stimulation (a food antigen), dams fed the buttermilk and the placebo diets had a higher production of IL-10 compared to the control diet ($p < 0.05$). There was also a lower production of IL-6 by OVA-stimulated splenocytes from dams fed the placebo diet compared to the control diet ($p < 0.05$). The production of IL-2, IFN-γ and TNF-α did not differ among diet groups.

Table 3-5 *Ex vivo* cytokine production by mitogen-stimulated splenocytes of Sprague-Dawley rat dams fed the three experimental diets.

Variable[1]	Control diet (n=7)	Placebo diet (n=7)	Buttermilk diet (n=8)
ConA (T cell mitogen)			
IL-2 (pg/ml)*	9680 ± 1180[b]	11500 ± 1040[b]	12100 ± 2290[a]
IL-10 (pg/ml)	1720 ± 316	1340 ± 107	1720 ± 269
TNF-α (pg/ml)*	233 ± 25.5[b]	315 ± 28.8[ab]	421 ± 54.9[a]
IFN-γ (pg/ml)	1140 ± 153[b]	1070 ± 115[b]	1910 ± 336[a]
IL-6 (pg/ml)	299 ± 54.8	197 ± 18.1	344 ± 63.6
LPS (Bacterial challenge)			
IL-1β (pg/ml)	144 ± 12.2	143 ± 18.4	167 ± 17
IL-10 (pg/ml)	753 ± 124[b]	1590 ± 286[a]	1410 ± 181[a]
TNF-α (pg/ml)	301 ± 58.3	232 ± 75.9	243 ± 21.7
IFN-γ (pg/ml)*	1040 ± 172[b]	1280 ± 242[ab]	2140 ± 520[a]
IL-6 (pg/ml)	463 ± 61.1	412 ± 47.4	413 ± 35.6
OVA (Food antigen)			
IL-2 (pg/ml)	33.1 ± 4.83	35.7 ± 5.40	36.9 ± 3.44
IL-10 (pg/ml)	554 ± 93.1[b]	1190 ± 254[a]	1170 ± 86.8[a]
TNF-α (pg/ml)	226 ± 45.3	184 ± 37.7	219 ± 24.9
IFN-γ (pg/ml)*	1150 ± 213	1050 ± 216	1510 ± 343
IL-6 (pg/ml)	210 ± 45.2[a]	123 ± 14.0[b]	191 ± 17.4[ab]

[1]Values are presented as mean ± SEM; IFN-γ, interferon gamma; IL, interleukin; TNF-α, tumor necrosis factor alpha; Labeled means in a row without a common letter differ, $p < 0.05$.
*Analysis performed on log-transformed values.

3.3.4 Splenocyte phenotypes

Immune cell phenotypes from dams fed the control, buttermilk and placebo diets are presented in **Table 3-6**. Dams fed the buttermilk diet had a lower proportion of T cells (CD3+) which was mainly attributable to a lower proportion of helper T cell (CD3+CD4+) compared to the control diet (both $p < 0.05$). Splenocytes from dams fed the buttermilk diet had a higher proportion of CD71+ (transferrin receptor) compared to the control diet ($p < 0.05$) which was mainly imputable to the cytotoxic T cell subset (% of CD8+ cell also expressing CD71+). The proportion of cytotoxic T cell expressing CD71+ was significantly lower in dams fed the placebo

diet compared with the buttermilk diet. Dams fed the placebo diet also had a higher proportion of the subset of cytotoxic T cells (CD3+CD8+) and cytotoxic T cells expressing CD152+ (cytotoxic T lymphocyte associated protein 4 (CTLA-4)) compared to the control diet ($p < 0.05$). There were no significantly differences in antigen presenting cell populations including total B cells (Total CD45RA+), activated B cells (CD45RA+CD86+), macrophages (Total CD68+ and CD11b/c), dendritic cells (OX6+OX62+) and NK cell (CD3-CD161+) among diet groups
(all $p > 0.05$).

Table 3-6 Splenocyte phenotype of Sprague-Dawley rat dams fed the three experimental diets.

Phenotype[1]	Control diet (n=7)	Placebo diet (n=7)	Buttermilk diet (n=8)
		% of gated cells	
Total CD3+ (T cell)*	45.7 ± 2.62[a]	44.8 ± 1.66[a]	39.1 ± 1.08[b]
CD3+CD4+ (helper T cell)*	27.7 ± 2.13[a]	26.2 ± 1.49[ab]	22.7 ± 0.60[b]
CD3+CD8+ (cytotoxic T cell)	16.5 ± 1.0[a]	17.9 ± 0.72[ab]	15.1 ± 0.86[b]
Total CD25+ (IL-2 receptor)	6.47 ± 0.34	6.33 ± 0.31	6.44 ± 0.32
Total CD71+ (Transferrin receptor)	19.3 ± 1.25[b]	21.9 ± 1.75[ab]	23.3 ± 2.17[a]
% of CD4+ expressing CD71+	23.8 ± 1.42	26.7 ± 1.79	25.8 ± 2.58
% of CD8+ expressing CD71+	15.6 ± 1.43[ab]	13.5 ± 0.92[b]	20.9 ± 2.53[a]
Total CD152+*	2.81 ± 0.40	3.66 ± 0.67	4.32 ± 0.78
% of CD4+ expressing CD152+*	5.12 ± 1.07	6.71 ± 0.53	7.18 ± 0.99
% of CD8+ expressing CD152+	6.10 ± 0.56[b]	9.20 ± 1.85[a]	7.97 ± 0.79[ab]
Total CD45RA+ (B cell)	38.2 ± 2.54	36.5 ± 1.83	40.5 ± 1.56
CD45RA+CD86+*	7.64 ± 0.68	9.24 ± 1.06	8.12 ± 0.40
Total CD68+ (macrophages)	14.6 ± 1.31	15.1 ± 2.60	13.3 ± 1.42
CD68+CD284+	10.2 ± 0.84	7.71 ± 0.63	8.87 ± 0.60
OX6+OX62+ (dendritic cell)*	5.07 ± 0.64	4.52 ± 0.63	3.74 ± 0.32
CD3-CD161+ (NK cell)	7.76 ± 0.91	6.54 ± 0.51	6.57 ± 0.56

[1]Values are presented as mean ± SEM; Labeled means in a row without a common letter differ, $P<0.05$. No significant differences were observed in CD4+CD25+ (mean 5.4 ± 0.5 %, n=22), CD8+CD25+ (mean 2.1 ± 0.3 %, n=22), Total CD27 (mean 48.9 ± 1.7 %, n=22), CD4+CD27+ (mean 25.5 ± 1.3 %, n=22), CD8+CD27+ (mean 17.6 ± 0.9 %, n=22), Total CD28 (mean 53.7 ± 2.4 %, n=22), CD4+CD28+ (mean 26.7 ± 1.6 %, n=22), CD8+CD28+ (mean 15.9 ± 1.2%, n=22), CD45RA+CD27+ (mean 29.3 ± 1.2 %, n=22), Total IgG^+ (mean 13.9 ± 1.1 %, n=22), Total IgM^+ (mean 11.4 ± 1.2 %, n=22) and Total IgA^+ (mean 10.8 ± 1.5 %, n=22).
*Analysis performed on log-transformed values.

3.4 Discussion

In this study, we investigated the effect of feeding whole buttermilk powder and its unique composition of choline metabolites (34% SM and 34% PC), on the immune system of lactating dams and its effect of the distribution of choline forms in breastmilk. We developed a "placebo" to match the nutritional composition of the buttermilk powder, but differed in the composition of choline forms, as well as other minor components (i.e. gangliosides, proteins, other lipids). Our data partly supports our initial hypotheses in that feeding buttermilk conferred greater immune benefit to the T cell population but not on antigen presenting cells population when compared to the placebo diet. However, both the buttermilk and the placebo diets enhanced the ability of antigen presenting cells to produce the regulatory cytokine IL-10 compared to standard rodent diet containing only FC.

We first demonstrated that feeding the same amount of total choline (1.7 g/kg diet) in the maternal diet did not significantly alter the total amount of choline in pups' stomach content (mainly representative of breastmilk). These results are consistent with our previous studies where we showed that providing the same amount of total choline in the maternal diet, regardless of the forms of choline, does not affect the total amount of choline found in breastmilk (Dellschaft, Richard *et al.,* 2017, Dellschaft, Ruth *et al.,* 2015, Lewis, Richard *et al.,* 2016, Richard, Lewis *et al.,* 2017). Also consistent with our previous studies, the results indicate that providing different forms of choline in the maternal diet modulates the distribution of choline forms in breastmilk (Lewis, Richard *et al.,* 2016, Richard, Lewis *et al.,* 2017). In the current study, we showed that feeding a diet containing buttermilk (high in SM and PC) during pregnancy and lactation led to a higher proportion of SM and Lyso-PC and lowered the proportion of FC in breastmilk compared to the standard rodent diet. The higher proportion of Lyso-PC in that group could be explained by

the action of phospholipase A in the mammary gland, which is the enzyme responsible for conversion of PC into Lyso-PC (Smoczynski 2017). Feeding the placebo diet, mainly composed of PC, led to a higher proportion of PC and Lyso-PC while reducing the proportion of FC compared with the control diet. In addition, the contribution of water and lipid metabolites of choline in the breastmilk was also affected by the diet. The buttermilk and placebo diets had higher and lower concentrations of lipid (PC, Lyso-PC, SM) and water (FC, GPC, Pcho) soluble forms of choline, respectively, compared with the control diet.

We demonstrated that feeding buttermilk in the maternal diet, providing high amounts of SM and PC, enhanced T cell function in lactating dams to a greater extent than the placebo diet devoid of SM. This suggest that dietary SM in particular, and/or specific bioactive peptides found in buttermilk specifically affect T cell function. T cell function was measured by the ability of splenocytes to produce IL-2, an important cytokine in T cell signaling and as a T cell growth factor plays a crucial role in modulating T cell differentiation and proliferation (Malek 2008). After ConA stimulation (T cell mitogen), IL-2 production was higher in the buttermilk group. In addition, there was also an overall higher Th1 response (both IFN-γ and TNF-α) in buttermilk-fed dams. IFN-γ is a key Th1 cytokine involved in the regulation of important immunological processes including the upregulation of major histocompatibility (MHC) class I and class II molecules expression on the cell surface and the induction of IL-2 production for proliferation which together can help mounting an appropriate response against viral and bacterial infections (Boehm, Klamp *et al.,* 1997, Kasahara, Hooks *et al.,* 1983, Schroder, Hertzog *et al.,* 2004). TNF-α is another crucial Th1 cytokine involved in several pro-inflammatory responses and it regulates the transcriptional factor NF-κB (Bradley 2008). Through the activation of NF-κB, TNF-α induces the expression of genes

involves in the regulation of inflammation, cell survival, proliferation and differentiation (Hayden & Ghosh 2014) which could also explain partly the higher IL-2 production after ConA stimulation.

T cell function was improved in buttermilk-fed dams compared to both the placebo and the control diets despite having a slightly lower proportion of total T cells (CD3+ cell). Buttermilk-fed dams had a higher proportion of cytotoxic T cells (CD8+) expressing the transferrin receptor (CD71+) which could explain partly the enhanced T cell response. CD71 is a receptor responsible for iron uptake and early activation of immune cells (Reddy, Eirikis *et al.,* 2004). Since cytotoxic T cells are mainly involved in recognizing intracellular viruses this might suggest a better immune response in case of a viral infection. Dietary SM is digested in the intestine and hydrolyzed to ceramide and sphingosine, which is further phosphorylated to sphingosine-1-phosphate (S1P). Both S1P and ceramides play important roles in the immune system (Maceyka & Spiegel 2014); S1P is involved in immune cell trafficking and development, whereas ceramides are involved in the regulation of TCR expression and cytokine production (Adam, Heinrich *et al.,* 2002, Ballou, Laulederkind *et al.,* 1996, Olesch, Ringel *et al.,* 2017). Although the most abundant phospholipids in buttermilk are PC and SM there are other minor components within the MFGM, such as gangliosides and cerebrosides, that have shown potential in modulating either negatively or positively T cell responses (Norris & Blesso 2017). Nevertheless, in our study, the consumption of whole buttermilk led to a beneficial effect on T cell function, meaning that the forms of choline in combination with sphingolipids and other minor components present in the MFGM had a positive effect on T cell responses.

We have previously reported some beneficial effects of feeding diets high in PC on maternal T cell function (Dellschaft, Richard *et al.,* 2017, Lewis, Richard *et al.,* 2016, Lewis, Richard *et al.,* 2017) whereas in the current study the placebo diet (containing 50% PC) did not

affect T cell response as compared to the control standard rodent diet. IL-2 and IFN-γ production were higher after ConA stimulation in dams that were fed a diet containing 100% PC vs. a control diet containing 100% FC (Lewis, Richard *et al.,* 2016). In another experiment, we showed that feeding a mixture of choline form (50% PC, 25% FC, 25% GPC) led to a more efficient T cell response after ConA stimulation by maintaining the IL-2 production while producing less Th1 cytokine (IFN-γ) that is known to increase IL-2 secretion (Lewis, Richard *et al.,* 2017). In the current study, although the placebo diet led to a trend toward a higher IL-2 and TNF-α production after ConA stimulation, it did not reach statistical significance. These unexpected results can be explained partly by the fact that dams fed the placebo diet had a higher proportion of cytotoxic T cells (CD8+) expressing the CTLA-4 receptor (CD152+) which inhibits CD28, the costimulatory molecule required for T cell activation and proliferation (Krummel & Allison 1995).

Following a bacterial challenge (LPS), dams that were fed the buttermilk and the placebo diets had an overall anti-inflammatory response by producing more IL-10. IL-10 is able to inhibit the pro-inflammatory response produced by the recognition of LPS by the pattern recognition receptors (PRRs) and therefore plays an important role in the resolution phase of inflammation (Fiorentino, Zlotnik *et al.,* 1991, Gerard, Bruyns *et al.,* 1993, Grutz 2005). Failing to produce this anti-inflammatory cytokine can result in an exagerated inflammatory response and subsequent tissue damage (Saraiva & O'Garra 2010). IL-10 is produced by a variety of cell types including macrophages, dendritic cells, B cells and T cells. Interestingly, we did not observe any significant differences in the proportions of monocytes (total CD68+), activated macrophages (CD68+CD284+), activated B cells (CD45RA+CD86+) and dendritic cells (OX6+OX62+). This suggests that lipid soluble forms of choline (i.e. PC and SM) might modulate intracellular signaling pathway in APCs leading to IL-10 production independently of the proportion and presence of

activation markers on the surface of APCs. The higher production of IFN-γ in buttermilk-fed dams after LPS stimulation also suggests that the response to a bacterial challenge was not attenuated but actually improved despite the higher IL-10 production. This is important since IL-10 downregulates the transcription of NF-κB, inhibiting the production of pro-inflammatory cytokines (Schottelius, Mayo *et al.,* 1999). Altogether, these results suggest that the lipid soluble forms of choline not only enhance the response of antigen presenting cells to LPS but could promote the resolution of inflammation.

Oral tolerance refers to the ability to suppress local and systemic immune responses to an antigen that is not harmful for the host (Garside & Mowat 2001). In response to a dietary antigen (OVA), the main allergic protein in eggs, we demonstrated that feeding both the buttermilk and the placebo diets to lactating dams led to a higher production of IL-10 and a lower production of IL-6 by splenocytes (although this only reached statistical significance in the placebo group). IL-10 plays an important role in oral tolerance and in preventing allergic reaction (Battaglia, Gianfrani *et al.,* 2004, Li & Flavell 2008) through downregulation of the pro-inflammatory signaling pathway, which inhibits IL-2 production and immune cell proliferation. IL-6 is a cytokine that can exert a dual role with either Th1 or Th2 properties. Th2 cytokine especially IL-13 favors a humoral response that can lead to an allergic reaction (Prescott & Dunstan 2007). Reduction in IL-6 production could be partly responsible for the higher IL-10 production which has been shown to downregulate IL-13 (Koya, Matsuda *et al.,* 2007). In this study, we did not observe any effect of the choline forms on the proportion of IgA^+, IgG^+ and IgM^+ cells in spleen. However, measurement of plasma OVA-specific IgG and IgE concentrations following OVA tolerization *in vivo* is required to confirm the effect of choline forms on humoral immune response, and this should be investigated in future studies. Overall, our data suggest that providing a higher proportion of lipid

soluble forms of choline in the maternal diet favors an anti-inflammatory response against a food antigen, which may in turn prevent food allergies.

3.5 Conclusion

Our results suggest that the proportion of choline forms in the maternal diet is able to modulate maternal immune function and breastmilk choline composition. Buttermilk is a rich source of dietary choline (PC and SM) which had a beneficial effect on T cell function and cytokine production during pregnancy and lactation. Moreover, providing a mixture of choline forms containing predominantly lipid-soluble forms (i.e. SM and PC in both the buttermilk and the placebo diets) to lactating dams enhanced the production of the regulatory cytokine IL-10 by immune cells, after both a bacterial challenge and stimulation with a dietary antigen, which is crucial for the resolution of inflammation. However, buttermilk also contains high amounts of MFGM which has a particular structure containing other minor components (i.e. PE, gangliosides, and bioactive peptides) besides choline that might also have a beneficial impact on the immune system and therefore further investigations are warranty. Overall, our results suggest that buttermilk could be an important source of choline during pregnancy and lactation.

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Chapter 4: Buttermilk: an important source of lipid-soluble forms of choline that influences the immune system development in Sprague-Dawley rat offspring.

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4.1 Introduction

In the early postnatal period, the immune system has a reduced ability to respond to infections and lacks immunological memory, a feature of the acquired immune system, which increases their vulnerability to infection (Basha, Surendran *et al.,* 2014). The lactation and weaning periods are critical stages for the appropriate development of the immune system. The development of the immune system can be influenced by the environment but also by the components of the diet (breastmilk, food) (Blumer, Pfefferle *et al.,* 2007). An important process that normally occurs during infancy is the establishment of oral tolerance (Peters, Dang *et al.,* 2016). Oral tolerance is the ability to suppress immune responses to orally administrated proteins that are not considered harmful to the host and it is characterized by a decrease in IgE response, T cell proliferation and the production of certain cytokines (Pabst & Mowat 2012). Failing to develop oral tolerance early in life could result in the development of allergic diseases that are characterized

by a predominantly T helper 2 (Th2) response (main cytokines involved are IL-4, IL-5 and IL-13) over a T helper 1 (Th1) response (Prescott 2003, Yaqoob & Calder 2011). Of note, at birth the immune system has a lower capacity to produce Th1 cytokines and maturation during infancy is associated with an increased proficiency to produce IFN-γ and TNF-α.

Choline is an essential nutrient present in all cells. It is important for the synthesis of phospholipids, such as phosphatidylcholine (PC and sphingomyelin (SM), that are present in the cell membrane and also play important roles in cell signaling (Hollenbeck 2012). During pregnancy and lactation, a considerable amount of choline is transferred from the placenta to the fetus to support brain and spinal cord development, which increases its demand substantially (Zeisel 2006). Furthermore, choline is also needed for optimal maternal and immune system development (Dellschaft, Ruth *et al.,* 2015). Choline can be obtained endogenously via the phosphatidylethanolamine methyltransferase pathway and also through the diet (Bremer J & Greenberg 1961, Zhu, Mar *et al.,* 2004). Choline is present in a wide variety of foods as water soluble forms (free choline (FC), glycerophosphocholine (GPC), phosphocholine (Pcho)) and lipid soluble forms (PC, SM) (Zeisel 2006). A recent study from our group showed that feeding a mixture of choline forms (50% PC, 25% FC, 25% GPC) during lactation and early development had a positive impact on immune system development in Sprague-Dawley rat pups (Richard, Lewis *et al.,* 2017). Nevertheless, studies suggest that pregnant and lactating women in North America are not meeting daily choline recommendations which stresses the importance of finding alternative dietary sources to increases the daily intake of choline in this population (Lewis, Subhan *et al.,* 2014).

Buttermilk is a dairy by-product originated from the churning of cream into butter. Its overall composition is similar to that of skim milk except that it contains a higher proportion of milk fat globule membrane (MFGM) consisting of a natural assembly of bioactive proteins and minor lipids (Conway, Gauthier *et al.,* 2014, Vanderghem, Bodson *et al.,* 2010). The lipid portion of MFGM is primarily composed of neutral lipids (70%; triglycerides, diglycerides, monoglycerides, cholesterol esters, and free cholesterol) and phospholipids (30%; phosphatidylethanolamine (31.1-42.0%), PC (19.2-34.5%) and SM (17.9-34.5%)) (Conway, Gauthier *et al.,* 2014, Lopez 2018). Gangliosides and free fatty acids can also be found within the MFGM but in minor amounts. Anti-inflammatory properties have been attributed to certain components of the MFGM, more specifically, SM and gangliosides have shown potential in lowering the production of pro-inflammatory cytokines in rodents (Norris, Porter *et al.,* 2017, Park, Suh *et al.,* 2007). In addition, we have recently demonstrated that feeding whole buttermilk during pregnancy and lactation beneficially modulated T cell responses in lactating dams (Azarcoya-Barrera, Goruk *et al.,* 2020). The unique lipid content high in PC and SM, compared to skim milk, makes buttermilk a valuable source of lipid soluble forms of choline.

The objective of this study was to determine the immunological impact of feeding whole buttermilk during lactation and weaning periods on the immune system development in rat pups. We hypothesized that due to the high content of lipid soluble forms of choline (PC and SM) present in the MFGM, feeding buttermilk during lactation and weaning periods would have a beneficial impact on the development of the immune system in rat pups.

4.2 Materials and Methods

4.2.1 Ethical statement

All animal care and experimental protocols were conducted in accordance with the Canadian Council on Animal Care guidelines and approved by the University of Alberta Animal Ethics Committee.

4.2.2 Animals and Diets

Pregnant primiparous female Sprague-Dawley rats (n=24) were obtained from Charles River Laboratories (Montreal, Quebec, Canada) at 7-10 days of gestation and were individually housed in a temperature and humidity controlled environment with a 12/12-h reversed light cycle. Dams were fed standard rat chow (Lab diet 5001; PMI Nutrition International, Brentwood, MO, USA) prior to the start of the study (i.e. until midway during the pregnancy period). At an estimated 10 days of gestation, dams were randomized to one of three experimental diets all containing 1.7g of choline/kg of diet: Control diet (100% FC; n=8), Buttermilk diet (BM; 34% SM, 34% PC, 17% GPC, 7% FC, 5% Pcho, 3% Lyso-PC); n=8) and Placebo diet (PB; 50% PC, 25% FC, 25% GPC; n=8). At birth, the litters were culled to ten pups per dam (5 males and 5 females when possible). Diets were fed *ad libitum* throughout pregnancy and lactation periods. Before the end of the pregnancy period, two dams (one from the control and one from the placebo diet groups) died from causes unrelated to the feeding experiment. Offspring were kept with their mothers for the duration of the suckling period (21 days). At 3 weeks, two pups from the same dam (1 male and 1 female) were terminated and pooled to represent a measure of the dam since the experimental unit in this study are the dams. At the end of the suckling period, two pups from each dam (1 male and 1 female) remained in the study and continued consuming the same diet as their mother for an

additional seven weeks until adulthood was reached. At 10 weeks of age, the two pups from the same dam were terminated and tissues were collected and pooled.

The nutrient composition of the experimental diets has been previously described (Azarcoya-Barrera, Goruk *et al.,* 2020). Briefly, the three experimental diets all contained 1.7 g/kg of total choline, were isocaloric and isonitrogenous and matched for the macronutrient content differing primarily in the forms of choline provided: 1-Control diet (100% FC), 2-Buttermilk diet (34 PC, 34% SM, 17% GPC, 7% FC, 5% Pcho), or 3-Placebo diet (50% PC, 25% FC, 25% GPC). The placebo formulation matched the nutritional composition of the buttermilk powder in terms of fat, protein and carbohydratesto attribute the possible benefits to the choline forms within the diet and not to other nutrients. The placebo diet did not contain MFGM and other minor lipids coming from milk, and it also differed on the choline composition which was based on what pregnant women reported consuming in a previous study (Azarcoya-Barrera, Goruk *et al.,* 2020). The three diets contained 40% fat, 33% carbohydrates and 27% protein. As PC and SM molecules provide fatty acids, the fatty acid composition of the three experimental diets was matched closely using a mixture of oil as previously published (Azarcoya-Barrera, Goruk *et al.,* 2020). Details about the experimental diets and the placebo formulation have been published in (Azarcoya-Barrera, Goruk *et al.,* 2020) and are presented in **Table 4-1** and **Table 4-2**. All diets met the essential fatty acid requirements of the rodent and had similar polyunsaturated fatty acid (PUFA)/saturated fatty acid (SFA) and n-6/n-3 ratios. Diets were prepared weekly and stored at 4°C until fed. Feed cups were weighed and replaced every 2-3 days to prevent oxidation. Dietary intake and body weights were monitored regularly throughout the intervention.

Table 4-1 Composition of experimental diets.

Ingredient (g/Kg diet)[1]	Control diet	PB diet	BM diet
Buttermilk powder	0	0	394
Calcium caseinate	0	131	0
Permeate of lactoserum	0	231	0
BiPRO™	0	14	0
Casein	0	135	135
Soy protein	268	0	0
Corn starch	171	171	171
Sucrose	195	0	0
Vitamin Mix (AIN-93-Vx)[2]	19	19	19
Mineral Mix[3]	50	50	50
Calcium Phosphate Dibasic	3.4	3.4	3.4
Inositol	6.3	6.3	6.3
Cellulose	80	80	80
L-cystine	1.8	1.8	1.8
Methionine	2	0	0
Fat Mixture			
Butter oil	0	17	0
Sunflower oil	0	12	0
Vegetable oil (soybean oil)	32	21.3	30
Corn oil	10	0	20
Lard	155	126	107
DHAsco	1.5	1.5	1.5
ARAsco	1.5	1.5	1.5
Choline mixture (providing 1.7g of total choline per kg of diet)			
PL700 mix	0	0	26.6
Soy Lecithin	0	31	0
Choline Bitartrate	4.2	0.6	0
GPC	0	0.6	0

[1]All ingredients were purchased from Harlan Teklad (Indianapolis, IN, USA), with the exception of the dietary oils that were all purchased from Safeway (Edmonton, AB, Canada). The hydrogenated canola oil was donated by Richardson Oilseed Limited (Lethbridge, AB, Canada), ARAsco and DHAsco were provided by DSM (Nutritional Products, Columbia, MD, USA) and PL700 mix was provided by Fonterra (Auckland, New Zeland); [2]AIN-93-VX Vitamin mix [1]; [3]Bernhart–Tomarelli salt mixture [2]. BM, buttermilk; GPC, glycerophosphocholine, PB, placebo.

Table 4-2 Nutritional composition of the ingredients used in the formulation of the placebo diet[1].

	Calcium caseinate	Whey permeate	BiPRO™	Butter oil	Buttermilk powder
			g/100g of product		
Total Protein	90.3	2.1	94.0	0.0	34.3
Casein	90.3	0.1	0.0	0.0	29.9
Serum protein	0.0	1.9	94.0	0.0	4.2
Lactose	0.1	84.9	0.02	0.0	49.6
Lipid	0.6	0.7	0.5	99.3	4.3
Ash	4.0	6.1	1.6	0.0	8.4
Humidity	3.6	1.4	6.6	0.0	2.7

[1]To match 100g of buttermilk powder, the "placebo" mix contained 33.1g of Calcium caseinate, 58.4g of Permeate of Lactoserum, 3.4g of BiPRO™ and 3.7g of butter oil. Ingredients were purchased as follow: Calcium caseinate 380 (Fonterra, New Zealand), Whey permeate (Agropur, Granby, Qc), Bipro (Davisco Foods International Inc, MN, USA) and butter oil (Gay Lea Foods Co-Operative, Mississauga, ON).

4.2.3 Tissue collection and immune cell isolation

At 3 and 10 weeks, pups were weighed and euthanized by CO_2 asphyxiation. Spleens were collected aseptically, weighed, and immune cells were isolated for further processing. Isolation of immune cells from the spleen has been previously described (Field, Wu *et al.,* 1990). Briefly, single cell suspensions were obtained by disrupting tissue through a nylon mesh screen in sterile Krebs-Ringer HEPES buffer with bovine serum albumin (5 g/l; Sigma-Aldrich Canada Ltd., Oakville, ON, Canada). Ammonium chloride lysis buffer (155 mM NH4Cl, 0.1 mM EDTA, 10mM KHCO3; Fisher Scientific, Edmonton, AB, Canada) was used to lyse erythrocytes. Cells were washed then re-suspended in complete culture medium (RPMI 1640 media; Life Technologies, Burlington, ON, Canada), supplemented with 5% (v/v) heat-inactivated fetal calf serum, 25 mM HEPES, 2.5 mM 2-mercaptoethanol and 1% antibiotic/antimycotic (pH 7.4; Fisher Scientific).

Prior to *ex vivo* analyses, a haemocytometer was used to count live cells using trypan blue dye exclusion (Sigma-Aldrich) to assess cell viability and was >90% for all treatment groups. All cell suspensions were then diluted to 1.25 x 10^6 cells/ml.

4.2.4 Immune cell phenotype analysis

Immune cell subsets present in freshly isolated splenocytes were identified by direct immunofluorescence assay, as previously described (Field, Thomson *et al.,* 2000). The use of four-color flow cytometry allowed identification of the following combinations of surface molecules in splenocytes: CD28/CD3/CD8/CD4, CD25/CD152/CD8/CD4, CD25/CD127/CD8/CD4, CD27/CD8/CD4, CD27/OX12/OX6/CD45RA, CD71/CD8/CD4, OX12/OX6/CD80, CD86/CD80/CD45RA, CD68/CD284/CD11b/c, OX62/CD25/OX6, CD161/OX62/CD3, CD3/CD4/CD25/FoxP3, IgG/IgM, IgA. All antibodies with the exception of IgG, IgM and OX6 (BD Biosciences, Mississauga, ON, Canada) were purchased from Cedarlane Laboratories, (Burlington, ON, Canada). After incubation, cells were washed and fixed in paraformaldehyde (10 g/l; Thermo Fisher Scientific) in phosphate-buffered saline. All of the samples were acquired within 72 h of preparation by flow cytometry (FACS Calibur; Becton Dickinson, San Jose, CA, USA) and analyzed according to their relative fluorescence intensity using Kaluza Software (Beckman Coulter, Mississauga, ON, Canada).

4.2.5 Ex vivo cytokine production by mitogen-stimulated cells

The measurement of the production of cytokines by mitogen-stimulated cells in spleen has been previously described (Blewett, Gerdung *et al*., 2009). Briefly, cells (1.25 x 10^6 cells/ml) were cultured in 3 ml RMPI-1640 medium (as above) for 48 h at 37ºC and 5% CO_2 without mitogen

(unstimulated) or with one of the following immune stimulators: concanavalin (ConA; 5 µg/ml; MP Biomedical), a T cell mitogen; lipopolysaccharide (LPS; 10 µg/ml; Sigma-Aldrich), a bacterial component that activates antigen presenting cells (APC); or ovalbumin (OVA), a food antigen. After incubation, cells were centrifuged for 10 minutes at 1000 rpm and supernatants were collected and stored at -80°C. The concentrations of cytokines interleukin (IL)-1β, IL-2, IL-6, IL-10, tumor-necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) were measured in the supernatants by commercial ELISA kits according to manufacturer's instructions, as previously described [22]. The detection limits for all cytokines were 15.6-4000 pg/ml (R&D Systems, Minneapolis, MN, USA). For the detection of TGF- β, LEGEND MAX™ Total TGF-β1 ELISA Kit was used following the manufacturer's instructions (BioLegend, San Diego, CA). Cytokine concentrations were quantified using a microplate reader (SpectraMax 190; Molecular Devices, Sunnyvale, CA, USA) and all measurements were conducted in duplicates, with CV <10%. IL-1β was only measured in the supernatant of LPS stimulated cells.

4.2.6 Statistical analyses

Data are reported as mean ± standard error of the mean (SEM) unless indicated otherwise. The study was powered to assess significant changes in immune function (i.e. *ex vivo* cytokine production as the primary outcome). Data were analyzed using one-way ANOVA in SAS (v9.4, Cary, NC) with diet as the main effect. In cases where a significant main effect of diet was found, post hoc analysis was performed using the DUNCAN adjustment to determine differences between diet groups. Variables that were not normally distributed were log-10 transformed prior to statistical analysis. Differences at $p \leq 0.05$ (two-sided) were considered significant.

4.3 Results

4.3.1 Growth Parameters and Food Intake

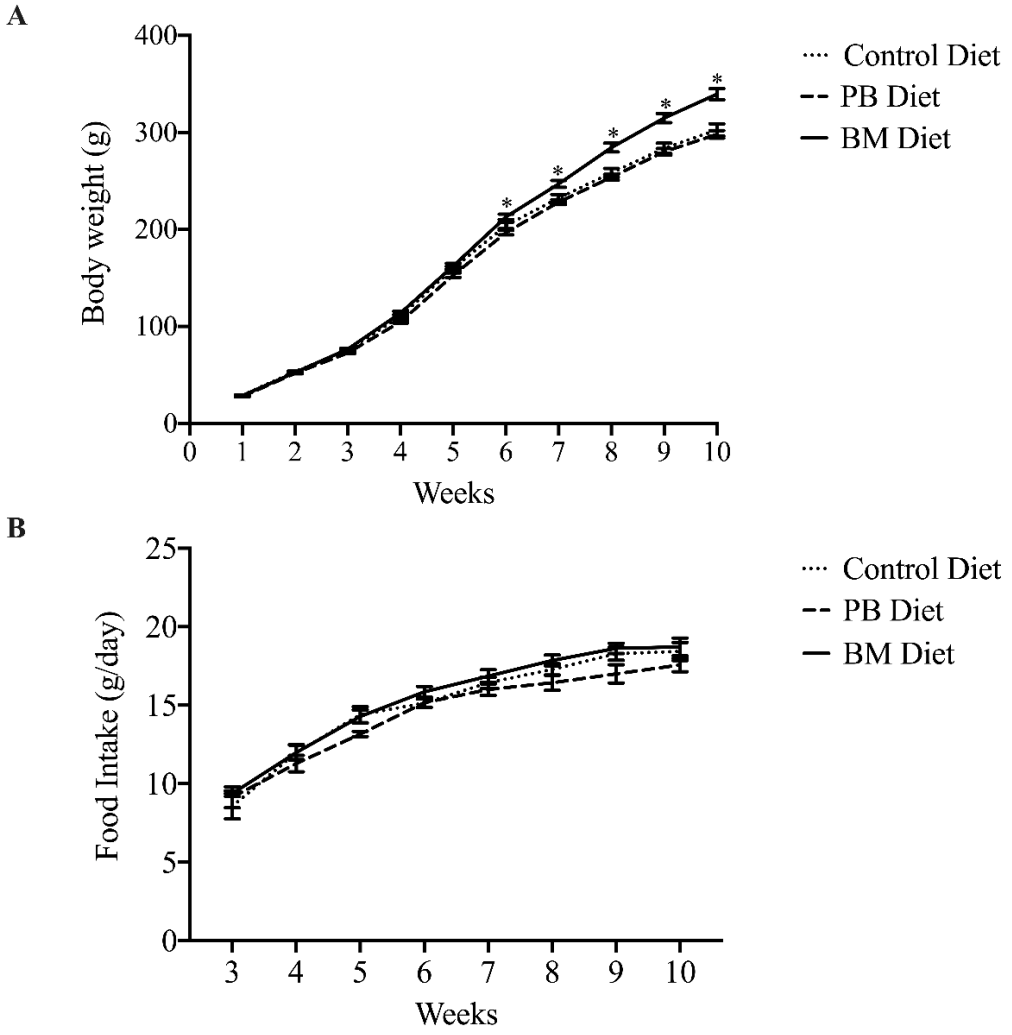
As previously reported (Azarcoya-Barrera, Goruk *et al.,* 2020), at the end of the lactation period there was no significant difference in the dams' body weight (mean 357.5±8.6 g) or food intake (mean 42.6 ± 0.6 g/day) amongst dietary groups. As presented in **Table 4-3**, there were no significant differences in organ weights or intestinal length among groups in both 3-week and 10-week-old pups (all $p > 0.05$). Starting from week 6 of age, pups fed the BM diet had a higher body weight compared to both the control and placebo diets (all $p < 0.05$, **Figure 4-1A**). Daily food intake of pups between weeks 3 to 10 was unaltered by dietary treatment (**Figure 4-1B**).

Table 4-3 Anthropometric data of 3- and 10-week-old Sprague-Dawley rat pups.

Variable[1]	Control diet (n=7)	PB diet (n=7)	BM diet (n=8)	*p* Value
		3-week-old pups		
Spleen weight (g)	0.38 ± 0.01	0.34 ± 0.01	0.35 ± 0.02	0.168
Spleen weight/BW (%)	0.52 ± 0.01	0.51 ± 0.02	0.51 ± 0.02	0.781
Splenocytes/g spleen (x10^6)	18.7 ± 0.55	17.8 ± 1.06	16.5 ± 1.11	0.301
Liver weight (g)	2.9 ± 0.15	2.6 ± 0.17	2.6 ± 0.17	0.221
Liver weight/BW (%)	4.11 ± 0.17	3.91 ± 0.14	3.74 ± 0.15	0.285
Intestine length (cm)	77.6 ± 1.77	74.1 ± 2.33	73.3 ± 1.49	0.236
		10-week-old pups		
Spleen weight (g)	0.79 ± 0.04	0.79 ± 0.04	0.84 ± 0.05	0.641
Spleen weight/BW (%)	0.25 ± 0.01	0.33 ± 0.01	0.39 ± 0.01	0.712
Splenocytes/g spleen (x10^6)	18.6 ± 1.37	16.7 ± 1.27	16.5 ± 1.06	0.449
Liver weight (g)	13.2 ± 0.25	12.7 ± 0.45	13.5 ± 0.27	0.255
Liver weight/BW (%)	4.11 ± 0.08	4.21 ± 0.14	4.08 ± 0.06	0.663
Intestine length (cm)	128.7 ± 2.15	130.0 ± 0.85	131.1 ± 1.61	0.593

[1]Values are presented as mean ± SEM. BM, buttermilk; BW, body weight; PB, placebo; Groups that do not share the same letter are significantly different based on the one-way ANOVA test with the Duncan adjustment for multiple comparisons test ($p < 0.05$).

Figure 4-1 Sprague-Dawley pups' body weight (A) and food intake (B) during the suckling and weaning periods. Values are presented as mean ± SEM. * means significantly different ($p < 0.05$) from both the control and placebo diets based on the one-way ANOVA test with the Duncan adjustment for multiple comparisons performed at each time point.



Abbreviations: BM, buttermilk; PB, placebo.

4.3.2 *Ex Vivo* Cytokine Production by Stimulated Splenocytes

Ex vivo cytokine production by splenocytes stimulated with ConA, LPS or OVA isolated from 3-weeks old pups is presented in **Table 4-4**. After ConA stimulation, the BM and PB diets led to a higher production of TNF-α and IFN-γ by splenocytes in 3-week-old pups compared to

the control diet (both $p < 0.05$), while no change was observed in IL-2, IL-6 and IL-10 production between groups. After LPS stimulation, splenocytes from pups from dams fed the BM diet produced more IL-1β and IL-6 compared to both the placebo and control diets (both $p < 0.05$). Compared to the control diet, LPS-stimulated splenocytes from pups from dams fed the BM and PB diets had a higher production of IL-10 and TNF-α (both $p < 0.05$). When stimulated with OVA, splenocytes from pups from dams fed the BM diet produced less TNF-α compared with the control diet only ($p < 0.05$). No change in IL-2 production was observed among groups.

Table 4-4 *Ex vivo* cytokine production by mitogen-stimulated splenocytes of 3-week-old pups from Sprague-Dawley dams fed the three experimental diets.

Variable[1]	Control diet (n=7)	PB diet (n=7)	BM diet (n=8)	*p* Value
ConA (T cell mitogen)				
IL-2 (pg/ml)	5081 ± 660	6492 ± 1410	7997 ± 1303	0.241
IL-10 (pg/ml)	101 ± 14.2	164 ± 25.6	134 ± 16.6	0.100
TNF-α (pg/ml)	71 ± 5.42^{b}	116 ± 8.97^{a}	110 ± 9.49^{a}	0.002
IFN-γ (pg/ml)	577 ± 123^{b}	1772 ± 166^{a}	1336 ± 202^{a}	0.005
IL-6 (pg/ml)*	280 ± 21.8	357 ± 39.9	339 ± 25.7	0.181
LPS (Bacterial challenge)				
IL-1β (pg/ml)	161 ± 17.2^{b}	215 ± 20.7^{b}	296 ± 27.1^{a}	0.002
IL-10 (pg/ml)	141 ± 7.93^{b}	260 ± 8.96^{a}	230 ± 19.2^{a}	<.001
TNF-α (pg/ml)	221 ± 17.7^{b}	361 ± 14.4^{a}	402 ± 24.8^{a}	<.001
IFN-γ (pg/ml)*	243 ± 61.5	361 ± 72.6	484 ± 176	0.483
IL-6 (pg/ml)	767 ± 43.3^{b}	885 ± 44.4^{b}	1157 ± 80.4^{a}	0.007
OVA (food antigen)				
IL-2 (pg/ml)	9.35 ± 1.01	10.2 ± 1.14	9.63 ± 0.63	0.825
IL-10 (pg/ml)	172 ± 10.5	154 ± 13.9	161 ± 17.3	0.731
TNF-α (pg/ml)	308 ± 17.1^{a}	279 ± 12.6^{ab}	238 ± 15.9^{b}	0.015
IFN-γ (pg/ml)	358 ± 83.7	287 ± 161	356 ± 93.8	0.887
IL-6 (pg/ml)	122 ± 18.9	112 ± 15.9	101 ± 15.2	0.669
TGF-β (pg/ml)*	1032 ± 56.5	927 ± 45.1	875 ± 72.6	0.245

[1]Values are presented as mean ± SEM; BM, buttermilk; IFN-γ, interferon gamma; IL, interleukin; PB, placebo; TNF-α, tumor necrosis factor alpha; Groups that do not share the same letter are significantly different based on the one-way ANOVA test with the Duncan adjustment for multiple comparisons test ($p < 0.05$).
*Analysis performed on log-transformed values.

Ex vivo cytokine production by splenocytes isolated from 10-week-old pups is presented in **Table 4-5**. Following ConA stimulation, splenocytes from BM- and PB-pups had a higher production of IL-2 along with IFN-γ and IL-6 compared with the control diet ($p < 0.05$). Splenocytes from BM-pups also produced more TNF-α compared with both the control and PB diets ($p < 0.05$). ConA-stimulated splenocytes from PB-pups produced more IL-10 compared with the control ($p < 0.05$), but not with the BM diet. Following LPS stimulation, BM- and PB- pups

had a higher production of IL-10 by splenocytes compared to the control diet ($p < 0.05$). There was also a higher production of IFN-γ by splenocytes from PB fed pups compared to both the BM and control diets ($p < 0.05$). There was no significant change in IL-1β, TNF-α and IL-6 production by LPS-stimulated splenocytes. After stimulation with OVA, splenocytes from 10-week-old pups fed the BM and PB diets produced less TNF-α compared with the control diet (both $p < 0.05$), while no change in IL-2 production or any other cytokines was observed among groups.

Table 4-5 *Ex vivo* cytokine production by mitogen-stimulated splenocytes of 10-week-old Sprague-Dawley rats fed the three experimental diets.

Variable[1]	Control diet (n=7)	PB diet (n=7)	BM diet (n=8)	*p* Value
ConA (T cell mitogen)				
IL-2 (pg/ml)	4914 ± 400[b]	7228 ± 486[a]	7098 ± 216[a]	0.004
IL-10 (pg/ml)	146 ± 11.9[b]	207 ± 15.2[a]	184 ± 14.4[ab]	0.025
TNF-α (pg/ml)	145 ± 9.67[b]	165 ± 20.3[b]	212 ± 12.8[a]	0.012
IFN-γ (pg/ml)	413 ± 44.1[b]	757 ± 69.1[a]	792 ± 73.9[a]	0.001
IL-6 (pg/ml)	136 ± 9.03[b]	283 ± 25.1[a]	273 ± 21.7[a]	0.001
LPS (Bacterial challenge)				
IL-1β (pg/ml)	120 ± 11.5	98 ± 12.8	128 ± 11.2	0.205
IL-10 (pg/ml)	137 ± 4.34[b]	235 ± 7.40[a]	256 ± 34.5[a]	0.003
TNF-α (pg/ml)	156 ± 11.8	157 ± 10.9	196 ± 18.9	0.116
IFN-γ (pg/ml)	399 ± 44.5[b]	586 ± 42.5[a]	312 ± 44.9[b]	0.001
IL-6 (pg/ml)	366 ± 43.6	422 ± 32.6	423 ± 23.8	0.408
OVA (Food antigen)				
IL-2 (pg/ml)	36.3 ± 6.04	39.4 ± 6.34	37.4 ± 5.29	0.931
IL-10 (pg/ml)	147 ± 26.4	128 ± 11.9	169 ± 18.1	0.334
TNF-α (pg/ml)*	258 ± 23.4[a]	178 ± 12.2[b]	182 ± 13.1[b]	0.004
IFN-γ (pg/ml)*	26.8 ± 3.63	28.6 ± 12.2	24.9 ± 5.85	0.782
IL-6 (pg/ml)	143 ± 17.1	126 ± 11.8	121 ± 11.2	0.496
TGF-β (pg/ml)	1148 ± 53.5	1151 ± 43.9	1194 ± 42.4	0.726

[1]Values are presented as mean ± SEM; BM, buttermilk; IFN-γ, interferon gamma; IL, interleukin; PB, placebo; TNF-α, tumor necrosis factor alpha; Groups that do not share the same letter are significantly different based on the one-way ANOVA test with the Duncan adjustment for multiple comparisons test ($p < 0.05$).
*Analysis performed on log-transformed values.

4.3.3 Splenocyte Immune Cell Phenotypes

As presented in **Table 4-6**, at 3 weeks of age, there was no change in the proportion of total T cells (CD3+) among groups. BM-pups had a higher proportion of cytotoxic T cells (CD3+CD8+) when compared to the control diet ($p < 0.05$). Pups from dams fed the BM or PB diet had a higher proportion of total cells expressing the co-stimulatory molecule (CD28+), CD86+ (CD28 ligand) and CD27+ (memory marker) (all $p < 0.05$). There was also a higher proportion of cytotoxic T

cells expressing CD27+ (CD8+CD27+) in the BM group compared to the control diet. Feeding dams the PB diet led to a higher proportion of macrophages (CD11b/c+), B cells (CD45RA+, OX12+) and activated B cell (CD45RA+ CD86+) in 3-week-old pups compared to both the BM and control diets, respectively (both $p < 0.05$) while no changes were observed in the proportion of CD45RA+CD80+ cells. There were no significant differences in the population of dendritic cells (DC; OX6+OX62+), natural killer cells (NK; CD3-CD161+), regulatory T cells (CD3+CD4+CD25+FoxP3+), CD68+ cells (monocytes and macrophages), OX62+ cells, or IgA^+, IgM^+ or IgG^+ cells among groups.

Table 4-6 Splenocyte phenotype of 3-week-old pups from Sprague-Dawley dams fed the three experimental diets.

Phenotype[1]	**Control diet (n=7)**	**PB diet (n=7)**	**BM diet (n=8)**	***p* Value**
	% of gated cells			
Total CD3+ (T cells)	20.9 ± 0.79	21.5 ± 0.75	19.0 ± 1.19	0.191
CD3+CD4+ (helper T cells)*	10.6 ± 0.37	10.8 ± 0.38	10.4 ± 0.64	0.800
CD3+CD8+ (cytotoxic T cells)	6.20 ± 0.51^{b}	7.27 ± 0.29^{ab}	8.17 ± 0.55^{a}	0.026
CD4+CD25+FoxP3+ (Treg**)**	3.09 ± 0.18	2.82 ± 0.15	3.31 ± 0.22	0.818
Total CD27+	23.5 ± 0.35^{b}	25.9 ± 0.75^{a}	26.5 ± 0.19^{a}	0.005
CD4+CD27+	8.63 ± 0.17	9.37 ± 0.39	9.04 ± 0.37	0.334
CD8+CD27	6.19 ± 0.25^{b}	7.32 ± 0.29^{a}	7.06 ± 0.28^{a}	0.027
Total CD28+ (Co-stimulatory molecule)	29.2 ± 0.34^{b}	30.9 ± 0.54^{a}	31.1 ± 0.32^{a}	0.005
CD4+CD28+	11.4 ± 0.23	11.7 ± 0.44	11.8 ± 0.11	0.641
CD8+CD28+	6.14 ± 0.43	7.12 ± 0.31	7.17 ± 0.43	0.155
Total CD71+	24.7 ± 1.25	21.8 ± 0.67	24.4 ± 1.12	0.135
CD4+CD71+	4.99 ± 0.21	5.02 ± 0.29	5.71 ± 0.24	0.089
CD8+CD71+	1.11 ± 0.12	1.25 ± 0.14	1.43 ± 0.14	0.263
Total CD86+	19.1 ± 0.44^{b}	20.3 ± 0.41^{a}	20.4 ± 0.24^{a}	0.038
Total CD80+	18.0 ± 0.75	18.6 ± 0.39	19.6 ± 0.41	0.109
Total CD45RA+ (B cells)	38.7 ± 1.01^{b}	41.7 ± 0.72^{a}	38.6 ± 1.05^{b}	0.052
CD45RA+CD86+	9.89 ± 0.63^{b}	11.9 ± 0.37^{a}	10.3 ± 0.57^{b}	0.034
OX12+ (Igκ chain)	18.7 ± 0.46^{b}	21.1 ± 0.38^{a}	19.9 ± 0.38^{b}	0.003
Total CD11b/c+ (macrophages)	8.34 ± 0.45^{b}	10.6 ± 0.63^{a}	9.68 ± 0.29^{ab}	0.012
Total OX6+ (MHC class II+)	31.6 ± 0.96^{b}	34.3 ± 0.58^{a}	32.6 ± 0.69^{ab}	0.065
OX62+OX6+ (dendritic cells)	5.67 ± 0.28	6.38 ± 0.39	5.85 ± 0.18	0.236

[1]Values are presented as mean ± SEM; BM, buttermilk; PB, placebo; Treg, T regulatory cell; Groups that do not share the same letter are significantly different based on the one-way ANOVA test with the Duncan adjustment for multiple comparisons test ($p < 0.05$). No significant differences were observed in CD3-CD161+ (average 3.0 ± 0.5), Total CD68+ (average 14.3 ± 0.34), Total OX62+[d] (average 15.3 ± 1.34), CD45RA+CD80+ (average 4.75 ± 0.26), OX12+CD80+ (average 5.42 ± 0.27), Total IgA^{+} (average 11.57 ± 0.3), Total IgM^{+} (average 43.3 ± 4.6) and Total IgG^{+} (average 11.3 ± 0.6).
*Analysis performed on log-transformed values.

At 10 weeks of age, there was no change in the proportion of total T cells, helper T cells (CD4+CD3+) or cytotoxic T cells (**Table 4-7**). BM- and PB-pups still had a higher proportion of cytotoxic T cells expressing CD27+ (memory marker, $p < 0.05$) compared with the control diet.

10-week-old pups fed the BM diet had a higher proportion of total cells expressing CD86+ and CD71+ (transferrin receptor) which was mainly attributable to the subset of helper T cells (CD4+CD71+) compared to the control diet (all, $p < 0.05$). PB-pups had a lower proportion of B cells (CD45RA+) compared to both the BM and control diets ($p < 0.05$), while no change was observed in the DC population (OX62+OX6+), macrophages (CD11bc+), NK cells (CD161+CD3-), regulatory T cells (CD3+CD4+CD25+FoxP3+), CD68+ cells (monocytes and macrophages), OX62+ cells or IgA^+, IgM^+ or IgG^+ cells amongst groups ($p > 0.05$).

Table 4-7 Splenocyte phenotype of 10-week-old Sprague-Dawley rats fed the three experimental diets.

Phenotype[1]	**Control diet (n=7)**	**PB diet (n=7)**	**BM diet (n=8)**	***p* Value**
	% of gated cells			
Total CD3+ (T cells)	45.7 ± 0.68	47.7 ± 0.78	46.2 ± 0.83	0.201
CD3+CD4+ (helper T cells)*	22.2 ± 0.45	23.6 ± 0.84	22.7 ± 0.47	0.295
CD3+CD8+ (cytotoxic T cells)	22.0 ± 0.84	22.7 ± 0.71	22.7 ± 0.98	0.795
CD4+CD25+FoxP3+ (Treg)	3.15 ± 0.21	3.25 ± 0.25	3.01 ± 0.21	0.908
Total CD27+	34.6 ± 1.46^{b}	38.9 ± 0.67^{a}	36.8 ± 0.34^{ab}	0.016
CD4+CD27+	15.5 ± 0.46	17.4 ± 0.89	16.2 ± 0.43	0.139
CD8+CD27+	12.9 ± 0.22^{b}	14.9 ± 0.78^{a}	14.9 ± 0.55^{a}	0.039
Total CD28+ (Co-stimulatory molecule)	40.9 ± 1.17	42.4 ± 1.21	42.3 ± 0.88	0.540
CD4+CD28+	20.9 ± 0.61	22.3 ± 0.98	21.7 ± 0.64	0.453
CD8+CD28+	16.8 ± 1.59	15.8 ± 0.74	18.0 ± 1.45	0.506
Total CD71+	16.4 ± 0.64^{b}	17.7 ± 0.61^{ab}	18.3 ± 0.28^{a}	0.049
CD4+CD71+	3.77 ± 0.19^{b}	3.65 ± 0.21^{b}	4.32 ± 0.18^{a}	0.178
CD8+CD71+	3.48 ± 0.19	3.89 ± 0.36	4.32 ± 0.33	0.034
Total CD86+	18.1 ± 0.62^{b}	18.1 ± 0.65^{b}	20.2 ± 0.64^{a}	0.041
Total CD80+	8.54 ± 0.42	8.45 ± 0.37	9.21 ± 0.58	0.473
Total CD45RA+ (B cells)	37.7 ± 1.34^{a}	31.2 ± 1.96^{b}	35.9 ± 1.09^{a}	0.018
CD45RA+CD86+	9.02 ± 0.47	8.63 ± 0.59	10.0 ± 0.35	0.114
OX12+ (Igκ chain)	21.1 ± 1.19	19.1 ± 1.01	21.9 ± 0.65	0.109
Total CD11b/c+ (macrophages)*	4.16 ± 1.12	4.81 ± 1.57	5.13 ± 1.41	0.877
Total OX6+ (MHC class II+)	38.2 ± 1.15	35.7 ± 0.85	38.4 ± 0.97	0.127
OX62+OX6+ (dendritic cells)	6.07 ± 0.58	6.48 ± 0.50	7.03 ± 0.7	0.525

[1]Values are presented as mean ± SEM; BM, buttermilk; PB, placebo; Treg, T regulatory cell; Groups that do not share the same letter are significantly different based on the one-way ANOVA test with the Duncan adjustment for multiple comparisons test ($p < 0.05$). No significant differences were observed in CD3-CD161+ (average 4.5 ± 0.2), Total CD68 (average 11.5 ± 0.83), Total OX62+ (average 13.3 ± 0.57), CD45RA+CD80+ (2.63 ± 0.46), OX12+CD80+ (average 4.63 ± 0.31), Total IgA^{+} (average 11.9 ± 1.1), Total IgM^{+} (average 41.6 ± 2.2) and Total IgG^{+} (average 15.1 ± 0.6).

*Analysis performed on log-transformed values.

4.4 Discussion

In this study, we aim to investigate the role of dietary choline metabolites supplied by buttermilk on the immune system's development early in life. We also designed a placebo diet to match the nutritional composition of the buttermilk diet except for the different forms of choline, which allowed us to assess more specifically the effects of SM on the immune system development. Both the BM and the PB diets contained higher proportions of lipid soluble forms of choline when compared to the control diet (containing only FC), the standard form of choline in rodents' diet (Reeves, Nielsen *et al.,* 1993). Overall, we demonstrated that feeding both the BM and PB diets enhanced immune function in suckled pups while the buttermilk diet had little additional effect on the development of the immune system compared to the PB diet. Similarly, feeding both the BM and PB diets from weaning to adulthood improved immune function.

First, we demonstrated that feeding dams the BM and PB diets had no effect on pups' growth parameters at 3 weeks of age. Previously, we demonstrated that feeding different mixtures of choline forms, similar to that in the PB diet (50% PC, 25%FC, 25% GPC) and one high in GPC (High GPC diet: 75% GPC, 12.5% PC, 12.5% FC) to lactating dams had a beneficial impact on offspring's growth (Richard, Lewis *et al.,* 2017). Although the placebo diet had the same distribution of choline forms as the Mixed Choline diet from our previous study, it differs in macronutrient and fatty acids composition since it was designed to match the buttermilk powder (Azarcoya-Barrera, Goruk *et al.,* 2020, Richard, Lewis *et al.,* 2017). Moreover, feeding lactating dams both the Mixed Choline and High GPC diets increased the proportion of GPC in breastmilk by 10% and 22%, respectively (Richard, Lewis *et al.,* 2017). GPC intake has been shown to increase plasma growth hormone levels which could play an important role in promoting growth during the suckling period (Kawamura, Okubo *et al.,*). While no change was observed in the

proportion of GPC in breastmilk in our current study, we showed that feeding the BM diet directly to weaned pups enhanced growth parameters starting from week 6 of age. Buttermilk is a rich source of MFGM, which is high in phospholipids including SM and PC. It has been demonstrated that supplementation of lipids extracted from MFGM during lactation and weaning period enhanced offspring growth in Wistar rats (Vickers, Guan *et al.,* 2009).

4.4.1 Three week old suckled pups

At birth, the immune system is considered "immature" as it is lacking the immunological memory that is acquired as the infant is exposed to different antigens in life making neonates more susceptible to infections (Simon, Hollander *et al.,* 2015). Moreover, the ability to mount an efficient Th1 response which includes the production of IFN-γ and TNF-α to combat infection is lower at birth compared to adults (Adkins, Bu *et al.,* 2001, Wilson, Westall *et al.,* 1986). We have demonstrated that suckled pups from dams fed the buttermilk and the placebo diets had a higher production of Th1 cytokines (IFN-γ and TNF-α) after T cell stimulation. Both IFN-γ and TNF-α play an important role as mediators of inflammatory responses after infection (Billiau 1996, Bradley 2008). IFN-γ contributes to the clearance of pathogens by upregulating antigen presentation, activating macrophages and DCs, attracting leukocytes to the site of infection and inhibiting pathogen replication (Schroder, Hertzog *et al.,* 2004). TNF-α regulates cellular proliferation and differentiation, leukocyte migration and blood coagulation (Baud & Karin 2001). In addition, we found that pups from the BM and PB groups had a higher population of cytotoxic T cells (CD3+CD8+) expressing a memory marker (CD8+CD27+) while also having a higher proportion of total cells in the spleen expressing CD28+, the co-stimulatory molecule required for T cell activation, and CD86+, the CD28 ligand. We had previously demonstrated that feeding

lactating dams the BM or the PB diet increased the proportion of SM and PC in breastmilk, with a reciprocal reduction in FC. Altogether, the pattern of cytokines produced (higher Th1 response) along with a higher expression of memory and activation markers on T cells suggest that providing a higher proportion of lipid soluble forms of choline (both PC and SM) to pups during suckling is consistent with the maturation of T cell population.

After LPS stimulation, splenocytes from pups from both the BM and PB groups had a higher production of TNF-α while pups in the BM group were also producing more IL1-β and IL-6. The recognition of LPS by the toll-like receptor 4 (TLR-4) can trigger both the innate and adaptive immune system, leading to the activation of the NF-κB pathway which induces the production of pro-inflammatory cytokines (Sweet & Hume 1996). TNF-α, IL1-β and IL-6 can act directly on the hypothalamus, muscle and fat cells leading to an increase in body temperature which decreases bacterial replication (Goldsby, Kindt *et al.,* 2003). Furthermore, they increase the production of antibodies which help the host orchestrate an adequate immune response. On the other hand, splenocytes from suckled pups from the BM and PB groups were producing more IL-10 after LPS stimulation. Although an adequate inflammatory response is crucial for the clearance of pathogens, an exaggerated response can result in tissue damage and complications and therefore the resolution phase of inflammation is as important (Cicchese, Evans *et al.,* 2018). IL-10 is a potent anti-inflammatory cytokine involved in the resolution phase by inhibiting the production of TNF-α, IL1-β and IL-6 (Couper, Blount *et al.,* 2008). Monocytes/macrophages, B cells and DCs are the cells that are mainly activated upon LPS stimulation. The proportion of activated B cells (CD45RA+CD86+) and macrophages (CD11b/c+) was only higher in the PB group while the response of immune cells in the BM group was slightly higher as measured by higher production of IL-1β and IL-6 after LPS stimulation. It therefore appears that APC of suckled pups from the

BM group have a greater ability to respond to a bacterial challenge which might be attributable to the higher SM content in breastmilk of BM-fed dams.

4.4.2 Ten week old pups

Feeding the BM and PB diets from weaning to adulthood further improved T cell response to ConA at 10 weeks of age with an increased production of IL-2, a T cell growth marker. This could be partially explained by the consistent higher production of major Th1 cytokines, including IFN-γ and TNF-α (although TNF-α only reached statistical significance in the BM group), known to stimulate IL-2 production (Kasahara, Hooks *et al.,* 1983). Consistent with results at 3 weeks of age, feeding the BM and PB diets was still associated with a higher proportion of cytotoxic T cells expressing CD27+ (memory marker) later in life, which suggests the possibility of a more efficient response to a pathogen if a second exposure occurs (Dutton, Bradley *et al.,* 1998). However, at 10 weeks of age, pups from the PB group were no longer expressing a greater proportion of activation markers (CD28+ and CD86+) while pups in the BM group had a higher proportion of cytotoxic T cells expressing CD71+ (transferrin receptor) and total cells expressing CD86 (CD28 ligand). CD71+ is responsible for early activation of immune cells (Reddy, Eirikis *et al.,* 2004) and could partially explain the improved T cell response in the buttermilk group (higher TNF-α production). On the other hand, feeding the BM and PB diets during weaning also improved the ability of T cells to produce a Th2 response by splenocytes by increasing IL-10 (although only reach statistical significance in the placebo group) and IL-6 production. IL-6 can act both as a Th1 or Th2 cytokine depending on the type of stimulation (Gabay 2006). Nevertheless, both IL-10 and IL-6 play an important role in the resolution phase of inflammation by inhibiting the secretion of pro-inflammatory Th1 cytokines (Fiorentino, Zlotnik *et al.,* 1991, Xing, Gauldie *et al.,* 1998). Overall,

our results suggest that although both the buttermilk and placebo diets improved T cell response to a mitogen, the buttermilk diet was associated with a slightly better Th1 response while the placebo diet favored a better Th2 response.

Following LPS stimulation, there was an overall anti-inflammatory response with a higher production of IL-10 by splenocytes in pups that received both the BM and PB diets. This is also consistent with our previous study where feeding a mixture of choline forms (50% PC, 25% GPC and 25% FC) also led to a higher IL-10 production by splenocytes stimulated with LPS in 10-week old pups (Richard, Lewis *et al.,* 2017). At 10 weeks, pups from the PB group had a higher production of IFN-γ by splenocytes when compared to both the control and the BM diets. The expression of IFN-γ can be mediated by the interaction of the T cell receptor (TCR) and CD28 with co-stimulatory molecules such as CD86 (Walker, Aste-Amezaga *et al.,* 1999). However, only pups from the BM group had a higher proportion of CD86+, in addition to a higher proportion of CD45RA+ cells. Overall, it appears that APC of pups from the PB group initiate a better immune response upon bacterial infection, while both the BM and PB group produced a similar Th2 response by increasing IL-10 production.

4.4.3 Response to a dietary antigen (ovalbumin)

We also investigated the effect of feeding different forms of choline on ex vivo markers of oral tolerance to a common food antigen (OVA in egg). In the current study, splenocytes were ex vivo stimulated with OVA at both 3 and 10 weeks of age. At 3 weeks, splenocytes of pups from the BM group had a significantly lower production of TNF-α, whereas at 10 weeks of age, feeding both the BM and the PB diet led to a lower production of TNF-α when compared to the control diet. When a food protein causes a hypersensitive reaction, T cells, macrophages and DCs can

increase the production of TNF-α and presumably contribute in orchestrating an allergic response (Ahmad, Azid *et al.,* 2018). Tregs play a key physiological role in preventing an inappropriate inflammatory response to dietary antigens by producing cytokines known to inhibit IL-2 production (IL-10 and TGF-β) and T cell differentiation of into Th2 thereby preventing an inappropriate humoral response (Wawrzyniak, O'Mahony *et al.,* 2017). In the current study, the proportion of Treg and the production of IL-10 and TGF-β after OVA stimulation was similar among group. Of note, since we did not use an allergic prone model, the three groups had a similarly low *ex vivo* IL-2 production (T cell proliferation marker) in response to OVA and therefore no exaggerated response to OVA was observed in any group. Overall, our findings suggest that both the PB and BM diets, which are high in lipid soluble forms of choline (SM and PC), potentially modulated the systemic immune response to a food antigen (lower TNF-α) in the direction that is believed to be beneficial for the establishment of oral tolerance.

Limitation pertaining to the current study include the lack of measurement of specific *in vivo* B cells function such as plasma levels of immunoglobulins. Further investigation that includes the measurement of Ova-IgG and Ova-IgE in plasma in orally tolerized pups after exposure to OVA is warranted in order to confirm the effect of feeding different choline forms early in life on the development of oral tolerance. In addition, the gut-associated immune system including the mesenteric lymph nodes and Peyer's Patches, plays a major role in the establishment of oral tolerance and therefore future studies should investigate the effect of choline forms on the gut-associated lymphoid tissue (GALT) (Wang, Sherman *et al.,* 2013). Several factors/mechanisms involved in the development of oral tolerance such as the mucosal microbiota and local secretory IgA response, should be assessed to better understand the effect of choline forms on systemic immunological responses (Cebra JJ, Jiang HQ *et al.,* 2005, Wambre & Jeong 2018).

4.5 Conclusion

In summary, the effect of feeding a mixture of choline forms high in lipid soluble forms (BM and PB diets), primarily modulated T cell function. At both 3 and 10 weeks of age, we found that splenocytes were better at mounting immune responses to a T cell mitogen (ConA) leading to a higher Th1 response over a Th2 response. Furthermore, both the PB and BM diets enhanced the production of the cytokine IL-10 after LPS stimulation, suggesting that there was an anti-inflammatory response after a bacterial challenge. Although both the BM and PB diets produced similar immune benefits, we demonstrated that feeding BM enhanced pups' growth starting from week 6 of age. Overall, data from this and our previous studies provide strong evidence that the lipid soluble forms of choline are important for the development of the immune system and that buttermilk is a rich source of not only choline but also lipid soluble forms of choline. Therefore, the proportion of lipid soluble forms of choline (PC and SM) in the maternal diet and infant formulas should be considered for the development of the immune system early in life.

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Chapter 5: The lipid-soluble forms of choline enhance *ex vivo* responses from the gut-associated immune system in young female rat offspring.

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5.1 Introduction

The gastrointestinal tract is constantly exposed to the external environment, dietary antigens, and potential pathogens. Intestinal homeostasis is maintained by the physical, chemical, and immune barriers which provide protection against pathogens but also provide tolerance towards dietary antigens and commensal bacterial (Huffnagle & Noverr 2008). The immune and tolerogenic responses in the gut are mainly orchestrated by the gut-associated lymphoid tissue (GALT). The GALT is the largest lymphoid tissue of the body and consists of organized lymphoid follicles, including the Peyer's patches (PP) and the mesenteric lymph nodes (MLN) (Koboziev, Karlsson *et al.,* 2010). The GALT is primarily inhabited by B and T cells (helper and cytotoxic). Helper T cells can be further subdivided depending on the cytokines they produce into T helper (Th) 1 (IL-12, IFN-γ and TNF-α), Th2 (IL-4, IL-5, IL-10) and Th17 (IL-17) (Yaqoob & Calder 2011).

In humans, the first years of life are an important period of rapid development for the GALT, which is considered "immature" and is highly influenced by environmental factors and diet (Blumer, Pfefferle *et al.,* 2007). The development of food allergies commonly occurs during the first two years of childhood due to a failure in developing oral tolerance to a specific dietary antigen (Tokuhara, Kurashima *et al.,* 2019). Oral tolerance is a state of immune unresponsiveness to dietary antigens and commensal bacteria (Tordesillas & Berin 2018). The mechanism by which oral tolerance is induced involves the deletion of activated T cells, decreased T cell proliferation, induction of regulatory T cells (Treg), and increased production of anti-inflammatory cytokines (IL-10) (Pabst & Mowat 2012).

Choline was officially recognized as an essential nutrient in 1998 by the Institute of Medicine (Institute of Medicine 1998). Choline has numerous roles in the body including structural integrity of cell membranes, lipoprotein formation and secretion, neurotransmission, cell signaling and optimal immune function (Hollenbeck 2012, Zeisel & da Costa 2009). Choline is particularly important during the pre- and post-natal periods where the demands of choline increase substantially to maintain maternal stores and meet the demands of the fetus/infant. Choline is found in the diet in a wide variety of animal and plant-based products as free choline (FC) or in one of the other choline moieties. Choline forms can be divided into two groups depending on their absorption. The water-soluble forms include FC, glycerophosphocholine (GPC) and phosphocholine (Pcho), and the lipid-soluble forms are phosphatidylcholine (PC and sphingomyelin (SM) (Lewis, Field *et al.,* 2015, Zeisel, Mar *et al.,* 2003).

Our group is interested in understanding the importance of the different forms of choline for the development of the immune system early in life (Azarcoya-Barrera, Field *et al.,* 2021, Azarcoya-Barrera, Goruk *et al.,* 2020, Dellschaft, Richard *et al.,* 2017, Lewis, Richard *et al.,* 2016,

Lewis, Richard *et al.,* 2017, Richard, Lewis *et al.,* 2017). First, we demonstrated that feeding 100% PC vs. 100% FC (comparing lipid- vs water-soluble forms of choline), as the dietary source of choline, improved peripheral T cell function in 3 week old offspring (Lewis, Richard *et al.,* 2016). Since no human diet only contains one form of choline, the next experiments were designed to provide a mixture of choline forms that is more representative of human consumption according to epidemiological cohort studies with a distribution of 50% PC, 25% GPC and 25% FC (Lewis, Subhan *et al.,* 2014). Moreover, to better understand the effect of specific choline forms on the development of the immune system, we also developed unique experimental diets that were high in GPC or SM.

Overall, evidence from our group suggests that diets containing a mixture of choline forms are of more benefit for the maturation of the peripheral immune system compared to a diet containing only water-soluble forms of choline (Azarcoya-Barrera, Field *et al.,* 2021, Lewis, Richard *et al.,* 2016, Lewis, Richard *et al.,* 2017). However, no studies have yet assessed the impact of the different choline forms on the development of the gut-associated immune system. Therefore, the objective of this study was to determine the impact of the forms of dietary choline during the lactation and weaning periods on the development of the GALT. We hypothesized that 1) the choline forms in the diet will differentially affect the development of the GALT, and 2) a diet higher in lipid-soluble forms of choline will beneficially impact the development of the GALT compared to a diet higher in water-soluble forms.

5.2 Materials and Methods

5.2.1 Animal handling and diets

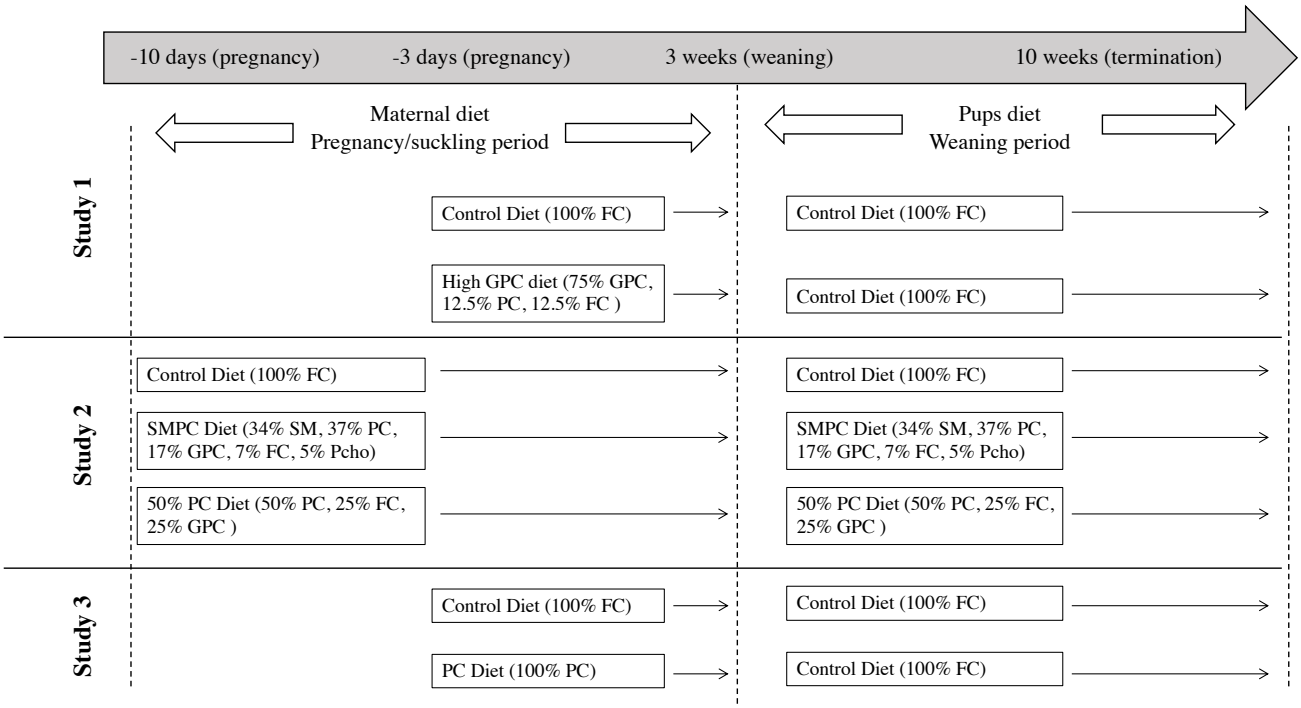
Three different studies were included to assess the impact of the forms of dietary choline on the development of the GALT (**Figure 1**). The effect of choline forms on the peripheral immune response in lactating dams (Azarcoya-Barrera, Goruk *et al.,* 2020, Dellschaft, Richard *et al.,* 2017, Lewis, Richard *et al.,* 2016, Lewis, Richard *et al.,* 2017) and suckled offspring at three weeks of age (Azarcoya-Barrera, Field *et al.,* 2021, Lewis, Richard *et al.,* 2016, Richard, Lewis *et al.,* 2017) has been published elsewhere. Pups at 10 weeks of age were assessed to understand either the programming effect of intervening during the suckling period (study 1 and 3) or the effect of intervening during both the suckling and weaning periods (study 2) on the development of the gut-associated immune system. Ten weeks of age was selected as it reflects early adulthood in rats. All three studies were conducted with the approval of the Institutional Animal Care Committee at the University of Alberta in accordance with the Canadian Council on Animal Care. Pregnant primiparous female Sprague–Dawley rats were obtained from Charles River Laboratories (Montreal, Quebec, Canada) at 7–10 days of gestation and were individually housed in a temperature and humidity-controlled environment with a 12/12-h reversed light cycle. Dams were fed standard rat chow diet (Lab diet 5001; PMI Nutrition International, Brentwood, MO, USA) prior to the start of the study.

In study 1, three days prior to parturition, dams were randomized to one of two experimental diets, both containing 1 g of total choline/kg of diet: Control diet (100% FC) or High GPC (HGPC, 75% GPC, 12.5% PC, 12.5% FC), which were consumed throughout the lactation period. At 3 weeks of age, two pups from each dam (both females) were pooled for MLN analysis (see below) and two other pups (both females) remained in the study and all consumed the Control

diet for an additional seven weeks (until 10 weeks of age). In study 2, at an estimated 10 days of gestation, dams were randomized to one of three experimental diets all containing 1.7 g of choline/kg of diet: Control diet (100% FC), SMPC diet (34% SM, 37% PC, 17% GPC, 7% FC, 5% Pcho) or 50% PC diet (50% PC, 25% FC, 25% GPC). Dams kept consuming the same diet during the lactation period and, at the end of the suckling period (3 weeks), two pups from each dam (both female) were pooled for MLN analysis (see below) and two other pups (both females) continued consuming the same diet as their mother for an additional seven weeks. In study 3, three days prior to parturition, dams were randomized to one of two experimental diets containing 1 g of total choline/kg of diet: Control diet (100% FC) or 100% PC diet (100% PC). At the end of the lactation period (3 weeks), two pups from each dam (both female) were pooled for MLN analysis (see below) and two other pups (both females) remained in the study and consumed the Control diet for seven weeks. At birth, litters were culled to 10 pups per dam. The nutrient composition of all the experimental diets has been previously described (Azarcoya-Barrera, Field *et al.,* 2021, Lewis, Richard *et al.,* 2016, Lewis, Richard *et al.,* 2017). All diets were isocaloric, isonitrogenous and matched for macro- and micronutrients as well as for fatty acid composition with their respective control diet in each study. The fat content of the experimental diets has been previously reported (Azarcoya-Barrera, Goruk *et al.,* 2020, Lewis, Richard *et al.,* 2016, Lewis, Richard *et al.,* 2017). The choline distribution in the experimental diets was confirmed by LC-MS-MS as prevously described (Azarcoya-Barrera, Goruk *et al.,* 2020, Zhao, Xiong *et al.,* 2011). Pups' stomach contents were also assessed at 3 weeks of age to reflect breastmilk composition and has been previously described (Azarcoya-Barrera, Field *et al.,* 2021, Lewis, Richard *et al.,* 2016, Lewis, Richard *et al.,* 2017). Diets were prepared weekly, and stored at 4 °C until fed. All diets

were fed *ad libitum*. Feed cups were weighed and replaced every 2–3 days to prevent oxidation. Dietary intake and body weights were monitored regularly throughout the intervention.

Figure 5-1 Animal study design. In study 1, Sprague-Dawley lactating dams were randomly assigned to the control diet (100% FC) or the High GPC diet (75% GPC, 12.5% PC, 12.5% FC). At weaning, rats were assigned to consume the control diet (100% FC, n=11-12). In study 2, midway pregnancy, dams were randomly assigned to consume the control diet (100% FC), the SMPC diet (34% SM, 37% PC, 17% GPC, 7% FC, 5% Pcho) or the 50%PC diet (50% PC, 25% FC, 25% GPC). At weaning, rats keep consuming the same diet as their mother (n=22). In study 3, lactating dams were randomly assigned to consume the control diet (100% FC) or the 100%PC diet (100% PC). At weaning, all rats were assigned to consume the control diet (100% FC, n=10).



Abbreviations: FC, free choline; GPC, glycerophosphocholine; PC, phosphatidylcholine; Pcho, phosphocholine; SM, sphingomyelin.

5.2.2 Tissue collection and immune cell isolation

At 10 weeks of age, rats were weighed and euthanized by CO_2 asphyxiation. Mesenteric lymph nodes (MLN) and Peyer's patches (PP) were collected aseptically, and immune cells were

isolated for further processing. Isolation of immune cells from the MLN and PP has been previously described (Field, Wu *et al.,* 1990). Prior to *ex vivo* analyses, a haemocytometer was used to count live cells using trypan blue dye exclusion (Sigma-Aldrich) to assess cell viability and was >90% for all treatment groups. All cell suspensions were then diluted to 1.25 x 10^6 cells/ml.

5.2.3 Immune cell phenotype analysis

Immune cell subsets present in freshly isolated MLN and PP were identified by direct immunofluorescence assay, as previously described (Field, Thomson *et al.,* 2000). Depending on the study, the following flow cytometry combinations allowed the identification of surface molecules in MLN cells: CD3/CD45RA, CD28/CD3/CD4/CD8, CD25/CD152/CD8/CD4, CD25/CD127/CD8/CD4, CD27/CD8/CD4, CD71/CD8/CD4, CD28/CD152/CD8/CD4, CD3/CD4/CD25/FoxP3, CD27/OX12/OX6/CD45RA. The following combinations of surface molecules were used for PP cells: CD28/CD3/CD4/CD8, CD25/CD152/CD8/CD4, CD27/OX12/OX6/CD45RA, CD71/CD8/CD4, CD68/CD284/CD11bc, OX62/CD25/OX6, OX62/CD3/CD161 (study 3 only). All antibodies, except for OX6 (BD Biosciences, Mississauga, ON, Canada), were purchased from Cedarlane Laboratories, (Burlington, ON, Canada). After incubation, cells were washed and fixed in paraformaldehyde (10 g/l; Thermo Fisher Scientific) in phosphate-buffered saline. All the samples were acquired within 72 h of preparation by flow cytometry (FACS Calibur; Becton Dickinson, San Jose, CA, USA) and analyzed with FlowJo v10.8.1 (USA).

5.2.4 Ex vivo cytokine production by mitogen-stimulated cells

The measurement of the production of cytokines by mitogen-stimulated cells in MLN has been previously described (Blewett, Gerdung *et al.,* 2009). The concentrations of cytokines interleukin (IL)-2, IL-6, IL-10, tumor-necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) were measured depending on the study in the supernatants by commercial ELISA kits according to manufacturer's instructions. The detection limits for all cytokines were 15.6-4000 pg/ml (R&D Systems, Minneapolis, MN, USA). Cytokine concentrations were quantified using a microplate reader (SpectraMax 190; Molecular Devices, Sunnyvale, CA, USA) and all measurements were conducted in duplicates, with CV <10%. IL-6, IFN-γ, TNF-α and IL-10 (only measured in study 2 and 3 since there was not enough supernatant to measure IL-10 in study 1) production by unstimulated MLN cells (no mitogen added) was also measured in all three studies.

5.2.5 Statistical analyses

Data are reported as mean ± standard error of the mean (SEM) unless indicated otherwise. The three studies were powered to assess significant changes in immune function as main effect (i.e. *ex vivo* cytokine production as the primary outcome). The sample size was based on previous experiments from our group for which n=5-6 is sufficient to assess a 20% significant difference in cytokine production. At three weeks of age, the experimental unit is the dam and therefore, pups from each dam are pooled for each analysis to obtain a measure of the dam. However, at 10 weeks of age, pups are considered their own experimental unit and are no longer considered a measure of the dams. For studies 1 and 3, data were analyzed using the Student's *t* test. Data from study 2 were analyzed by one-way ANOVA with diet as the main effect and, in cases where a significant main effect of diet was found, post hoc analysis was performed using the DUNCAN adjustment

to determine differences between diet groups. Statistical analyses were performed using SAS version 9.4 (SAS Institute Inc, Cary, NC, USA). Variables that were not normally distributed were log-10 transformed prior to statistical analysis. Differences at $p \leq 0.05$ (two-sided) were considered significant.

5.3 Results

5.3.1 Growth parameters and food intake

Growth parameters and food intake data from studies 1 and 2 have been previously published and discussed elsewhere (Azarcoya-Barrera, Field *et al.,* 2021, Richard, Lewis *et al.,* 2017). Briefly, there were no differences in body weight or food intake between groups fed the HGPC and control diets at 10 weeks of age. Starting from week 6 up to 10 weeks of age, rats fed the SMPC diet had a higher body weight when compared to both the control and the 50%PC diets but no differences in food intake was observed (Azarcoya-Barrera, Field *et al.,* 2021). In study 3, body weight (mean 291 ± 8.4 g) and food intake (30.0 ± 0.72 g/d) did not differ between the 100% PC and control groups at 10 weeks of age. In study 3, the 100%PC groups had a lower intestinal length (111.4 cm ± 1.28) when compared to the control group (118.6 ± 1.72; $p = 0.005$) despite having similar body weights. Overall, maternal diets with a higher content of lipid-soluble forms of choline were associated with a higher proportion of lipid-soluble forms of choline in breastmilk. In addition, pups from the 100% PC groups had a higher plasma PC concentration at 3 weeks of age (Lewis, Richard *et al.,* 2016).

5.3.2 Ex vivo cytokine production by mesenteric lymph nodes

In study 1 and following ConA stimulation, feeding dams a diet high in GPC during the lactation period led to a decrease in IL-2, IFN-γ and TNF-α production by MLN in their offspring when compared to the control group (**Table 5-1**, all $p \leq 0.05$). In study 2, there were no significant differences between the HGPC and control groups in IL-6 and IL-10 production by MLN stimulated with ConA (both $p > 0.05$). Rats from the 50%PC and SMPC groups had a higher production of IL-2, IL-10 and TNF-α when compared to the control group (**Table 5-2,** all $p \leq$

0.05). In addition, the SMPC group had an increase in production of IFN-γ when compared to the control group only ($p = 0.029$). In study 3, feeding dams a 100%PC diet during the lactation period led to an increase in production of IFN-γ, in MLN, ($p \leq 0.05$) in their offspring when compared to the control group, while no significant changes were found in IL-2, IL-10, and TNF-α between groups (**Table 5-3**, both $p > 0.05$).

Following LPS stimulation (Study 1 and 3 only), MLN from rats from the HGPC group during the suckling period had a lower production of TNF-α ($p \leq 0.05$) when compared to the control group, while no changes were found in IL-6 and IL-10 (both $p > 0.05$) production between groups (Table 5-1). There were no significant differences between the 100% PC and the control groups after LPS stimulation (Table 5-3). In response to a dietary antigen (OVA, only study 2), both the 50%PC and SMPC groups led a to a lower production of IL-2, TNF-α, and IL-6 by MLN and a higher production of IL-10 when compared to the control diet (Table 5-2, all $p \leq 0.05$). Cytokine production by unstimulated MLN cells were below detection for IL-6, TNF-α, and IFN-γ in all three studies. IL-10 production in unstimulated cells was undetectable in most cases while approximatively 40% of the samples showed minimal stimulation (in average 20.0 ± 8.86 pg/mL) that was unevenly distributed across groups (data not shown).

Table 5-1 *Ex vivo* cytokine production by mesenteric lymph nodes 10-week-old female offspring fed the high GPC and control diets in study 1.

Variable[1]	Control diet (n=12)	High GPC diet (n=11)	*p* Value
	ConA (T cell mitogen)		
IL-2 (pg/ml)	2820 ± 135	1580 ± 264	0.003
IL-6 (pg/ml)	51.0 ± 7.01	39.9 ± 4.88	0.204
IL-10 (pg/ml)*	97.3 ± 35.9	50.6 ± 26.5	0.208
TNF-α (pg/ml)	220 ± 17.9	100 ± 10.2	<0.001
IFN-γ (pg/ml)*	630 ± 64.0	415 ± 33.4	0.007
	LPS (bacterial antigen)		
IL-6 (pg/ml)	61.9 ± 6.62	53.7 ± 10.0	0.507
IL-10 (pg/ml)	110 ± 18.8	120 ± 15.3	0.759
TNF-α (pg/ml)	51.4 ± 4.42	35.5 ± 3.78	0.013

[1]Values are presented as mean ± SEM; GPC, glycerophosphocholine; HGPC, high GPC; IFN-γ, interferon gamma; IL, interleukin; TNF-α, tumor necrosis factor alpha. A p value < 0.05 was considered statistically different from control group based on Student's t test analysis.
*Analysis performed on log-transformed values.

Table 5-2 *Ex vivo* cytokine production by mesenteric lymph nodes of 10-week-old female offspring fed the 50%PC, SMPC and control diets in study 2.

Variable[1]	Control diet (n=22)	50%PC diet (n=22)	SMPC diet (n=22)	*p* Value
		ConA (T cell mitogen)		
IL-2 (pg/ml)*	2250 ± 110^{b}	4940 ± 1050^{a}	4800 ± 590^{a}	0.002
IL-10 (pg/ml)*	77.5 ± 6.73^{b}	150 ± 15.2^{a}	180 ± 18.1^{a}	<0.001
TNF-α (pg/ml)	66.7 ± 5.36^{c}	110 ±8.31^{b}	140 ± 10.6^{a}	<0.001
IFN-γ (pg/ml)	1802 ± 369^{b}	2590 ± 392^{ab}	4080 ± 532^{a}	0.029
		OVA (food antigen)		
IL-2 (pg/ml)*	94.5 ± 12.6^{a}	52.2 ± 3.73^{b}	49.5 ± 4.26^{b}	<0.001
IL-10 (pg/ml)	48.9 ± 2.48^{c}	74.5 ± 4.44^{b}	107 ± 6.36^{a}	<0.001
TNF-α (pg/ml)*	32.7 ± 9.53^{a}	18.4 ± 1.27^{b}	17.6 ± 1.76^{b}	0.032
IL-6 (pg/ml)*	123 ± 35.2^{a}	43.8 ± 7.74^{b}	39.4 ± 6.56^{b}	0.042

[1]Values are mean ± SEM, n=22. Labeled means in a row without a common letter differ, $p \leq 0.05$; IFN-γ, interferon gamma; IL, interleukin; PC, phosphatidylcholine; SM sphingomyelin; TNF-α, tumor necrosis factor alpha.
*Analysis performed on log-transformed values.

Table 5-3 *Ex vivo* cytokine production by mesenteric lymph nodes of 10-week-old female offspring fed the 100%PC and control diets in study 3.

Variable[1]	Control diet (n=10)	100%PC diet (n=10)	*p* Value
	ConA (T cell mitogen)		
IL-2 (pg/ml)	1590 ± 140	1990 ± 190	0.099
IL-10 (pg/ml)	120 ± 15.2	150 ± 35.8	0.545
TNF-α (pg/ml)	340 ± 44.3	390 ± 44.4	0.443
IFN-γ (pg/ml)*	330 ± 58.3	690 ± 122	0.009
	LPS (bacterial antigen)		
IL-10 (pg/ml)	80.5 ± 21.4	130 ± 14.9	0.075
TNF-α (pg/ml)*	340 ± 44.3	390 ± 44.4	0.546

[1]Values are presented as mean ± SEM. IFN-γ, interferon gamma; IL, interleukin; PC, phosphatidylcholine; TNF-α, tumor necrosis factor alpha. A p value ≤ 0.05 was considered statistically different from control group based on Student's t test analysis.
*Analysis performed on log-transformed values.

5.3.3 Mesenteric lymph nodes immune cell phenotypes

Immune cell phenotypes from MLN isolated from suckled pups (3 weeks old) are presented in **Tables 5-4, 5-5 and 5-6**. Overall, feeding the HGPC diet (study 1) led to a higher proportion of helper T cells expressing CD25+ when compared to the control group. In study 2, pups from the 50%PC and SMPC groups had a higher proportion of cytotoxic T cells at 3-weeks of age compared to the control group. In addition, the 50%PC group had an increased proportion of both helper and cytotoxic T cells expressing CD25+ (IL-2 receptor) and cytotoxic T cells expressing CD28+ compared to both the control and the SMPC groups. In study 3, there were no significant changes on immune cell phenotypes between groups (100%PC vs. control) at 3 weeks of age.

Table 5-4 Mesenteric lymph node immune cell phenotypes of 3-week-old offspring fed the high GPC and control diets in study 1.

Phenotype[1]	Control diet (n=6)	High GPC diet (n=5)	*p* Value
	% of gated cells		
Total CD3+ (T cells)	59.6 ± 2.71	62.9 ± 1.47	0.308
CD3+CD4+ (helper T cells)	41.9 ± 2.77	44.3 ± 1.12	0.449
CD3+CD8+(cytotoxic T cells)*	18.1 ± 1.16	18.8 ± 0.625	0.521
Total CD25+ (IL-2 receptor α chain)	6.14 ± 0.229	6.81 ± 0.385	0.934
CD4+CD25+*	4.69 ± 0.113	5.72 ± 0.367	0.028
CD8+CD25+	1.85 ± 0.187	1.89 ± 0.137	0.861
Total CD45RA+ (B cells)	26.7 ± 1.81	25.5 ± 1.24	0.616

[1]Values are presented as mean ± SEM. Two pups from the same dam were pooled for this analysis since the experimental unit at 3 weeks of age is the dam.
CTLA-4, cytotoxic T-lymphocyte-associated protein 4; GPC, glycerophosphocholine; HGPC, high GPC; IL, interleukin; Treg, T regulatory cell. A p value ≤ 0.05 was considered statistically different from control group based on Student's t test analysis.
*Analysis performed on log-transformed values.

Table 5-5 Mesenteric lymph node immune cell phenotypes of 3-week-old offspring fed the 50%PC, SMPC and control diets in study 2.

Phenotype[1]	Control diet (n=7)	50%PC diet (n=7)	SMPC diet (n=8)	*p* Value
	% of gated cells			
Total CD3+ (T cells)	57.9 ± 2.92	65.2 ± 1.76	63.1 ± 2.01	0.106
CD3+CD4+ (helper T cells)	42.3 ± 2.36	45.4 ± 1.58	43.1 ± 1.46	0.512
CD3+CD8+ (cytotoxic T cells)	14.6 ± 0.925[b]	19.6 ± 0.995[a]	18.0 ± 1.06[a]	0.009
Total CD25+ IL-2 receptor α chain)	9.78 ± 0.476[ab]	11.4 ± 0.694[a]	9.16 ± 0.521[b]	0.032
CD4+CD25+	5.97 ± 0.408[b]	7.81 ± 0.492[a]	5.59 ± 0.454[b]	0.008
CD8+CD25+	2.33 ± 0.222[b]	3.56 ± 0.219[a]	2.21 ± 0.068[b]	<.001
Total CD45RA+ (B cells)	24.8 ± 0.995	27.8 ± 1.44	23.2 ± 1.98	0.158

[1]Values are mean ± SEM, n=22. Labeled means in a row without a common letter differ, $p \leq 0.05$. Two pups from the same dam were pooled for this analysis since the experimental unit at 3 weeks of age is the dam. CTLA-4, cytotoxic T-lymphocyte-associated protein 4; MHC, major histocompatibility complex; PC, phosphatidylcholine; SM sphingomyelin; Treg, regulatory T cells.

Table 5-6 Mesenteric lymph node immune cells phenotypes of 3-week-old offspring fed the 100% PC and control diets in study 3.

Phenotype[1]	Control diet (n=5)	100% PC diet (n=5)	*p* Value
	% of gated cells		
Total CD3+ (T cells)	56.2 ± 2.36	55.4 ± 3.28	0.847
CD3+CD4+ (helper T cells)*	42.3 ± 2.14	43.7 ± 3.77	0.860
CD3+CD8+(cytotoxic T cells)	25.3 ± 2.86	24.5 ± 3.58	0.739
Total CD25+ (IL-2 receptor α chain)*	9.97 ± 0.999	9.34 ± 0.363	0.630
CD4+CD25+	8.16 ± 0.397	7.18 ± 0.577	0.210

[1]Values are presented as mean ± SEM. Two pups from the same dam were pooled for this analysis since the experimental unit at 3 weeks of age is the dam. CTLA-4, cytotoxic T-lymphocyte-associated protein 4; GPC, glycerophosphocholine; HGPC, high GPC; IL, interleukin; Treg, T regulatory cell. A p value ≤ 0.05 was considered statistically different from control group based on Student's *t* test analysis.
*Analysis performed on log-transformed values.

At 10-weeks of age, in study 1, there were no significant changes between the HGPC group and the control group on the proportion of T cell (**Table 5-7**). In study 2, the proportion of cytotoxic T cells was higher in MLN of rats from the SMPC group **(Table 5-8)** and lower in rats from the 100%PC groups **(Table 5-9)** when compared to their respective controls (both $p \leq 0.05$). In study 3, the 100%PC group had a lower proportion of T cells (CD3+) ($p \leq 0.05$) compared to the control group. The Treg populations were similar between the HGPC and the control groups (study 1). In study 2, the 50%PC group had an increased proportion of Treg cells when compared to the control group only. In contrast, feeding a diet containing only PC (100%PC) decreased the proportion of Treg vs. the control group in study 3. In study 2, MLN of rats from both the SMPC and the 50%PC groups had an increased proportion of total CD25+ cells, and both helper and cytotoxic T cells expressing CD25+, when compared to the control group ($p \leq 0.05$). In addition, rats from the SMPC group had a higher proportion of cytotoxic T cells expressing the co-inhibitory molecule (CD152+) when compared to the 50%PC group only (all $p \leq 0.05$). In study 3, rats from the

100%PC diet group had an increased proportion of total cells expressing CD152+ in MLN ($p \leq 0.05$). The proportion of B cells (CD45RA+) was lower in the HGPC group, from study 1, when compared to the control group. In study 2, the proportion of OX6+ cells (MHC class II) was higher in the 50%PC groupwhen compared to the SMPC group only (Table 6, $p \leq 0.05$). No changes were found in the proportion of antigen presenting cell populations between the 100%PC and control groups in study 3 (all $p > 0.05$).

Table 5-7 Mesenteric lymph node immune cells phenotypes of 10-week-old female offspring fed the high GPC and control diets in study 1.

Phenotype[1]	**Control diet (n=12)**	**High GPC diet (n=11)**	***p* Value**
	% of gated cells		
Total CD3+ (T cells)	62.3± 1.59	65.2 ± 1.13	0.157
CD3+CD4+ (helper T cells)	40.7 ± 1.77	42.6 ± 1.65	0.442
CD3+CD8+(cytotoxic T cells)	20.1 ± 0.619	19.0 ± 0.623	0.218
CD4+CD28+	3.61 ± 0.146	4.08 ± 0.163	0.047
CD8+CD28+	2.16 ± 0.197	2.24 ± 0.037	0.719
CD4+CD25+FoxP3+ (Treg)	1.98 ± 0.241	1.56 ± 0.131	0.152
Total CD25+ (IL-2 receptor α chain)	5.82 ± 0.210	6.17 ± 0.278	0.489
CD4+CD25+	4.67 ± 0.156	4.83 ± 0.215	0.513
CD8+CD25+	1.71 ± 0.093	1.81 ± 0.081	0.465
Total CD152+ (CTLA-4)	2.71 ± 0.305	3.24 ± 0.210	0.185
CD4+CD152+	1.15 ± 0.145	1.41 ± 0.068	0.150
CD8+CD152+*	0.723 ± 0.082	0.768 ± 0.061	0.583
Total CD45RA+ (B cells)	37.1 ± 1.61	32.5 ± 0.956	0.032

[1]Values are presented as mean ± SEM. CTLA-4, cytotoxic T-lymphocyte-associated protein 4; GPC, glycerophosphocholine; HGPC, high GPC; IL, interleukin; Treg, T regulatory cell. A *p* value ≤ 0.05 was considered statistically different from control group based on Student's *t* test analysis. *Analysis performed on log-transformed values.

Table 5-8 Mesenteric lymph node immune cell phenotypes of 10-week-old female offspring fed the 50%PC, SMPC and control diets in study 2.

Phenotype[1]	**Control diet (n=22)**	**50%PC diet (n=22)**	**SMPC diet (n=22)**	***p* Value**
	% of gated cells			
Total CD3+ (T cells)	50.7 ± 2.52	46.7 ± 1.23	48.9 ± 1.71	0.297
CD3+CD4+ (helper T cells)	26.2 ± 1.44	25.5 ± 1.07	26.5 ± 0.977	0.806
CD3+CD8+ (cytotoxic T cells)	11.3 ± 0.628^{b}	12.4 ± 0.363^{ab}	13.5 ± 0.381^{a}	0.005
CD4+CD28+	4.61 ± 0.131^{c}	5.38 ± 0.174^{b}	5.81 ± 0.138^{a}	<.001
CD8+CD28+	2.76 ± 0.083^{b}	2.77 ± 0.056^{b}	3.05 ± 0.057^{a}	0.005
CD4+CD25+FoxP3+ (Treg)	1.66 ± 0.146^{b}	2.23 ± 0.168^{a}	1.91 ± 0.127^{ab}	0.030
Total CD25+ IL-2 receptor α chain)	5.74 ± 0.283^{c}	8.21 ± 0.306^{b}	9.7 ± 0.342^{a}	<.001
CD4+CD25+	3.05 ± 0.241^{c}	3.71 ± 0.145^{b}	4.36 ± 0.184^{a}	<.001
CD8+CD25+	1.12 ± 0.099^{b}	1.51 ± 0.113^{a}	1.79 ± 0.126^{a}	0.003
Total CD152+ (CTLA-4)*	3.52 ± 0.217	2.96 ± 0.087	3.4 ± 0.232	0.124
CD4+CD152+*	1.67 ± 0.155	1.39 ± 0.069	1.58 ± 0.103	0.316
CD8+CD152+*	1.08 ± 0.099^{a}	0.842 ± 0.034^{b}	1.12 ± 0.074^{a}	0.020
Total CD45RA+ (B cells)	29.9 ± 1.87	29.4 ± 1.64	26.9 ± 1.08	0.355
Total OX6+ (MHC class II+)	25.8 ± 1.12^{ab}	27.8 ± 1.014^{a}	23.9 ± 0.838^{b}	0.026

[1]Values are mean ± SEM, n=22. Labeled means in a row without a common letter differ, $p \leq 0.05$. CTLA-4, cytotoxic T-lymphocyte-associated protein 4; MHC, major histocompatibility complex; PC, phosphatidylcholine; SM sphingomyelin; Treg, regulatory T cells.
*Analysis performed on log-transformed values.

Table 5-9 Mesenteric lymph node immune cell phenotypes of 10-week-old female offspring fed the 100%PC and control diets in study 3.

Phenotype[1]	**Control diet (n=10)**	**100%PC diet (n=10)**	***p* Value**
	% of gated cells		
Total CD3+ (T cells)	69.4 ± 1.33	65.4 ± 1.23	0.040
CD3+CD4+ (helper T cells)*	44.8 ± 1.83	42.2 ± 0.777	0.231
CD3+CD8+(cytotoxic T cells)	18.8 ± 0.644	14.8 ± 1.15	0.008
CD4+CD28+*	3.95 ± 0.241	3.38 ± 0.146	0.227
CD8+CD28+	3.66 ± 0.232	2.08 ± 0.194	<0.001
CD4+CD25+FoxP3+ (Treg)	4.34 ± 0.122	3.39 ± 0.174	0.003
Total CD25+ (IL-2 receptor α chain)*	5.37 ± 0.262	5.61 ± 0.405	0.709
CD4+CD25+*	4.09 ± 0.241	4.86 ± 0.564	0.274
CD8+CD25+	1.56 ± 0.074	1.57 ± 0.147	0.991
Total CD152+ (CTLA-4)	1.87 ± 0.056	2.46 ± 0.148	0.003
CD4+CD152+*	0.873 ± 0.046	1.01 ± 0.079	0.176
CD8+CD152+*	0.461 ± 0.021	0.536 ± 0.055	0.227
Total CD45RA+ (B cells)	31.2 ± 2.49	35.5 ± 1.38	0.151

[1]Values are presented as mean ± SEM. CTLA-4, cytotoxic T-lymphocyte-associated protein 4; IL, interleukin; PC, phosphatidylcholine; TLR-4, toll-like receptor 4. A p value ≤ 0.05 was considered statistically different from control group based on Student's t test analysis.
*Analysis performed on log-transformed values.

5.3.4 Peyer's patches immune cell phenotypes

There was no impact on the proportion of cytotoxic and helper T cell populations in PP of rats from HGPC group (**Table 5-10**) from study 1 and 100%PC group (**Table 5-11**) from study 3. In study 2, rats from the SMPC group had a higher proportion of helper T cells when compared to both the 50%PC and the control groups (**Table 5-12**). There were no significant differences between groups across the studies in the proportion of cytotoxic and Th cells expressing CD28+ (all $p > 0.05$). In study 1, feeding dams with the HGPC diet led to a higher proportion of cytotoxic T cells expressing CD152+ whereas, in study 3, the 100%PC group had a lower proportion when compared to their respective control group (both $p \leq 0.05$). In study 2, ratsfrom the 50%PC group had a higher proportion of Th cells expressing CD152+ when compared to both the control and

SMPC groups ($p \leq 0.05$). In study 1, no significant changes were found in the proportion of B cell populations (CD45RA+) between the HGPC and control groups. There was a lower proportion of dendritic cells (OX62+OX6+), macrophages (CD68+), and both total cells expressing TLR-4 (CD284) and activated macrophages (CD11bc+CD284+) in the HGPC group when compared to the control group (Table 7, all $p \leq 0.05$). In study 2, PP of rats from both the 50%PC and SMPC groups had a higher proportion of B cells (CD45RA+), and a higher proportion of total cells expressing TLR-4 (CD284) when compared to the control group (both $p \leq 0.05$). There was also a lower proportion of macrophages (CD68+) in both 50%PC and SMPC groups when compared to the control group while no significant differences were found in the proportion of dendritic cells between groups. In study 3, no changes were found in the proportions of B cells and dendritic cells between the 100%PC and the control groups (both $p > 0.05$). Feeding rats with a diet containing 100% PC increased the proportion of activated macrophages expressing the TLR-4 (CD11bc+CD284+) and decreased the proportion of total macrophages (CD68+) when compared to the control group (both $p \leq 0.05$).

Table 5-10 Peyer's patches immune cell phenotypes of 10-week-old female offspring fed the high GPC and control diets in study 1.

Phenotype[1]	**Control diet (n=12)**	**HGPC diet (n=11)**	***p* Value**
	% of gated cells		
Total CD3+ (T cells)	24.1 ± 0.859	23.6 ± 1.11	0.734
CD3+CD4+ (helper T cells)	7.73 ± 0.525	8.87 ± 0.532	0.143
CD3+CD8+(cytotoxic T cells)	5.04 ± 0.392	5.67 ± 0.394	0.268
CD4+CD28+	0.901 ± 0.093	0.939 ± 0.095	0.783
CD8+CD28+	0.671 ± 0.067	0.716 ± 0.049	0.588
Total CD25+ (IL-2 receptor α chain)	8.71 ± 0.473	7.25 ± 0.361	0.022
CD4+CD25+	1.45 ± 0.173	1.14 ± 0.089	0.134
CD8+CD25+*	3.16 ± 0.303	2.49 ± 0.171	0.055
Total CD152+ (CTLA-4)	3.78 ± 0.335	3.82 ± 0.379	0.944
CD4+CD152+	0.494 ± 0.053	0.422 ± 0.042	0.299
CD8+CD152+	0.725 ± 0.063	0.514 ± 0.055	0.020
Total CD45RA+ (B Cells)	58.3 ± 2.17	63.8 ± 1.43	0.099
Total CD284+ (TLR-4)	5.38 ± 0.185	4.36 ± 0.193	0.001
Total CD11bc+ (macrophages)	11.7 ± 0.602	10.7 ± 0.556	0.294
CD11bc+CD284+ ((TLR-4+ macrophages)	2.46 ± 0.203	1.94 ± 0.129	0.041
Total CD68+ (macrophages)*	5.32 ± 0.469	3.59 ± 0.205	0.002
OX62+OX6+ (dendritic cells)	4.31 ± 0.252	3.41 ± 0.194	0.010

[1]Values are presented as mean ± SEM. CTLA-4, cytotoxic T-lymphocyte-associated protein 4; GPC, glycerophosphocholine; HGPC, high GPC; IL, interleukin; TLR-4, toll-like receptor 4. A *p* value ≤ 0.05 was considered statistically different from control group based on Student's *t* test analysis.

*Analysis performed on log-transformed values.

Table 5-11 Peyer's patches immune cell phenotypes of female 10-week-old female offspring fed the 50%PC, SMPC and control diets in study 2.

Phenotype[1]	Control diet (n=22)	50%PC diet (n=22)	SMPC diet (n=22)	*p* Value
	% of gated cells			
Total CD3+ (T cells)	25.1 ± 1.26	25.5 ± 1.23	25.1 ± 1.27	0.971
CD3+CD4+ (helper T cells)	5.49 ± 0.152[b]	5.34 ± 0.241[b]	6.41 ± 0.167[a]	<.001
CD3+CD8+(cytotoxic T cells)	5.71 ± 0.261	5.63 ± 0.188	6.15 ± 0.216	0.431
CD4+CD28+	1.96 ± 0.076	2.11 ± 0.065	2.16 ± 0.082	0.162
CD8+CD28+	1.27 ± 0.051	1.31 ± 0.064	1.38 ± 0.065	0.451
Total CD25+ (IL-2 receptor α chain)	13.1 ± 0.461	13.5 ± 0.346	13.6 ± 0.301	0.558
CD4+CD25+	4.62 ± 0.164	5.08 ± 0.138	5.01 ± 0.184	0.119
CD8+CD25+	2.71 ± 0.095	2.72 ± 0.061	2.57 ± 0.081	0.376
Total CD152+ (CTLA-4)	9.05 ± 0.225	9.15 ± 0.261	8.84 ± 0.272	0.679
CD4+CD152+	3.21 ± 0.108[b]	3.63 ± 0.124[a]	3.23 ± 0.129[b]	0.027
CD8+CD152+	2.23 ± 0.112	2.05 ± 0.112	2.29 ± 0.092	0.260
Total CD45RA+ (B Cells)	47.6 ± 1.016[b]	52.3 ± 0.465[a]	50.9 ± 1.26[a]	0.004
Total CD284+ (TLR-4)	9.81 ± 0.368[b]	11.9 ± 0.241[a]	12.1 ± 0.378[a]	<.001
Total CD11bc+ (macrophages)	12.5 ± 0.252	12.7 ± 0.223	12.5 ± 0.285	0.681
CD11bc+CD284+ ((TLR-4+ macrophages)*	3.24 ± 0.052	3.44 ± 0.066	3.54 ± 0.159	0.106
Total CD68+ (macrophages)*	7.62 ± 0.417[a]	6.58 ± 0.233[b]	6.39 ± 0.318[b]	0.029
OX62+OX6+ (dendritic cells)	3.79 ± 0.107	3.83 ± 0.092	3.72 ± 0.115	0.797

[1]Values are mean ± SEM, n=22. Labeled means in a row without a common letter differ, $p \leq 0.05$.
CTLA-4, cytotoxic T-lymphocyte-associated protein 4; IL, interleukin; PC, phosphatidylcholine; SM sphingomyelin; TLR-4, toll-like receptor 4.
*Analysis performed on log-transformed values.

Table 5-12 Peyer's patches immune cell phenotypes of female 10-week-old female offspring fed the 100%PC and control diets in study 3.

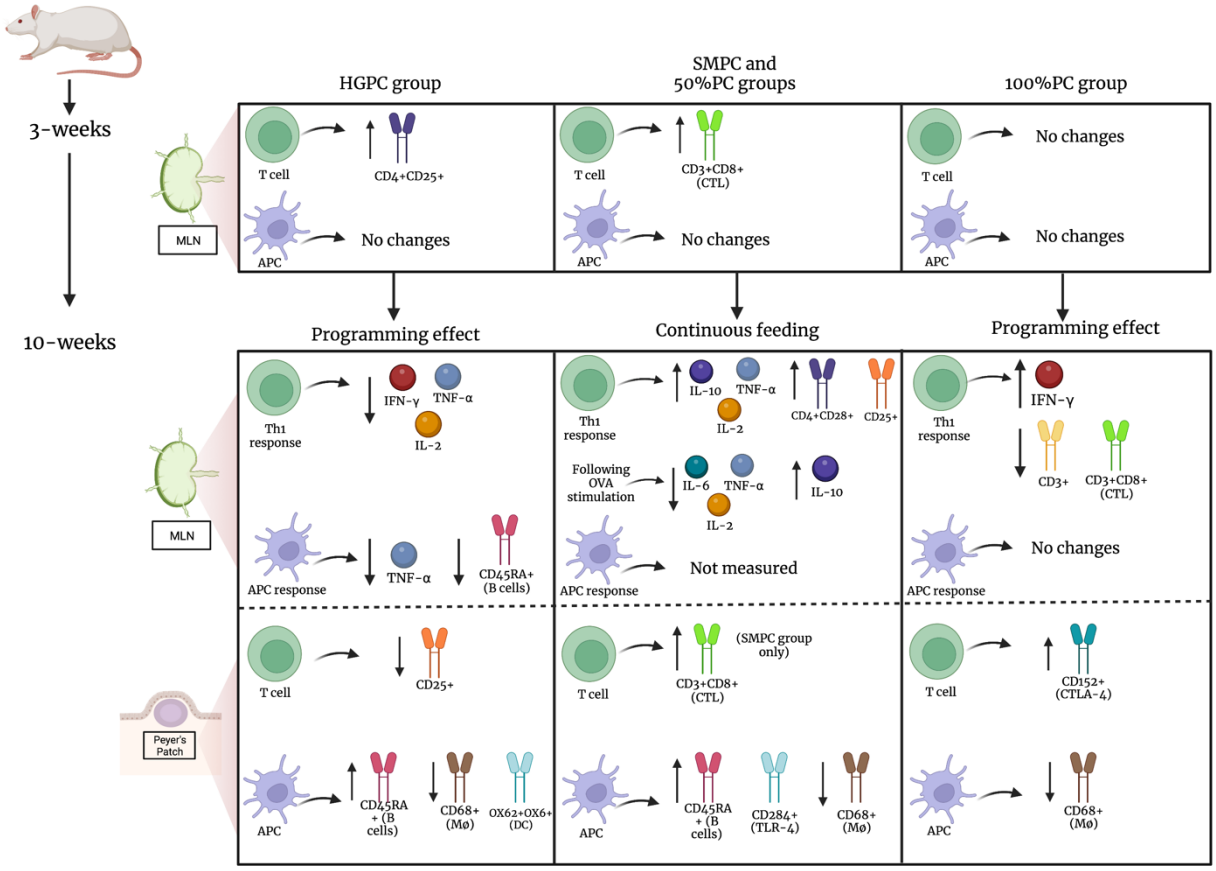
Phenotype[1]	**Control diet (n=6)**	**100%PC diet (n=5)**	***p* Value**
	% of gated cells		
Total CD3+ (T cells)	24.6 ± 0.971	22.5 ± 1.61	0.296
CD3+CD4+ (helper T cells)*	11.4 ± 1.27	9.8 ± 0.451	0.261
CD3+CD8+(cytotoxic T cells)	4.08 ± 0.878	3.84 ± 0.401	0.810
CD4+CD28+	2.51 ± 0.398	1.52 ± 0.186	0.062
CD8+CD28+*	1.37 ± 0.163	1.24 ± 0.044	0.553
Total CD25+ (IL-2 receptor α chain)	9.61 ± 0.658	11.2 ± 0.343	0.075
CD4+CD25+	1.62 ± 0.218	1.91 ± 0.166	0.319
CD8+CD25+	1.73 ± 0.164	2.45 ± 0.424	0.157
Total CD152+ (CTLA-4)	3.73 ± 0.377	5.34 ± 0.472	0.024
CD4+CD152+	0.847 ± 0.117	1.16 ± 0.131	0.108
CD8+CD152+	0.525 ± 0.036	0.746 ± 0.083	0.028
Total CD45RA+ (B Cells)	55.4 ± 3.39	57.3 ± 2.91	0.683
Total CD284+ (TLR-4)	9.60 ± 0.801	10.6 ± 0.572	0.368
Total CD11bc+ (macrophages)	13.1 ± 0.212	13.1 ± 1.16	0.990
CD11bc+CD284+ ((TLR-4+ macrophages)	2.12 ± 0.565	4.24 ± 0.567	0.019
Total CD68+ (macrophages)	11.4 ± 0.600	9.46 ± 0.261	0.025
OX62+OX6+ (dendritic cells)	4.54 ± 0.572	5.91 ± 0.513	0.117

[1]Values are presented as mean ± SEM. CTLA-4, cytotoxic T-lymphocyte-associated protein 4; IL, interleukin; PC, phosphatidylcholine; TLR-4, toll-like receptor 4. A p value ≤ 0.05 was considered statistically different from control group based on Student's t test analysis.
*Analysis performed on log-transformed values.

5.4 Discussion

In studies 1 and 3, we aimed to determine the potential programming effect of feeding different choline forms during the suckling period on the gut-associated immune responses once adulthood is reached. In study 2, we determined the effect of feeding higher proportions of lipid-soluble forms of choline during both the lactation and weaning periods on immune responses in the gut. Overall, we showed that feeding different proportions of choline forms during the suckling period has a programming effect on gut-associated immune function and immune cell phenotypes at 10 weeks of age (summarized in **Figure 5-2**). However, feeding a mixture of choline forms during both the suckling and weaning periods modulated to a greater extent the development of the gut-associated immune system suggesting that the lipid-soluble forms of choline should also be consumed during the weaning period to confer greater immune benefits. We partly confirmed our hypothesis in that a higher proportion of lipid-soluble forms of choline will have a greater impact on the development of the GALT than a diet containing mostly water-soluble forms. However, since the HGPC and 100%PC diets were only provided during the suckling period, it is likely that providing a mixture of choline forms is also needed during the weaning period for optimal GALT development. Understanding the critical window where providing a mixture of choline forms favors the development of the immune system should be investigated in future studies to confirm the importance of suckling vs. weaning periods.

Figure 5-2 Summary of the effects of feeding diets early in life with different proportion of choline forms on ex vivo immune cell phenotypes and function from the gut-associated lymphoid tissue of 3-weeks and 10-weeks old female offspring. Briefly, in study 1 and 3, the programing effect of intervening during the lactating period only was assessed later in life at 10 weeks of age when pups consume a control diet during the weaning period whereas in study 2, the continuous effect of feeding different proportion of choline forms throughout mid-gestation, lactation and weaning periods was assessed. Created with http:BioRender.com.



Abbreviations: APC, antigen-presenting cells; CD, cluster of differenciation; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; CTL, cytotoxic T cells; DC, dendritic cells; GPC, glycerophosphocholine; IFN-γ, interferon gamma; IL, interleukin; Mø, macrophages; MLN, mesenteric lymph nodes; OVA, ovalbumin; PC, phosphatidylcholine; SM, sphingomyelin; Th1, T helper 1; TLR-4, toll-like receptor 4; TNF-α, tumor necrosis factor alpha.

5.4.1 Effect of the different forms of choline on the gut associated-immune system when fed during both the lactation and weaning periods

Feeding the SMPC and 50%PC diets during both the lactation and weaning periods improved T cells responses in MLN by increasing the production of IL-2 and TNF-α in addition

to IFN-γ (SMPC group only) when compared to the control group. Both TNF-α and IFN-γ are key Th1 cytokines known to induce IL-2 production (Hayden & Ghosh 2014, Kasahara, Hooks *et al.,* 1983). In addition, we found a higher proportion of both helper and cytotoxic T cells (both at 3 and 10 weeks of age) expressing the IL-2 receptor (CD25+) in MLN, which could partly explain the higher IL-2 production in these groups. Feeding the SMPC diet during the suckling and weaning periods led to a higher proportion of helper and cytotoxic T cells in PP and MLN, respectively. Helper T cells in PP assist B cells in the secretion of immunoglobulins (mainly immunoglobin A (IgA) (Gardby, Kagrdic *et al.,* 1998). It has been suggested that cytotoxic T cells play a role in the suppression of immune responses to orally administrated antigens, and therefore assist in the induction of oral tolerance (Lagoo, Eldridge *et al.,* 1994). In addition, there was a higher proportion of cells expressing MHC class II (OX6+) in MLN of rats fed the 50%PC diet and a higher proportion of B cells in PP in both the SMPC and 50%PC diets. Feeding diets high in lipid-soluble forms of choline during the lactation period led to few changes on immune cell phenotypes at 3 weeks of age, however, they were mostly maintained when diets were fed through the weaning period. Altogether, our data suggests a better maturation of the GALT towards a more efficient Th1 response that could be attributable to an enhanced antigen presentation ability within the GALT.

5.4.2 Programming effect of the forms of choline on the gut-associated immune sytem

We showed that feeding a diet high in GPC during the suckling period lowers IL-2 and Th1 cytokines (IFN-γ and TNF-α) production after ConA stimulation. Consistently, there was lower TNF-α production following LPS stimulation. The increased proportion of helper T cells expressing CD25+ in the HGPC group at 3-weeks of age was no longer observed at 10 weeks of

age. At birth, the immune system favors a skewed Th2 response over a Th1 response and the maturation of the immune system is associated with an increased ability to produce Th1 cytokines (Hartel, Adam *et al.,* 2005). The HGPC group also had a lower proportion of B cells (CD45RA+) in MLN and a lower proportion of antigen presenting cell populations (APC, macrophages, dendritic cells (DC) in PP. DC navigate between and within MLN and PP and other components of the GALT such as the lamina propria. It is thought that microfold (M)-cells, specialized epithelial cells of the mucosa, in the PP can transfer antigens to DC, and these could either prime T cells within the PP or migrate to the MLN (van Wijk & Cheroutre 2010). Once in the MLN, the priming of T cells by DC leads to activation and expansion of these cells that result in Th1 and/or Th17 effector cells (Huffnagle & Noverr 2008, Koboziev, Karlsson *et al.,* 2010). Altogether, this could partially explain the lower cytokine production after mitogen stimulation by MLN in the HGPC group. Interestingly, we had previously shown that feeding lactating rats with the HGPC diet increased the production of IL-2 and IFN-γ by mitogen-stimulated MLN when compared to a diet only containing FC (Lewis, Richard *et al.,* 2017). This suggests that feeding GPC throughout the weaning period might be required to confer the same immunological benefits as previously observed in the dams and/or that different choline forms are more important during different life stages.

Feeding a diet containing only PC (100%PC) during the suckling period increased IFN-γ production and tended to increase IL-2 production after T cell mitogen stimulation in MLN. A trend towards an increased TNF-α production after LPS stimulation was also observed. We found that rats on the 100%PC diet had a lower proportion of T cells (CD3+) in MLN and a higher proportion of CD152+ cells in both PP and MLN. At 3 weeks of age, there was no difference in MLN immune cell phenotypes assessed, suggesting a programming effect of PC on MLN later in

life. T cell activation is a complex process that requires a stimulatory signal. CD152 or CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) is a CD28 homolog that provides an inhibitory signal when binding, with much higher affinity, to CD80 and/or CD86 that may counteract the co-stimulatory signal provided by CD28 therefore, inhibiting early T cell activation and possibly IL-2 production (Buchbinder & Desai 2016). This, along with the lower proportion of CD3+ could potentially explain the lower T cell response to stimulation when compared to the 50%PC and SMPC groups. Furthermore, in study 3, the 100%PC diet was only fed during the suckling period and rats were fed the control diet at weaning, where the immune system is still developing and lacks maturation. Therefore, similarly to the HGPC diet, we cannot exclude the possibility that feeding a 100%PC diet during both the suckling and weaning periods would have led to greater immune benefits as we have observed in the 50% and SMPC groups. The lipid-soluble forms of choline are primarily incorporated into chylomicrons, once absorbed by the enterocyte, and enter the bloodstream where they can be delivered to peripheral organs first (i.e spleen) (Lewis, Field *et al.,* 2015). We have previously demonstrated *in vitro* that increasing the availability of lyso-PC (the form of PC readily accessible to cells) increased the PC content in splenocyte membranes which was associated with an increased IL-2 production and T cell proliferation after stimulation (Lewis, Richard *et al.,* 2016). SM is the most abundant sphingolipid in cell membranes and the products of its metabolism (ceramide, sphingosine-1-phosphate) have shown to modulate cell differentiation, apoptosis, cell proliferation and inflammatory immune responses (Albeituni & Stiban 2019, Chi 2011). Therefore, it is possible that increasing the content of lipid-soluble forms of choline during both the lactation and weaning diets is required to enhance the maturation of the GALT.

5.4.3 Response to a dietary antigen (ovalbumin)

The GALT maintains homeostasis by providing protection to pathogens that are harmful to the host while providing an environment that is tolerant towards dietary antigens and gut bacteria, a process known as oral tolerance. Oral tolerance is characterized by an increased production of anti-inflammatory cytokines, such as IL-10 and TGF-β, which inhibit Th1 responses, and immunoglobulin (Ig) E production and induce IgA production (Commins 2015). In that regard, we found that after stimulation with a dietary antigen (OVA), rats fed with the SMPC and 50%PC diets had a lower production of pro-inflammatory cytokines TNF-α, IL-6 and IFN-γ along with a decrease in IL-2 production in MLN. Moreover, we found that MLN from rats in the 50%PC and SMPC groups had a higher production of IL-10. Although we did not stimulate the MLN of rats from studies 1 and 3 with a dietary antigen, we analyzed the Treg population in MLN in all three studies. Treg function as suppressors of pro-inflammatory responses towards food antigens or commensal bacterial by increasing the production of IL-10 and TGF-β (Harrison & Powrie 2013). Providing a diet high in GPC early in life had no programming effect on the proportion of regulatory T cells in MLN. Feeding the 50%PC during the lactation and weaning periods increased the proportion of Treg whereas the 100%PC group had a lower proportion of Treg when compared to their respective controls. Even though no significant changes were observed in the proportion of Treg in the SMPC group, the production of IL-10 was higher than both the control and 50%PC groups suggesting that their Treg may have a greater ability to suppress cell proliferation after OVA stimulation. Altogether, our findings suggest that feeding diets containing between 50-70% lipid-soluble forms of choline favors the establishment of oral tolerance as measured by a lower *ex vivo* cytokine production after OVA stimulation.

5.4.4 Limitations

The current study has several limitations. This was a secondary analysis of three previous studies with slightly different study design. Therefore, comparison across studies is not possible especially since LPS and OVA were not tested consistently across studies. Furthermore, the nutritional intervention started at different stages (i.e. at day 10 of pregnancy for study 2 or at day 19 of pregnancy for studies 1 and 3) which could have affected the results since the immune system developed during the pregnancy period. Moreover, the amount of total choline varied slightly between studies for which study 1 and 3 had 1g/kg of diet and study 2 had 1.7g/kg of diet. Although we cannot exclude the possibility that the total amount of choline would have affected the results, our group had previously demonstrated that feeding diets that contain either 1g/kg or 2g/kg had the same effect on the peripheral immune system (Dellschaft, Ruth *et al.,* 2015). Secretory IgA (sIgA) is an important feature of the gut-associated immune system that plays a role in both the protection against pathogens and the induction of oral tolerance and was not assessed in our studies. In addition, we did not collect gut samples to assess gut morphology and integrity, which is a limitation of our study. Furthermore, we only assessed cytokine production in response to OVA in the second study and therefore we cannot conclude about the effect of the other proportions of choline forms on the development of oral tolerance, which should be confirmed in future studies. In the three studies, only female rats were use to investigate the impact of dietary choline on the GALT. This is an important limitation since biological sex affects immune responses (Braga Tibaes, Azarcoya-Barrera *et al.,* 2022), therefore, sex differences should be investigated in future studies. Key enzymes involved in *de novo* synthesis of choline (Kennedy or phosphatidylethanolamine N-methyltransferase (PEMT) pathways) were not assessed which should be investigated in future studies (van der Veen, Kennelly *et al.,* 2017). Finally, a dose-

response study to determine the optimal proportions between lipid- and water-soluble form is required since we did not have diets containing 25-30% lipid-soluble forms.

5.5 Conclusion

In summary, our results suggest that consuming a mixture of choline forms in the diet mainly composed of lipid-soluble forms favors the development and maturation of the gut-associated immune system and oral tolerance when compared to diets containing only water-soluble forms of choline. Moreover, feeding different proportions of choline forms during the suckling period had a programming effect on the GALT later in life, but greater immune benefits were observed when the lipid-soluble forms of choline were fed during both the suckling and weaning periods, highlighting the importance of the weaning period in the development of the GALT. Collectively, our findings highlight the importance of consuming a mixture of choline forms during early developmental period for the development of the immune system as compared to only one form of choline (i.e. FC, the form of choline primarily used in infant formula).

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Chapter 6: Egg-phosphatidylcholine attenuates T cell dysfunction in high-fat diet fed male Wistar rats.

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6.1 Introduction

Obesity is defined as an excessive accumulation of body fat that increases the risk of developing other chronic diseases . Adipocyte hypertrophy is common in obesity and leads to hypoxia and cell death both contributing to an increase recruitment of immune cells within the adipose tissue (Gonzalez-Muniesa, Martinez-Gonzalez *et al.,* 2017). This is associated with a shift from M2-like macrophages exerting anti-inflammatory properties to M1-like macrophages secreting pro-inflammatory cytokine (i.e. TNF-α and IL-6) that contribute to a state of chronic low-grade systemic inflammation commonly documented in obesity. Many studies have shown that, both in humans and obese rodent models, obesity impairs immune cell function and is associated with poor response to vaccination (Ghanim, Aljada *et al.,* 2004, Hsu, Aronoff *et al.,* 2007, Nieman, Henson *et al.,* 1999, Ordway, Henao-Tamayo *et al.,* 2008, Richard, Wadowski *et al.,* 2017, Weber, Rutala *et al.,* 1986, Weber, Rutala *et al.,* 1985).

Our group has previously reported that individuals with obesity and type 2 diabetes (T2D) have additional immune dysfunction when compared to normoglycemic BMI-matched individuals. Indeed, individuals with obesity and T2D had a lower production of IL-2 (proliferation marker), IL-6 and TNF-α by peripheral blood mononuclear cells (PBMCs) stimulated with phytohaemagglutinin, a T cell mitogen. Similarly, in Wistar rats fed a cafeteria diet, Lamas et al. showed a lower IL-2 production by splenocytes stimulated with Concanavalin A (ConA, another T cell mitogen) along with a reduction in the number of T helper cells (Lamas, Martinez *et al.,* 2002). Therefore, Wistar rats fed a diet-induced obesity appear to be a relevant model to study the obesity-related immune dysfunction in humans.

Choline is an essential nutrient needed for a wide variety of processes in the body (Zeisel 2000, Zeisel 2004, Zeisel & da Costa 2009). Choline can be found in the diet as water-soluble forms (free choline (FC), glycerophosphocholine (GPC) and phosphocholine) and lipid-soluble forms (phosphatidylcholine (PC), sphingomyelin (SM)) (Zeisel 2006). The two forms of choline mostly consumed in North America are PC and FC (Lewis, Subhan *et al.,* 2014). Although PC can be found in a variety of foods (soy, dairy, nuts etc.), meat and eggs are the major sources of PC in the diet (Lewis, Subhan *et al.,* 2014, Patterson, Bhagwat *et al.,* 2008). PC plays an important role in cell membrane integrity, intestinal barriers function (i.e. mucus) and assembly and secretion of lipoproteins (Cole, Vance *et al.,* 2012).

Studies from our group have consistently reported that feeding diets containing a higher proportion of PC (relative to FC, the standard form of choline in rodent diet) beneficially modulates immune function during both the lactation period in dams and suckling and weaning periods in offspring (Azarcoya-Barrera, Field *et al.,* 2021, Azarcoya-Barrera, Goruk *et al.,* 2020, Lewis, Richard *et al.,* 2016). Specifically, feeding a maternal diet containing 100% egg-PC led to a higher

IL-2 production after T cell stimulation (ConA) compared to a diet containing 100% FC in splenocytes of suckled pups. We further demonstrated *in vitro* that incubating splenocytes with lyso-PC, the form of PC readily available to cell, was associated with an increase proliferation rate and IL-2 secretion (Lewis, Richard *et al.,* 2016). This suggests that PC can positively modulate T cell function and could potentially counteract some of the obesity-related immune dysfunctions. Therefore, the main objective of this study was to determine the impact of feeding egg-PC on immune function in the context of a high-fat diet (HFD) when compared to a low-fat and a high-fat control diets containing only FC. We hypothesized that feeding egg-PC will attenuate obesity-related immune dysfunction by increasing IL-2 production after T cell stimulation**.**

6.2 Materials and Methods

6.2.1 Animals and diets

All animal care and experimental protocols were conducted in accordance with the Canadian Council on Animal Care guidelines and approved by the University of Alberta Animal Ethics Committee. Three-week-old male (n=6) Wistar rats were obtained from Charles River Laboratories (Montreal, Quebec, Canada) and were housed 2 rats per cage in a temperature and humidity-controlled environment with a 12/12-h reversed light cycle. During the first week acclimatization period, rats were fed standard rat chow (Lab diet 5001; PMI Nutrition International, Brentwood, MO, USA). At 4 weeks of age, rats were randomized to one of three experimental diets all containing 1.5g of total choline/kg of diet: Control-low fat (CLF, 100% FC), Control-high fat (CHF, 100% FC), PC-high fat (PCHF, 100% PC). Diets were fed *ad libitum* for the duration of the study (9 weeks). Before the end of the study, one rat (on the PCHF diet) died from complications unrelated to the study protocol.

The three experimental diets all contained the same amount of total choline differing primarily in the forms of choline provided. The CHF and the PCHF diets were matched for the macronutrient content (50% fat, 23% carbohydrates and 27% protein) whereas the CLF diet was composed of 20% fat, 53% carbohydrates and 27% protein. The nutrient composition of the experimental diets is presented in **Table 6-1**. Even though the total content of fat was different between the low-fat and high-fat diets, the fatty acids composition of the three experimental diets were matched closely using a mixture of oils (**Table 6-2**). The fat mixture added to the rodent diet was composed of flax oil, lard, corn oil and PC from egg (for PCHF only; Lipoid E 80). Lipoid E 80 is a phospholipid extract from egg containing about 85% PC+LysoPC, 9.5% phosphadityl-ethanolamine and 3% sphingomyelin (Lipoid GmbH, Frigenstr. 4 D-67065 Ludwigshafen, Germany). All diets met the essential fatty acid requirements for rodent and had similar polyunsaturated fatty acid (PUFA)/saturated fatty acid (SFA) and n-6/n-3 PUFA ratios. Diets were prepared weekly and stored at 4°C until fed. Feed cups were weighed and replaced every 2-3 days to prevent oxidation. Dietary intake and body weights were monitored regularly throughout the intervention.

Table 6-1 Composition of experimental diets.

Ingredient (g/Kg diet)[1]	CLF diet	CHF diet	PCHF diet
Casein	261.9	261.9	261.9
Corn starch	273.9	123.9	125.6
Sucrose	200	200	200
Vitamin Mix (AIN-93-Vx)[2]	19	19	19
Mineral Mix[3]	50	50	50
Calcium Phosphate Dibasic	3.4	3.4	3.4
Inositol	6.3	6.3	6.3
Cellulose	80	80	80
L-cystine	1.8	1.8	1.8
Choline bitartrate	3.7	3.7	0
Fat Mixture			
Total fat mix	100	250	250
Flax Oil	10	25	25
Corn oil	40	100	99
Lard	50	125	112
Egg-PC	0	0	14

[1]All ingredients were purchased from Harlan Teklad (Indianapolis, IN, USA), with the exception of the dietary oils that were all purchased from Safeway (Edmonton, AB, Canada); [2]AIN-93-VX Vitamin mix (45); [3]Bernhart–Tomarelli salt mixture (46). Egg-PC was purchased from Lipoid GmbH. CLF, control low-fat; CHF, control high-fat; PCHF, PC high-fat; PC, phosphatidylcholine.

Table 6-2 Fatty Acid composition of experimental diets.

Fatty Acid[1]	CLF diet	CHF diet	PCHF diet
	(% of total fatty acids)		
14:0	0.72 ± 0.0	0.76 ± 0.0	0.69 ± 0.04
16:0	20.3 ± 0.68	19.4 ± 0.83	20.6 ±1.34
16:1	1.14 ± 0.12	1.24 ± 0.16	1.17 ± 0.16
C18:0	10.5 ± 1.47	10.8 ± 1.54	11.5 ± 0.02
C18:1	33.3 ± 0.63	33.9 ± 0.41	33.6 ± 0.09
C18:2n6	28.3 ± 1.90	28.4 ± 0.78	27.8 ± 1.14
C18:3n3 (ALA)	5.64 ± 0.57	5.47 ± 0.65	4.79 ± 0.34
Total SFA	31.6 ± 2.15	30.9 ± 0.71	32.7 ± 1.40
Total MUFA	34.4 ± 0.74	35.2 ± 0.57	34.8 ± 0.07
Total PUFA	33.9 ± 1.32	33.9 ± 0.13	32.5 ± 1.48
Ratio n-6/n-3	5.06 ± 0.85	5.24 ± 0.76	5.80 ± 0.17
Ratio PUFA/SFA	1.08 ± 0.12	1.09 ± 0.03	1.00 ± 0.09

[1]Analysis of the fatty acid composition of the three experimental diets collected weekly (n=2 batches). ALA, α-linolenic acid; MUFA, monounsaturated fatty acids; n, omega; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; CLF, control low-fat; CHF, control high-fat; PCHF, PC high-fat; PC, phosphatidylcholine.

6.2.2 Lipid analysis

The fatty acid composition of the three experimental diets was measured by gas chromatography (GC). Briefly, total lipids from diets were extracted using the Folch method (Folch, Lees *et al.,* 1957), saponified, and then methylated with hexane and BF3 (boron trifluoride). For the liver, a modified Folch method was used to extract total lipids and phospholipids as previously described (Field, Ryan *et al.,* 1988, Layne, Goh *et al.,* 1996). Fatty acid methyl esters were separated and identified by automated GLC (GLC7890A; Agilent Technologies) on a 100-m CPSIL 88 fused capillary column (100 m × 0.25 mm; Agilent) as described previously (Cruz-Hernandez, Deng *et al.,* 2004).

6.2.3 Tissue collection and immune cell isolation

At 13 weeks, male rats were weighed and euthanized by CO_2 asphyxiation in the morning hours. Spleens were collected aseptically, weighed, and immune cells were isolated for further processing. Isolation of immune cells from the spleen have been previously described (Field, Wu *et al.,* 1990). Briefly, single cell suspensions were obtained by disrupting tissue through a nylon mesh screen in sterile Krebs-Ringer HEPES buffer with bovine serum albumin (5 g/l; Sigma-Aldrich Canada Ltd., Oakville, ON, Canada). Ammonium chloride lysis buffer (155 mM NH4Cl, 0.1 mM EDTA, 10mM KHCO3; Fisher Scientific, Edmonton, AB, Canada) was used to lyse erythrocytes. Cells were washed then re-suspended in complete culture medium (RPMI 1640 media; Life Technologies, Burlington, ON, Canada), supplemented with 5% (v/v) heat-inactivated fetal calf serum, 25 mM HEPES, 2.5 mM 2-mercaptoethanol and 1% antibiotic/antimycotic (pH 7.4; Fisher Scientific, see above). Prior to *ex vivo* analyses, a haemocytometer was used to count live cells using trypan blue dye exclusion (Sigma-Aldrich, as above) to assess cell viability and was >90% for all treatment groups. All cell suspensions were then diluted to 1.25 x 10^6 cells/ml.

6.2.4 Plasma metabolites measurement

Blood glucose was measured in the non-fasting state from the saphenous vein of 12-weeks-old rats with a glucometer (Accu-chek, Roche, Switzerland). At the end of the study, blood was collected by cardiac puncture in tubes containing EDTA, dipeptidyl peptidase 4 inhibitor (EMD Millipore, MA), and Complete® general protease inhibitor (Sigma) before being centrifuged at 3,000 g for 10 min to obtain plasma. Plasma adiponectin and leptin were measured by ELISA according to the manufacturer's instructions. Plasma cytokines and chemokines concentration were measured using a Proinflammatory Panel 1 V-PLEX electrochemiluminescent multiplex

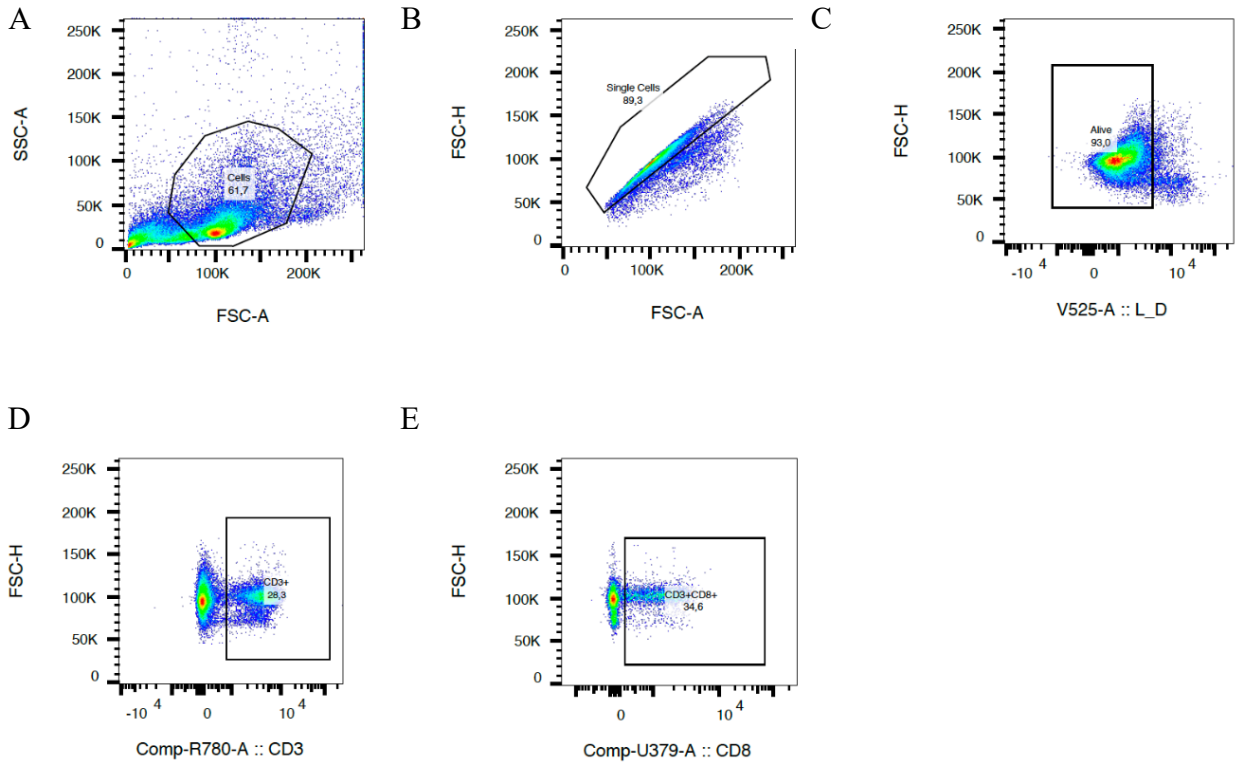
cytokine kit (Meso Scale Discovery, USA) to determine circulating concentrations of IFN- γ, IL-10, IL-13, IL-1β, IL-4, IL-5, IL-6, and TNF-α and chemokine KC/GRO (keratinocyte chemoattractant/human growth-regulated oncogene) following the manufacturer's instructions.

6.2.5 Immune Cell Phenotype Analysis

Four multicolor flow cytometry panels were design and splenocytes were stained with different antibodies as follow: T cell panel: CD4 (FITC), CD3(APC-Vio770), CD8 (BUV395), CD28 (PE-Vio770), CD25 (PercP-eFluor710), CD127 (AlexaFluor594), CD27 (BUV737); B cell panel: CD45RA (AlexaFluor40), CD80 (PE), CD27 (BUV737), CD74 (OX6, PerCP); Natural killer cells panel: CD161 (Alexa647), OX62 (PE), CD74 (OX6, PerCP), CD3 (APC-Vio770); Monocytes and macrophages panel: CD68 (APC-Vio770), CD11bc (PE-Vio770). The proportion of CD3+CD8- cells were considered as CD3+CD4+ cells because the CD4-fluorescein isothiocyanate did not stain properly. All panels contained viability dye (Zombie Yellow-V525). After incubation, cells were washed with PBS and fixed in paraformaldehyde (10 g/l; Thermo Fisher Scientific) in phosphate-buffered saline. Cell events were collected within 72 h of preparation on a BD LSRFortessa X20 SORP flow cytometer and data was analyzed using FlowJo v10 (USA). Regarding the gating strategy, lymphocyte populations were first gated based on forward scatter (FSC) area versus side scatter (SSC) area. Debris and doublets were then excluded, and viable cells were identified in all stained panels based on FSC area versus FSC height. For live lymphocytes, total T cells were defined as CD3+ and within CD3+ cells, helper and cytotoxic T cells were then identified by gating on CD8- and CD8+, respectively. Helper and cytotoxic T cells expressing IL-2 α chain receptor were identified as CD3+CD8-CD25+ and CD3+CD8+CD25+. An example of a gating strategy is presented in **Figure 6-1**. B cells were identified as CD45RA+.

Activated B cells were identified from CD45RA+ as CD45RA+CD80+. Macrophages were gated as CD68+. Within OX62+, dendritic cells were defined as OX62+OX6. Natural killer cells were identified as CD161+CD3-. The median fluorescence intensity (MFI) of CD25+ in CD3+, CD4+ and CD8+ was determined. Since there was no difference in the number of splenocytes between groups, only the proportion of immune cells are presented.

Figure 6-1 Gating strategy. (A) Lymphocyte population based on forward scatter area versus side scatter area; (B) Single cells selected, doublets excluded; (C) cells alive were identified using viability dye (Zombie Yellow-V525); (D) total T cells population identified as CD3+ within the live lymphocytes population; (E) cytotoxic T cells were gated within the CD3+ population and identified as CD8+ cells.



Abbreviations: CD, cluster of differentiation; SSC-A, side scatter area; FSC-A, forward scatter area; FSC-H, forward scatter height.

6.2.6 *Ex vivo* cytokine production by mitogen-stimulated cells

The measurement of cytokines production by mitogen-stimulated cells in spleen has been previously described (Blewett, Gerdung *et al.,* 2009) Briefly, cells (1.25 x 10^6 cells/ml) were cultured in 3 ml RMPI-1640 medium (as above) for 48 h at 37ºC and 5% CO_2 without mitogen (unstimulated) or with mitogens including phorbol 12-myristate 13-acetate plus ionomycin (PMA+I; 2 μg/ml); lipopolysaccharide (LPS; 5 μg/ml); pokeweed (PWM; 55 μg/ml). PMA+I activates T cells, LPS activates primarily antigen presenting cells (APC), and PWM activates T cells and APC. After incubation, cells were centrifuged for 10 minutes at 1000 rpm and supernatants collected and stored at -80°C until analyses. Concentrations of interleukin (IL)-1β, IL-2, IL-6, IL-10, tumor-necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) were measured by commercial ELISA kits according to manufacturer's instructions (R&D Systems, Minneapolis, MN, USA) and as previously described (Blewett, Gerdung *et al.,* 2009). The detection limits for all cytokines were 15.6-4000 pg/ml (R&D Systems, Minneapolis, MN, USA). Cytokine concentrations were quantified using a microplate reader (SpectraMax 190; Molecular Devices, Sunnyvale, CA, USA) and all measurements were conducted in duplicates, with CV <10%. IL-1β was only measured in the supernatant of LPS and PWM stimulated cells and IL-2 was only measured in the supernatant of PMA+I and PWM stimulated cells.

6.2.7 *Ex vivo* T cell proliferation assay

96-well plates were coated with 1 μg /mL of anti-CD3 and were incubated overnight at 4°C. Frozen splenocytes were warm thawed for 10 minutes at 37°C before being washed with DMSO in 10% RPMI 1640 medium (Life Technologies, Burlington, ON, Canada). Afterwards, cells were transferred in 5mL 10% RPMI to 10mL culture tubes and incubated for 4 hours at 37°C.

After incubating, cells were washed and resuspended in 10% RPMI. A haemocytometer was used to count live cells using trypan blue dye exclusion (Sigma-Aldrich) to assess cell viability. The 96-well plates were set up to get 1.25x10^6 cells/mL in 200uL. Samples were added in triplicate, with anti-CD28 stimulation (0.3 ug/mL) of previously coated anti-CD3 wells along with unstimulated cells (Pearson, Mensah *et al.,*). Plates were then incubated at 37°C for 72 hours. After incubation, cell numbers were fixed with 100uL 4% PFA for 20 minutes, followed by a 20 minute incubation with crystal violet (Acros Organic) solution (0.1%, w/v, with ethanol 2%, v/v in 0.5 M Tris-C1, pH 7.8; 100 ul per well) at room temperature. The stained cell layer was rinsed thoroughly with deionized water, vacuum aspirated and incubated with sodium dodecyl sulfate (SDS) solution (0.1%, w/v, with ethanol 50%, v/v, in 0.5 M Tris-C1, pH 7.8; 100 uL per well) for 30 minutes at room temperature. Meanwhile, crystal violet was completely released from the cells into the supernatant. The supernatant was scanned in a microplate reader (SpectraMax 190; Molecular Devices, Sunnyvale, CA, USA) and read at a fixed wavelength of 590 nm. All samples were measured in triplicate. One male rat fed the LFD did not have enough viable cells to perform the assay and its data was excluded from the analysis.

6.2.8 Statistical Analysis

Data are reported as mean ± standard error of the mean (SEM) unless indicated otherwise. Data was analyzed using one-way ANOVA in SAS (v9.4, Cary, NC) with diet as the main effect. The study was powered to assess significant differences in immune function (i.e. *ex vivo* cytokine production as the primary outcome) as main effect. In cases where a significant main effect was found, post hoc analysis was performed using the DUNCAN adjustment to determine differences

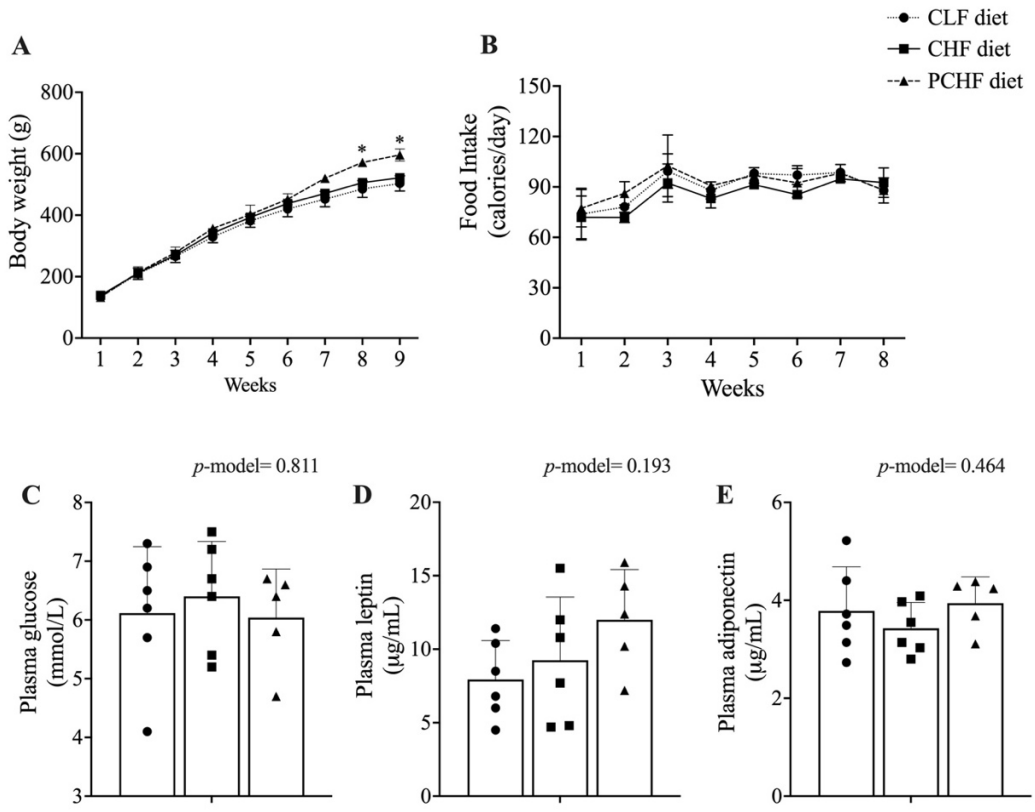
between diet groups. Variables that were not normally distributed were log-10 transformed prior to statistical analysis. Differences at $P \leq 0.05$ (two-sided) were considered significant.

6.3 Results

6.3.1 Anthropometric characteristics and daily food intake

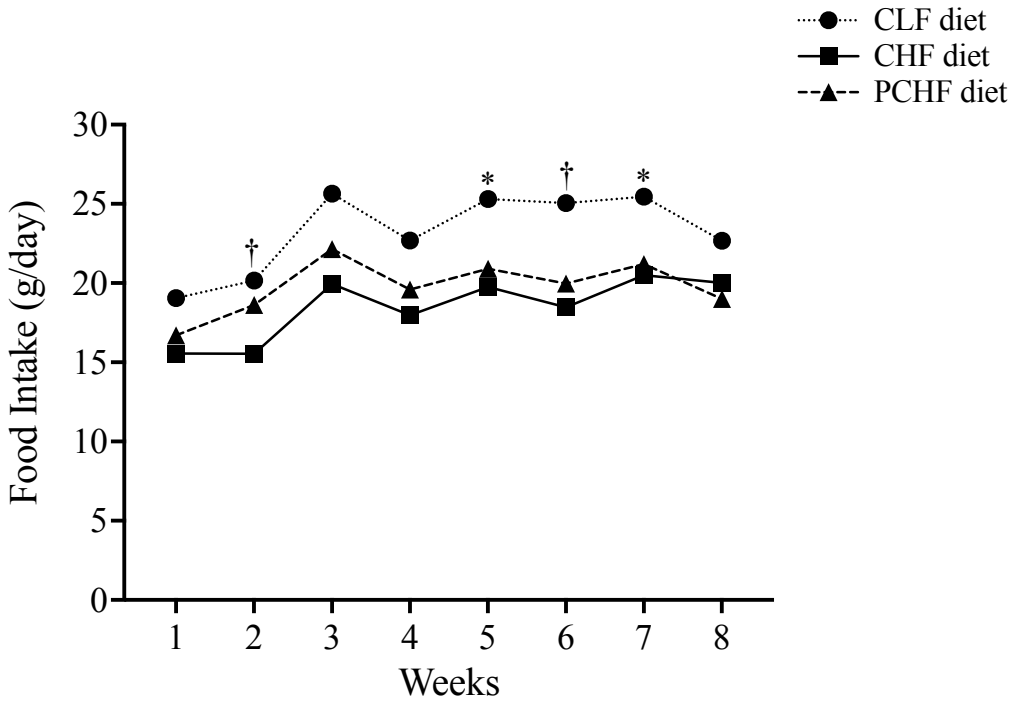
Male Wistar rats fed the PCHF diet had a significantly higher body weight at the end of the feeding experiment when compared to the CLF diet (all $p < 0.05$, **Figure 6-2A**). No changes in body weight were found between the CLF and the CHF diets (all $p > 0.05$). Rats from the CLF group had a significant higher food intake (g/day) when compared to the CHF and PCHF groups ($p < 0.05$, **Figure 6-3**. Consequently, the calorie intake per day was similar between the groups (**Figure 6-2B**). Animal and intestinal length, organ's weight and splenocytes count (all $p > 0.05$, **Table 6-3**) were all unaffected by the dietary treatments.

Figure 6-2 Body weight, caloric intake and plasma metabolites of Wistar Rats fed the three experimental diets. (A) body weight; (B) caloric intake; (C) non-fasting plasma levels of glucose; (D) leptin and (E) adiponectin of male Wistar rats fed the three experimental diets. Values are presented as mean ± SEM. * means significantly different ($p < 0.05$) from the CLF diet based on the one-way ANOVA test with the Duncan adjustment for multiple comparisons.



Abbreviations: CLF, control low-fat; CHF, control high-fat; PC, phosphatidylcholine; PCHF, PC high-fat.

Figure 6-3 Food intake in g/day of male Wistar rats fed the three experimental diets. Values are presented as mean ± SEM. * CLF significantly different ($p < 0.05$) from both the CHF and the PCHF diet; † CLF significantly different from the CHF diet only based on the one-way ANOVA test with the Duncan adjustment for multiple comparisons performed at each time point.



Abbreviations: CLF, control low-fat; CHF, control high-fat; PCHF, PC high-fat; PC, phosphatidylcholine.

6.3.2 Plasma metabolites

There was no significant difference in plasma glucose levels amongst dietary groups (all $p > 0.05$, **Figure 6-2C**). Plasma level of adiponectin and leptin were similar between groups (all $p > 0.05$, **Figures 6-2D, 6-2E**). In addition, no significant differences were found in the concentration of plasma cytokines and chemokines between groups (Table 6.3).

Table 6-3 Anthropometric and plasma cytokine data of male Wistar rats fed the three experimental diets.

Variables[1]	CLF diet (n=6)	CHF diet (n=6)	PCHF diet (n=5)	*p* model
Animal length (cm)	25.2 ± 0.31	25.0 ± 0.36	25.4 ± 0.51	0.779
Spleen weight (g)*	1.36 ± 0.12	1.49 ± 0.08	1.65 ± 0.04	0.166
Splenocytes, n x 10^6/100 g tissue*	12.8 ± 3.22	7.62 ± 0.84	9.08 ± 1.33	0.230
Liver weight (g)	26.1 ± 1.33	25.7 ± 1.13	28.3 ± 1.30	0.325
Intestine length (cm)	123.2 ± 4.14	121.3 ± 3.14	125.6 ± 0.6	0.662
Plasma cytokines				
IL-13 (pg/mL)	1.81 ± 0.31	2.78 ± 0.27	1.83 ± 1.16	0.381
IL-1β (pg/mL)	32.4 ± 4.28	38.5 ± 8.88	18.3 ± 13.0	0.360
IL-4 (pg/mL)	1.67 ± 0.48	1.19 ± 0.24	0.98 ± 0.50	0.512
IL-5 (pg/mL)	110.9 ± 7.62	116.9 ± 13.9	83.4 ± 22.2	0.298
IL-6 (pg/mL)	58.3 ± 9.38	60.1 ± 11.2	37.0 ± 11.1	0.328
TNF-α (pg/mL)	10.6 ± 1.09	10.8 ± 1.24	9.69 ± 0.83	0.765
IL-10 (pg/mL)	16.1 ± 0.60	12.7 ± 2.53	12.9 ± 4.52	0.614
IFN-γ (pg/mL)	8.98 ± 2.13	8.09 ± 1.72	8.36 ± 4.64	0.965
KC/GRO (pg/mL)*	65.4 ± 19.8	54.6 ± 16.8	29.7 ± 5.03	0.345

[1]Values are means ± SEM, n=6. Groups that do not share the same letter are significantly different based on the one-way ANOVA test with the Duncan adjustment for multiple comparisons test ($p < 0.05$). CLF, control low-fat; CHF, control high-fat; IFN-γ, interferon gamma; IL, interleukin; KC/GRO, keratinocyte chemoattractant/human growth-regulated oncogene; PCHF, PC high-fat; PC, phosphatidylcholine; TNF-α, tumor necrosis factor alpha. *Analysis performed on log-transformed values.

6.3.3 Liver total fatty acid composition

Despite the fact that all three diets were closely matched in term of fatty acids composition, several changes in liver total fatty acid composition were noted (**Table 6-4**). The PCHF diet led to an increased proportion of docosapentaenoic acid (DPA) and total PUFA when compared to the CLF diet. Both the PCHF and CHF diets had a higher concentration of α-linolenic acid (ALA) and total n-6 PUFA and a lower proportion of total SFAs when compared to the CLF. The PUFA/SFA ratio was higher in PCHF when compared to both the CHF and CLF diets. The CHF had a higher

PUFA/SFA ratio compared to the CLF. No changes were found in the proportion of arachidonic acid (AA) or docosahexaenoic acid (DHA) among groups.

Table 6-4 Liver total lipids fatty acid composition in male Wistar rats fed the three experimental diets.

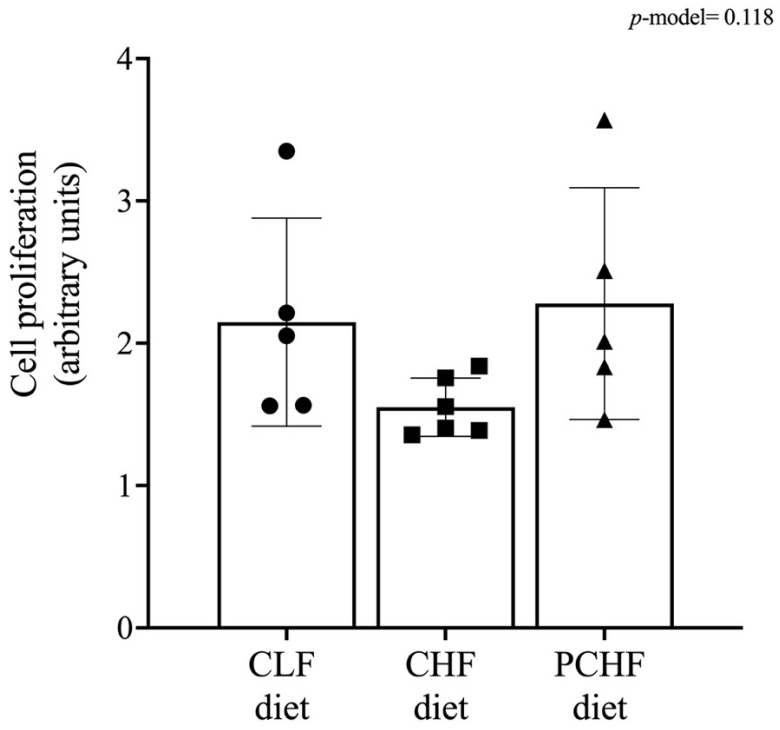
Fatty Acid[1]	CLF diet (n=6)	CHF diet (n=6)	PCHF diet (n=5)	*p* model
	(% of total fatty acids)			
14:0	0.51 ± 0.07[a]	0.39 ± 0.03[b]	0.33 ± 0.03[b]	0.081
14:1	0.19 ± 0.02[a]	0.15 ± 0.01[ab]	0.13 ± 0.01[b]	0.068
16:0	22.7 ± 0.64[a]	20.4 ± 0.33[b]	19.9 ± 0.69[b]	0.008
16:1n9	0.33 ± 0.02	0.41 ± 0.03	0.36 ± 0.03	0.173
16:1n7	1.59 ± 0.28[a]	0.75 ± 0.06[b]	0.69 ± 0.14[b]	0.005
17:0	0.30 ± 0.01[a]	0.26 ± 0.02[a]	0.20 ± 0.01[b]	0.003
18:0	19.1 ± 1.06	17.4 ± 0.61	16.3 ± 1.66	0.277
18:1n9	13.4 ± 1.28	17.0 ± 0.95	15.7 ± 2.35	0.297
18:1n7	2.50 ± 0.14[a]	1.37 ± 0.09[b]	1.20 ± 0.10[b]	<.001
18:2n6	13.9 ± 0.37[b]	18.5 ± 0.72[a]	20.1 ± 1.64[a]	0.003
20:0	0.15 ± 0.02	0.14 ± 0.03	0.13 ± 0.04	0.939
18:3n3 (ALA)	0.90 ± 0.07[b]	1.48 ± 0.13[a]	1.81± 0.26[a]	0.009
20:2n6	0.36 ± 0.02[b]	0.43 ± 0.02[ab]	0.44 ± 0.03[a]	0.060
20:3n6	0.66 ± 0.06	0.62 ± 0.04	0.76 ± 0.09	0.284
20:4n6 (AA)	16.8 ± 1.22	14.9 ± 0.73	15.2 ± 2.13	0.612
20:5n3 (EPA)	0.33 ± 0.04	0.40 ± 0.03	0.43 ± 0.01	0.155
24:0	0.01 ± 0.01	0.01 ± 0.0	0.01± 0.0	0.481
24:1n9	0.30 ± 0.02[b]	0.38 ± 0.02[ab]	0.49 ± 0.07[a]	0.017
22:4n6	0.11 ± 0.01[a]	0.07 ± 0.01[b]	0.12 ± 0.01[a]	0.002
22:5n6	0.02 ± 0.00[a]	0.01 ± 0.00[b]	0.01± 0.00[b]	0.003
22:5n3 (DPA)	0.63 ± 0.09[b]	0.81 ± 0.08[ab]	0.98 ± 0.10[a]	0.062
22:6n3 (DHA)	5.23 ± 0.52	4.17 ± 0.27	4.73 ± 0.64	0.320
Total SFA	42.8 ± 0.68[a]	38.6 ± 0.62[b]	36.9 ± 1.14[b]	0.001
Total MUFA	18.3 ± 1.50	20.1 ± 0.94	18.6 ± 2.49	0.728
Total PUFA	38.9 ± 1.46[b]	41.3 ± 0.85[ab]	44.6 ± 1.52[a]	0.029
Total n-6 PUFA	31.8 ± 0.90[b]	34.5 ± 0.59[a]	36.6 ± 1.05[a]	0.006
Total n-3 PUFA	7.10 ± 0.57	6.86 ± 0.29	7.95 ± 0.50	0.242
Ratio n-6/n-3	4.56 ± 0.24	5.06 ± 0.16	4.65 ± 0.17	0.162
Ratio PUFA/SFA	0.91 ± 0.04[c]	1.07 ± 0.03[b]	1.21 ± 0.03[a]	0.001

[1]Values are means ± SEM. Groups that do not share the same letter are significantly different based on the one-way ANOVA test with the Duncan adjustment for multiple comparisons test ($p < 0.05$). AA, arachidonic acid; ALA, α-linolenic acid; CLF, control low fat; CHF, control high-fat; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid MUFA, monounsaturated fatty acids; PCHF, PC high-fat; PC, phosphatidylcholine; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

6.3.4 T cell proliferation assay and *ex vivo* cytokine production by splenocytes after mitogen stimulation

Diet had little impact on T cell proliferation by splenocytes after stimulation with anti-CD3/anti-CD28 ($p = 0.118$) as shown in **Figure 6-4.** *Ex-vivo* cytokine production by splenocytes isolated from male Wistar rats is presented in **Table 6-5**. After PMA+I stimulation, rats fed with the CHF diet had a significantly lower production of IL-2 and TNF-α when compared to the CLF and PCHF diets (both $p < 0.05$) and a trend towards a lower IL-6 production when compared to the CLF diet only ($p = 0.055$). No changes were observed in IFN-γ and IL-10 production between groups (both $p > 0.05$). Following LPS stimulation, there was no significant change in cytokine production among diet groups (all $p > 0.05$). After PWM stimulation, there was an increased production of IL-6 in the CHF group when compared to the CLF group, although it did not reach significance ($p > 0.05$). There were no significant differences between the PCHF and the CLF groups after PWM stimulation (all $p > 0.05$).

Figure 6-4 Effect of diet on T cell proliferation following stimulation with anti-CD3/anti-CD28 in male Wistar rats. Values are means ± SEM. p-model = 0.118.



Abbreviations: CLF, control low-fat; CHF, control high-fat; PC, phosphatidylcholine; PCHF, PC high-fat.

Table 6-5 Ex vivo cytokine production by mitogen-stimulated splenocytes of male Wistar rats fed the three experimental diets.

Variable[1]	CLF diet (n=6)	CHF diet (n=6)	PCHF diet (n=5)	*p* model
PMA+I (T cell mitogen)				
IL-2 (pg/ml)	2146.4 ± 173.2[a]	883.3 ± 223.4[b]	2246.8 ± 385.0[a]	0.005
IL-10 (pg/ml)	166.8 ± 37.9	207.6 ± 24.6	225.6 ± 13.9	0.407
TNF-α (pg/ml)	102.1 ± 13.8[a]	61.2 ± 8.5[b]	131.5 ± 12.8[a]	0.004
IL-6 (pg/ml)	220.1 ± 52.8[a]	54.1 ± 18.7[b]	137.7 ± 46.8[ab]	0.055
LPS (Bacterial challenge)				
IL-1β (pg/ml)	28.0 ± 4.68	33.5 ± 5.27	34.4 ± 3.93	0.595
IL-10 (pg/ml)	487.8 ± 110.3	569.9 ± 75.6	663.0 ± 42.9	0.445
TNF-α (pg/ml)*	189.0 ± 18.9	172.7 ± 21.7	226.0 ± 28.7	0.288
IL-6 (pg/ml)	518.9 ± 57.1	565.7 ± 82.6	592.6 ± 90.5	0.814
PWM (T and APC cells mitogen)				
IL-1β (pg/ml)	110.6 ± 13.24	152.9 ± 34.3	123.8 ± 25.6	0.537
IL-2 (pg/ml)	432.8 ± 71.0	357.6 ± 74.6	425.9 ± 26.7	0.673
IL-10 (pg/ml)	385.2 ± 52.8	527.2 ± 61.6	514.0 ± 66.6	0.199
TNF-α (pg/ml)*	311.8 ± 45.8	268.0 ± 31.9	398.8 ± 68.1	0.536
IFN-γ (pg/ml)*	119.0 ± 49.3	315.5 ± 172.6	311.9 ± 199.2	0.200
IL-6 (pg/ml)	1258.6 ± 215.6[b]	2043.7 ± 323.0[a]	1612.7 ± 149.6[ab]	0.109

[1] Values are means ± SEM. Groups that do not share the same letter are significantly different based on the one-way ANOVA test with the Duncan adjustment for multiple comparisons test ($p < 0.05$). CLF, control low-fat; CHF, control high-fat, PCHF, PC high-fat; PC, phosphatidylcholine; IFN-γ, interferon gamma; IL, interleukin; TNF-α, tumor necrosis factor alpha.
*Analysis performed on log-transformed values.

6.3.5 Splenocyte phenotypes

Proportions of immune cell phenotypes of male Wistar rats fed the three experimental diets are presented in **Table 6-6**. Rats fed with the PCHF diet had a lower proportion of CD3+ cells when compared to both the CLF and CHF diets ($p < 0.05$). There were no significant differences in the proportion of helper T cells (CD3+CD4+) and cytotoxic T cells (CD3+CD8+) as well as activated T cells (CD3+CD25+) among groups (both $p > 0.05$). The MFI of CD25+ in CD4+ was higher in the CHF when compared to the CLF group only. No changes were found in

the MFI of CD25+ in CD3+ and CD8+. The proportions of total CD127+ cells were lower in the PCHF diet when compared to the CHF diet. No significant changes were found in the proportion of B cells between groups nor in the proportion of activated B cells (CD45RA+CD80+). In addition, no significant differences in the proportion of CD28+ (co-stimulatory molecule), CD27+ (memory marker), natural killer cells (NK, CD3-CD161+) and macrophages (CD11bc+), OX6+ (MHC II cells) were noted.

Table 6-6 Splenocyte phenotype of male Wistar rats fed the three experimental diets.

Phenotype[1]	CLF diet (n=6)	CHF diet (n=6)	PCHF diet (n=5)	*p* model
		% of total gated cells		
Total CD3+ (T cells)	38.9 ± 1.94[a]	36.9 ± 1.54[a]	31.7 ± 1.19[b]	0.024
		% of CD3+ gated cells		
CD4+ (helper T cells)	80.9 ± 0.91	78.7 ± 1.68	79.1 ± 1.96	0.556
CD8+ (cytotoxic T cells)	20.1 ± 1.66	23.1 ± 2.79	20.2 ± 2.24	0.575
CD25+ (activated T cells)*	14.0 ± 1.39	15.9 ± 1.10	16.7 ± 0.56	0.280
CD4+CD25+	23.2 ± 2.37	22.7 ± 3.01	20.8 ± 2.30	0.814
CD8+CD25+	16.6 ± 1.42	18.4 ± 1.33	20.5 ± 0.67	0.137
		% of total gated cells		
Total CD28+ (Co-stimulatory molecule)	19.9 ± 4.90	31.5 ± 4.22	23.8 ± 4.30	0.235
Total CD127+	4.03 ± 0.31[ab]	4.36 ± 0.41[a]	3.05 ± 0.42[b]	0.079
Total CD80+	11.3 ± 0.71	10.4 ± 0.70	11.1 ± 1.30	0.758
Total CD45RA+ (B cells)	35.5 ± 2.05	38.9 ± 1.81	31.5 ± 5.40	0.411
CD45RA+CD80+	10.6 ± 0.91	10.2 ± 1.15	11.1 ± 1.26	0.839
Total CD68+ (macrophages)*	9.55 ± 0.90	9.48 ± 0.42	7.90 ± 1.12	0.319
Total OX6+ (MHC class II+)	40.8 ± 1.16	42.2 ± 3.30	39.5 ± 4.98	0.851
Total OX62+	10.7 ± 1.63	11.6 ± 2.00	9.92 ± 2.00	0.818
OX62+OX6+ (dendritic cells)	1.82 ± 0.31	2.78 ± 0.27	1.83 ± 1.16	0.381
CD161+CD3- (natural killer cells)	9.36 ± 0.64	11.2 ± 0.89	10.8 ± 1.16	0.317
		CD25+ MFI		
CD3+	2829.5 ± 296.9	3364.3 ± 300.7	2595.6 ± 270.3	0.202
CD4+	2280.2 ± 238.9[b]	3099.5 ± 117.4[a]	2573.2 ± 280.6[ab]	0.045
CD8+*	1105.7 ± 242.0	1147.8 ± 121.1	1060.8 ± 172.5	0.845

[1]Values are presented as mean ± SEM. Groups that do not share the same letter are significantly different based on the one-way ANOVA test with the Duncan adjustment for multiple comparisons test ($p < 0.05$). CD3+CD8-CD28+ (average 58.5 ± 13.6); CD3+CD8+CD28+ (average 71.9 ± 3.80); CD3+CD8-CD127+ (4.60 ± 0.77); CD3+CD8+CD127+ (6.17 ± 0.82); Total CD11bc+ (average 16.0 ± 1.43). CLF, control low-fat; CHF, control high-fat, MFI, median fluorescence intensity; PCHF, PC high-fat; PC, phosphatidylcholine.
*Analysis performed on log-transformed values.

6.4 Discussion

In this study, we aim to determine the effect of egg-PC supplementation on immune function in male Wistar rats fed a HFD. Our data support our hypothesis that feeding a HFD impairs immune responses and that adding PC as part of a HFD attenuates the obesity-related T cell dysfunction. Of note, this is the first study where the proportion of different fatty acid in the diets were matched between the low-fat and high-fat diets to assess immune responses in this model. This is crucial since fatty acids are known to influence immune function in that saturated and omega-3 polyunsaturated fatty acids exert pro- and anti-inflammatory properties, respectively (Calder 1998, Calder 2009). Therefore, matching the proportion of fatty acids when assessing the effect of choline forms (water vs. lipid-soluble) on the immune system is important. Despite diets being matched for the fatty acid composition, in liver the PCHF diet led to a lower proportion of SFAs and a higher proportion of DPA, PUFAs and an overall higher PUFA/SFA ratio. A higher PUFA/SFA ratio has been associated with a lower risk of cardiovascular disease (Kang, Shin *et al.,* 2005).

We first demonstrated that providing a HFD with PC increased rats' body weight compared to the CLF at the end of the feeding experiment (weeks 8 and 9). We previously showed that feeding PC and other lipid soluble form of choline early in life improve pups' growth (Azarcoya-Barrera, Field *et al.,* 2021). In order to be absorbed by the enterocyte, PC is first broken down to Lyso-PC by Phospholipase A2 and once in the enterocyte can be reacetylated to PC or further be broken down into GPC (Lewis, Field *et al.,* 2015). GPC has been shown to increased plasma levels of growth hormone, which can increases growth, tissue regeneration, and cell production (Kawamura, Okubo *et al.,* 2012). The CHF diet had no impact on body weight compared to the

CLF diet through the duration of the experiment. This is mainly attributable to the same caloric intake by the CLF group when compared to the HFD diets.

We demonstrated that feeding a HFD containing only FC impairs T cell responses in male Wistar rats after PMA+I stimulation by decreasing the production of IL-2 and TNF-α, a Th1 pro-inflammatory cytokine. In addition, rats fed the HFD had a lower T cell proliferation rate after stimulation with anti-CD3/anti-CD28, although it did not reach significance (p = 0.118). These results are consistent with previous studies where feeding Wistar rats with a cafeteria diet also impaired IL-2 production after ConA stimulation (a T cell mitogen) (Lamas, Martinez *et al.,* 2002). We also showed that the CHF group produced less IL-2 despite having an increase expression of CD25 in helper T cells as measured by MFI. This is consistent to some extent with a previous study from our group where we demonstrated that PBMCs isolated from individuals with obesity and T2D produced less IL-2, TNF-α and IL-6 when stimulated with phytohemagglutinin (another T cell mitogen) than individuals with obesity who were metabolically healthy and this, despite having a higher proportion of helper T cell expressing CD278 which plays a role in IL-2 synthesis and T cell proliferation (Dong, Juedes *et al.,* 2001, Richard, Wadowski *et al.,* 2017). The underlying mechanism as to how PC modulates T cell function are still unclear. PC, as a lipid-soluble form of choline, is mostly absorbed and secreted through chylomicrons and enter the bloodstream first to be potentially delivered to peripheral organs (i.e. including the spleen) instead of reaching the portal vein first and going to the liver (Lewis, Field *et al.,* 2015). Feeding a diet containing primarily PC could potentially affect the incorporation of PC into cell membranes (ex. splenocytes) and therefore modulate its composition. In that regard, we have demonstrated that a diet containing a 100% PC during the suckling period increased the proportion of PC in splenocyte cell membranes of suckled pups (Lewis, Richard *et al.,* 2016). How the incorporation of PC into

the lipid rafts occurs which might affect immune responses remains to be investigated. In addition, in vitro studies by our group have shown that incubating splenocytes with Lyso-PC (the form of PC readily available to cell) increased the proliferation rate and IL-2 production as measured by water-soluble tetrazolium salt assay, suggesting that PC can indeed modulate T cell function (Lewis, Richard *et al.,* 2016).

In our previous studies we have noted that PC supplementation early in life primarily affects T cell function and to a lesser extent B cell function. Moreover, in humans, we have also reported that obesity and T2D affect particularly T cells and neutrophil function, while no effect on B cells function was observed as measured by *ex vivo* cytokine production after LPS stimulation. Consistent with previous study, we showed that a HFD primarily affects T cells function whereas no changes were seen in APC (B cells, monocytes, and dendritic cells) function. Although obesity is associated with a higher proportion of pro-inflammatory M1-like macrophages in adipose tissue (Reilly & Saltiel 2017), the proportion of circulating B cell, monocyte and NK cells appears to be unaltered as reported in some studies (Bahr, Jahn *et al.,* 2018, Richard, Wadowski *et al.,* 2017). In the current study, we did not find significant differences in the frequency of monocytes/macrophages (CD68+), B cells (CD45RA+), activated B cells (CD45RA+CD80+), OX6+ cells (MHC II), dendritic cells (OX6+OX62+) or NK cells (CD3-CD161+) in spleen among groups.

Chronic systemic low-grade inflammation is characterized by elevated circulating levels of CRP, TNF-α and IL-6 (Calder, Ahluwalia *et al.,* 2011). Adipose tissue appears to be an important contributor to systemic inflammation and its related metabolic complications such as insulin resistance. Indeed, M1-like macrophages are known to promote the secretion of cytokines such as TNF-α and IL-6 (Reilly & Saltiel 2017) and both IL-6 and TNF-α have been linked to

insulin resistance by impairing insulin receptor signaling (Kim, Bachmann *et al.,* 2009, Nieto-Vazquez, Fernandez-Veledo *et al.,* 2008). Insulin resistance is thought to play a key role in modulating immune cell responses since immune cells that lack the insulin receptor have been shown to have an impaired cytokine secretion and lower antigen presentation activity upon influenza infection in a rodent model (Tsai, Clemente-Casares *et al.,* 2018). High-fat feeding has also been reported to promote intestinal inflammation and gut permeability, which can contribute to systemic inflammation (Rohr, Narasimhulu *et al.,* 2020). In addition, high-fat feeding has also been associated with higher leptin and lower adiponectin concentrations in rodents (Handjieva-Darlenska & Boyadjieva 2009, Ribot, Rodriguez *et al.,* 2008). Yet in our study, plasma cytokine levels were unaffected by dietary treatments along with non-fasting glucose and leptin and adiponectin concentrations, although we observed trends towards an "obesity phenotype". This could be explained at least in part by the fact that rats fed the CLF diet were consuming slightly more g/day of diet and that no differences in body weight were observed between the CLF and the CHF group. Pair-feeding the animal on the CLF to the ones on the HF diets would have most likely led to differences in those parameters. Another explanation could be that all our diets were matched closely for the proportion of fatty acids whereas most diets used to induce obesity are higher in SFAs compared to typical low-fat diets. Previous studies have shown that SFAs, such as palmitic acid and lauric acid, are capable of initiating an inflammatory response by binding to Toll-like receptor 4 (TLR-4) (Rogero & Calder 2018). Finally, glucose measurements were done in the non-fasting state and since the CLF diet was much higher in carbohydrate, this could explain the similar plasma glucose values between groups. Overall, our results suggest that the changes observed in T cell function in the CHF group are likely attributable to other mechanisms which could include increased gut permeability and increased bacterial translocation.

Although our study has strengths which include the design of our diets being closely matched for choline content and fatty acid composition and the assessment of several factors that have been proposed to be involved in modulating immune function, some limitations should also be stressed. Although our sample size was enough to see differences in our primary outcome (i.e. *ex vivo* cytokine production), we might have been underpowered for some of our secondary analysis with higher inter-individual variations. Other potential mechanism, including gut permeability, were not assessed which could have provided a better understanding of how PC modulate immune function in the context of a HFD. Moreover, body composition was not measured in this study to determine if there were difference in body fat mass regardless of the similarities in body weight between our diet groups. In addition, a diet containing 100% PC is not physiologically relevant to human consumption, therefore, a dose-response study is needed in order to establish the optimal proportion of PC to modulate T cell responses while being of relevance for human consumption. Finally, although *ex vivo* cytokine production of different mitogens provides an overall understanding of how different immune cells populations respond to a challenge, other methods such as intracellular analysis of cytokines by flow cytometry could provide a better understanding of specific immune cells population response.

6.5 Conclusion

In summary, our results suggest that feeding a HFD impairs T cells function while having little effect on antigen-presenting cells function in male Wistar rats. We showed that providing egg-PC as part of a HFD can normalize T cell responses, as measured by *ex vivo* cytokine production, to some extent in Wistar rats by increasing the production of IL-2 and Th1 cytokines, known to promote T cell proliferation.

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Chapter 7: Final discussion

7.1 Executive summary of findings

7.1.1 Feeding buttermilk-derived choline forms during gestation and lactation modulates *ex vivo* T cell response in rat dams

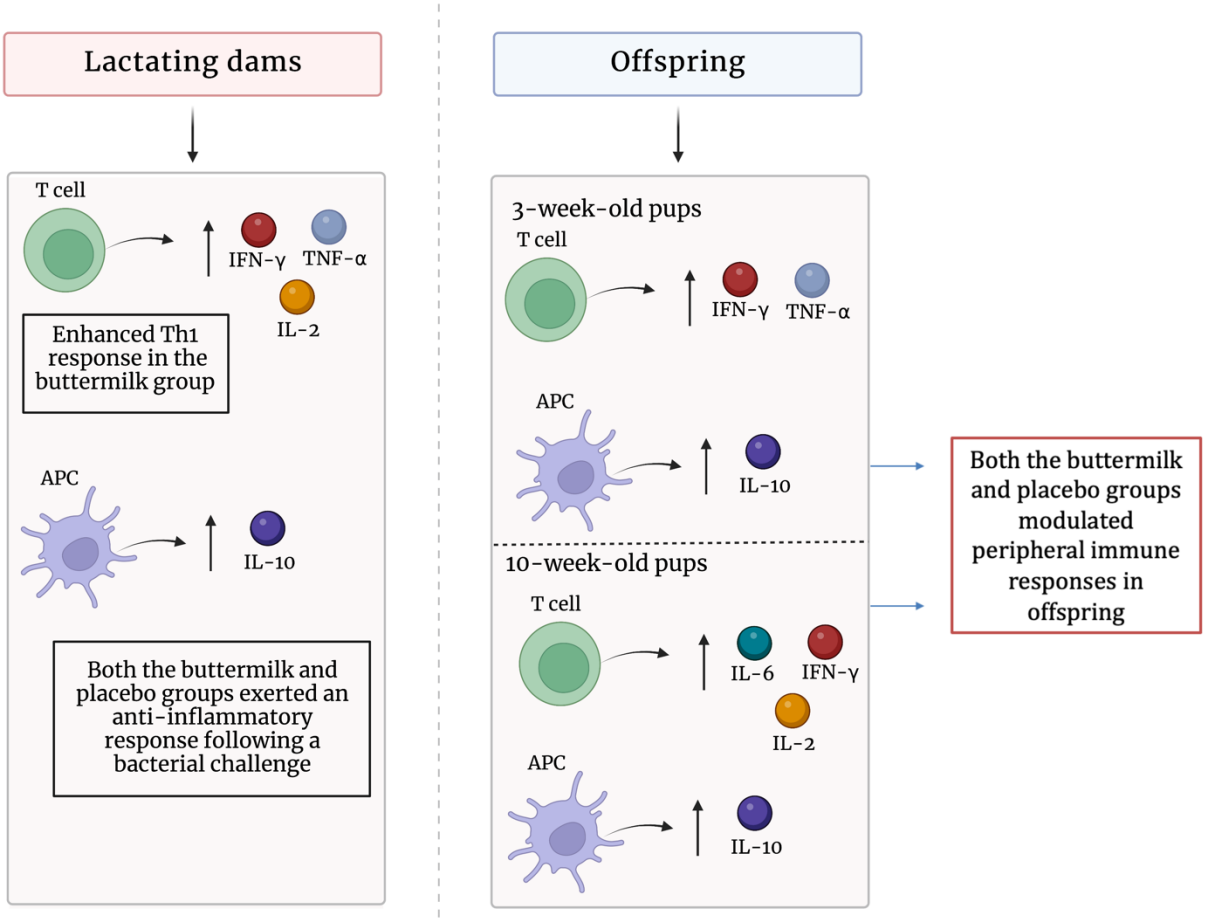
In Chapter 3, we investigated the impact of feeding buttermilk-derived choline forms on immune responses in lactating dams. We hypothesized that feeding whole buttermilk to dams during pregnancy and lactation 1) will have a greater beneficial effect on immune responses of lactating dams compared to our placebo and control diets; 2) will modulate the choline composition of breastmilk in lactating dams. Our findings partially support our hypothesis in that dams that were fed with the buttermilk diet produced more IL-2 and Th1 cytokines following splenocytes stimulation with a T cell mitogen, suggesting better T cell function in dams fed the buttermilk diet (**Figure 7-1**). When stimulated splenocytes with either LPS or OVA, both the placebo and buttermilk diets led to an overall anti-inflammatory response by increasing the production of IL-10. Our second hypothesis was supported by the results presented in Chapter 3. The pups' stomach content, which was reflective of breastmilk composition, was modulated by the choline composition in the diet. Diets that were high in lipid-soluble forms of choline, had a higher proportion of lipid-soluble forms in breast milk.

7.1.2 Buttermilk: an important source of lipid-soluble forms of choline that influences the immune system development in Sprague-Dawley rat offspring

In Chapter 4, we examined the impact of feeding buttermilk-derived choline forms during both the lactation and weaning periods on the immune system development in rat offspring. We hypothesized that feeding whole buttermilk during the lactation and weaning periods will have a

beneficial effect on immune system development in rat pups when compared to our control and placebo diets. Our hypothesis was partially confirmed by the results obtained in this study. Both the buttermilk and the placebo diets increased the proportion of CD28+ cells, Th1 cytokines following T cell mitogen stimulation, and IL-10 following LPS stimulation in suckled pups. At weaning, pups from the buttermilk and placebo groups increased the production of IL-2, IFN-γ and IL-6 following T cell mitogen stimulation and were still producing more IL-10 after LPS stimulation when compared to the control diet only containing FC (**Figure 7-1**). Following a dietary antigen stimulation (ovalbumin), both the placebo and buttermilk diets decreased the production of TNF-α at 10 weeks of age. Our results suggest that both the buttermilk and placebo diets promote a Th1 responses following T cell mitogen stimulation which is associated with a better maturation of immune system. Moreover, diets with a high content of lipid-soluble forms could also be of benefit for the establishment of oral tolerance by decreasing immune responses upon stimulation with a dietary antigen.

Figure 7-1 Summary of the impact of feeding buttermilk during preganancy and lactation on peripheral T cell immune responses of rat dams and their offspring. Created with http:BioRender.com.



Abbreviations: APC, antigen presenting cells; IFN-γ, interferon gamma; IL, interleukin; Th1, T helper 1; TNF-α, tumor necrosis factor alpha.

7.1.3 The lipid-soluble forms of choline enhance the function of the gut-associated immune system.

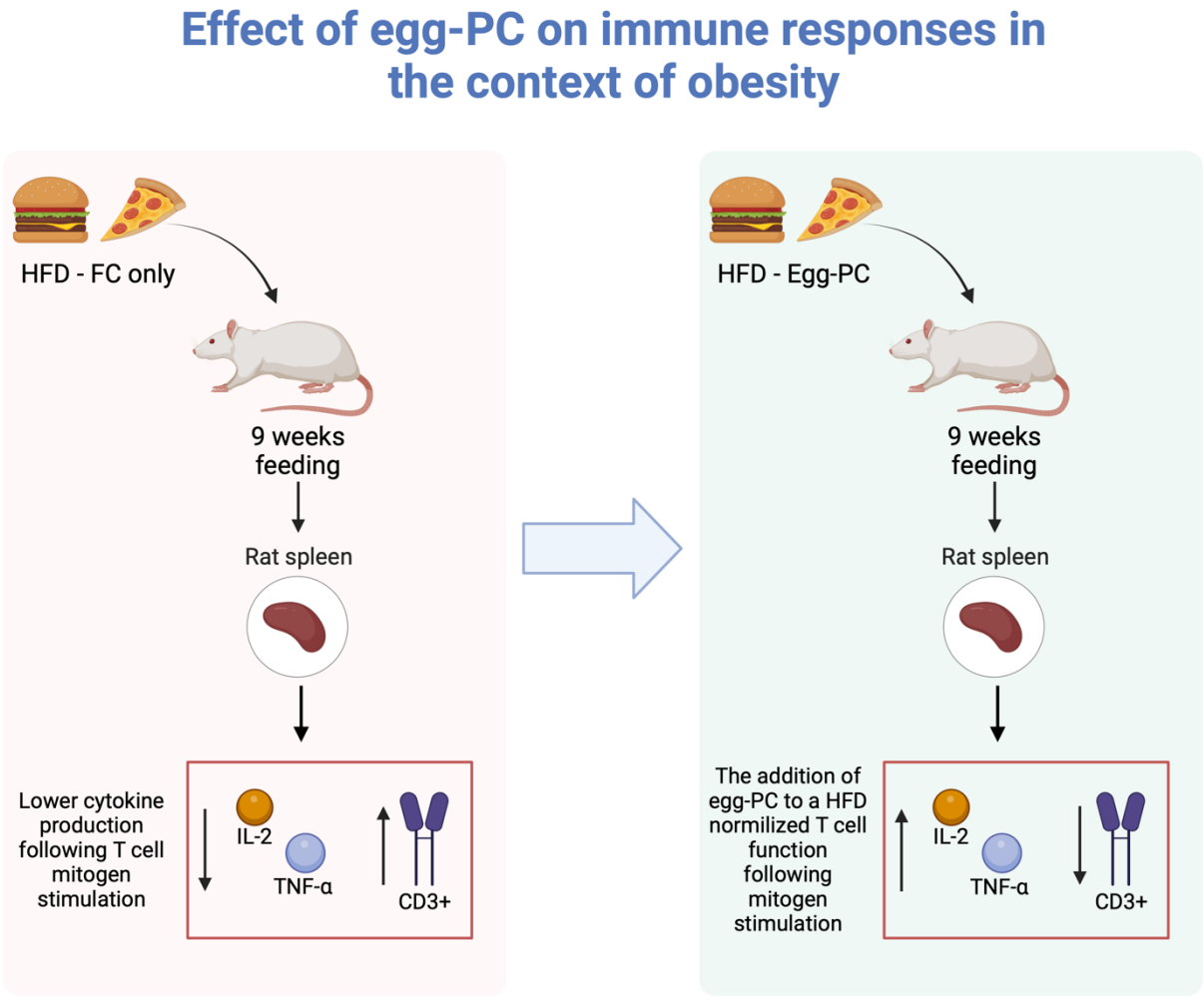
In Chapter 5, we investigated the effect of dietary choline on the development of the GALT. We hypothesized that 1) the form of choline in the diet will affect differently the development of the GALT and, 2) a diet high in lipid-soluble forms of choline content will have a greater impact of the development of the GALT when compared to a diet containing only or mostly water-soluble

forms of choline. Results from this study showed that diets containing at least 50% of lipid soluble forms enhanced T cell responses in the GALT by increasing Th1 cytokine production upon mitogen stimulation. In addition, there was a lower production of IL-2, IL-6 and TNF-α after food antigen stimulation when diets with at least 50% lipid-soluble forms of choline were fed to rats (summarized in Figure 6-2). Moreover, greater immune benefits were observed when the diets were fed during both the suckling and the weaning periods as compared to only during the suckling period. Our results suggest that a mixture of choline forms mostly composed of lipid-soluble forms enhanced immune responses in the GALT.

7.1.4 Egg-phosphatidylcholine attenuates T cell dysfunction in high-fat diet fed male Wistar rats.

In Chapter 6, we examined the effect of providing different sources of choline in the context of a HFD on immune responses in Wistar rats. We hypothesized that 1) feeding a HFD will impaired immune responses and 2) providing PC as part of a HFD will attenuate the obesity-related immune dysfunction. Our hypotheses were confirmed by our results in that feeding a HFD decreased IL-2 and TNF-α production following T cell mitogen stimulation and the addition of PC to a HFD counteracted these effects (**Figure 7-2)**. Our results suggest that feeding a HFD impaired T cells responses, which was attenuated by the addition of PC to the HFD.

Figure 7-2 Summary of the impact of egg-PC on immune responses in the context of obesity. Created with http:BioRender.com.



Abbreviations: CD, cluster of differentiation; HFD, high-fat diet; egg-PC, egg phosphatidylcholine; FC, free choline; IL, interleukin; TNF- α, tumor necrosis alpha.

7.2 General discussion and future directions

7.2.1 Different effects of choline forms on maternal immune function and the development of immune system in offspring.

In a series of animal experiments, we showed that feeding a diet containing choline forms derived from buttermilk and high in SM and PC content improved peripheral T cells responses in

lactating dams. In offspring, we showed that our diet containing buttermilk-derived choline forms along with a diet containing 50% lipid-soluble forms improve Th1 responses which is associated with greater maturation of the immune system. Consistently, previous studies have shown that providing a diet containing a mixture of choline forms during lactation modulated maternal immune responses and had a programming effect on the immune system later in life (Lewis, Richard *et al.,* 2017, Richard, Lewis *et al.,* 2017). Although our initial hypothesis was that our buttermilk diet would have a greater impact on both maternal and offspring immune responses due to its high content of SM, our results suggest that both lipid-soluble forms of choline can positively affect the immune system to similar extent. However, we demonstrated that SM is not only able to modulate anti-inflammatory responses in the context of obesity but is also able to modulate T cell responses upon mitogen stimulation (Mazzei, Zhou *et al.,* 2011, G. Norris, C. Porter *et al.,* 2017, Norris, Jiang *et al.,* 2016, G. H. Norris, C. M. Porter *et al.,* 2017).

Studies presented in this thesis provide evidence that buttermilk is not only a good source of dietary choline during pregnancy, lactation and early in life that can help the population achieving the AI in choline, but also provide a unique distribution of choline forms that can affect beneficially the immune system during lactation and early in life. Moreover, our findings add to the body of evidence suggesting that dairy products should be favored over plant-based beverages, especially in early developmental period, where plant-based beverages are not recommended according to Canada's Food Guide. We demonstrated for the first time that the form of choline in the diet can affect the gut-associated immune responses early in life. Similar to the peripheral immune system, diets containing at least 50% of lipid-soluble forms of choline (PC and SM) and fed during both the lactation and weaning periods modulated to a greater extend immune responses in the gut. However, our study did not assess diets with proportions of lipid-soluble forms of

choline raging from 25% to 50%. Therefore, dose response studies should be considered to determine the optimal lipid/water soluble forms proportion in the diet. In addition, the gut microbiota has emerged as an important regulator of the immune system development. Norris *et al*., (2016) demonstrated that the addition of SM to a HFD increased the relative gram-positive phyla and, in contrast, decreased the relative abundance of gram-negative phyla in mice (Norris, Jiang *et al.,* 2016). In the current study we did not investigate the impact of dietary choline on the composition of the gut microbiota, and therefore, this warrants further investigation.

Our study was the first in demonstrate that the choline form in the diet (specially the lipid-soluble forms) can modulate immune responses to a food antigen to what it appears would be beneficial for the establishment of oral tolerance early in life. However, we did not work with an allergy prone model, and therefore we were not expecting an exaggerated response to a dietary antigen from our animals. Future studies should aim to investigate the role of dietary choline in a model like the Brown Norway rats, which are more susceptible to an Ig-E mediated response to a food allergen (Abril-Gil, Garcia-Just *et al.,* 2015). In addition, the measurement of plasma OVA-specific immunoglobulins concentration should also be considered in future studies to confirm the effect on oral tolerance *in vivo*.

Studies from this thesis presented evidence that the lipid-soluble forms of choline should be consumed in adequate amount during pregnancy and lactation. Epidemiological data from pregnant and lactating women showed that the choline coming from the diet was mainly coming in the form of PC (50%), which aligns with our results (Lewis, Subhan *et al.,* 2014). However, women were most likely to meet choline requirements when consuming eggs and/or dairy products which are a good source of lipid-soluble forms of choline and, therefore, these type of foods should be recommended during these periods (Lewis, Subhan *et al.,* 2014). In contrast, humans

consuming a plant-based diet will be consuming higher proportion of FC relative to other lipid-soluble forms of choline, as is the choline form mainly found in legumes and vegetables (Lewis, Kosik *et al.,* 2014). In addition, our research provides evidence that the forms of choline in breastmilk influence the immune system development and therefore, infant formulas should aim to provide a distribution of choline forms that is representative of breastmilk. In that regard, the choline content in infant formulas has been shown to vary greatly across different formulas, both in term of total choline content and in the forms of choline present (Elde & Van Parys 2022). Therefore, infant formulas would benefit from revisiting their choline content and forms in order to confer greater immune benefit.

Finally, our studies were mostly done in females (lactating dams) and we did not account for sex differences in offspring. Recently, our research group published a study comparing sex differences on the immune system in the context of HFD feeding (see appendix 1) in Wistar rats. We showed that females developed a milder obesity phenotype and that they were able to maintain a higher cytokine production when compared to male rats. Future studies should explore the sex differences on the immune system development early in life to determine whether dietary choline forms modulate females and males' immune responses similarly.

7.2.2 Phosphatidylcholine and obesity

Using an animal model, we demonstrated that a HFD impaired T cell responses by lowering IL-2 production and, that the addition of egg-PC in the diet counteract the effects of feeding a HFD on immune responses in male Wistar rats. Although our study showed some promising results some limitations should be addressed in future studies. Our diet was providing only PC (100%) and no other forms of choline. This would not be physiologically achievable in a human diet where

one always consume a mixture of choline forms. A dose-response study with different doses of PC should be performed in the future to examine if a range more closely to what it has been reported previously in humans (50% PC) could confer the same immune benefits. In addition, examining the effect of a HFD containing a mixture of choline high in lipid soluble forms (buttermilk-derived choline forms also containing SM) should also be investigated in future studies since we did observe greater benefit with the buttermilk diet on T cells responses of lactating dams. Therefore, providing a combination of PC and SM may lead to greater immune benefit in the context of obesity. On the other hand, gut permeability is known to be increased in obesity and whether PC can prevent the negative effects of a HFD feeding on gut permeability remains to be investigated (Portincasa, Bonfrate *et al.,* 2021). PC administration has shown to have therapeutic effects on patients with ulcerative colitis, improved intestinal barrier against *Clostridium difficile* toxin and modulated gut microbial composition (Gao, Du *et al.,* 2021, Olson, Diebel *et al.,* 2014). This would be a possible mechanism by which PC and SM can attenuate the immune dysfunction seen in obesity by potentially improve gut permeability and/or change gut microbiota composition. It can be hypothesized that if the lipid-soluble forms of choline can improve gut permeability, and that serum LPS and circulating pro-inflammatory cytokines concentrations would be reduced as well.

PC has been shown to consistently increase IL-2 production; however, the possible mechanism by which PC is able to modulate T cells responses remains unclear. Lewis, *et al*, (2016) showed *in vitro* that increasing the availability of PC in the form of Lyso-PC increased T cell proliferation and IL-2 production in splenocytes (Lewis, Richard *et al.,* 2016). The lipid-soluble forms, in this specific case PC, are packed into chylomicrons after absorption by the enterocyte and secreted into the lymphatic system and therefore could reach peripheral organs (i.e. spleen) *en route* to the liver (Lewis, Field *et al.,* 2015). It is possible that increasing the availability of lipid-

soluble forms of choline increased the uptake by the cells. PC in the most abundant phospholipid in cells membranes and an important component of the lipid rafts. Lipid rafts are involved in signal transduction, including T cell activation (Simons & Toomre 2000). We could hypothesize that increasing the availability of PC could be modulating the composition of the rafts. There have been some studies made with other lipids (gangliosides) that showed that the type of ganglioside matter for T cell activation (Nagafuku, Okuyama *et al.,* 2012). Here we only tested PC from eggs, and, even though, it showed promising results, other PC molecule coming from different sources (MFGM, soy) should be considered as there may be different effects depending on the sources. In addition, other forms of choline should also be considered in further studies such as SM. Furthermore, no study to our knowledge has assessed whether dietary PC modulated gene expression of genes relates to T cell activation. As component of the cell membrane, including T cells, we could speculate that PC is modulating the expression of genes such as lymphocyte-specific protein tyrosine kinase (LCK), linker for activation of T cells (LAT) or RAS.

Adipocytes respond to overnutrition by increasing in size and number. This will result in constant stress that will end in adipocyte hypoxia and/or death that will lead to the recruitment of M1-macrophages (pro-inflammatory) and release of inflammatory cytokines (Reilly & Saltiel 2017). Han, *et al*., (2021) demonstrated in their study that Lyso-PC extracted from lentils decreased body weight gain, prevented adipocyte hypertrophy and the infiltration of crown-like structures (M1-macrophages) (Han, Park *et al.,* 2021). However, some questions remain to be answered, will PC from other sources (eggs) have the same effect on adipose tissue? Is adipocyte function also modulated by the form of choline? To finalize, we demonstrated that eggs are a potential good source of choline in the form of PC and should be considered as main sources of dietary PC when translating our findings into human randomized control trials.

7.3 Conclusion

Overall, the research presented in this thesis demonstrated that a diet high in lipid-soluble forms of choline modulated immune responses in different life stages. We showed that buttermilk is a valuable source of dietary choline that provides a unique composition of choline forms that was able to improve T cells responses in lactating dams and improve the maturation of both the peripheral and local immune systems in offspring. The evidence suggests that the form of choline should be carefully considered when designing infant formulas but also in the maternal diet to provide a distribution of choline forms that will favors the immune system development. This thesis provided evidence that PC can not only promote better T cell responses early in life or in healthy conditions but also in the context of obesity. PC is a promising form of choline to counteract the effects of feeding a HFD in Wistar rats and, therefore, there is a need for more studies to understand the mechanism behind PC and other lipid-soluble forms of choline at modulating T cell responses.

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Appendix

Appendix 1 Sex Differences Distinctly Impact High-Fat Diet-Induced Immune Dysfunction in Wistar Rats.

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Introduction

The immune system consists of different compartments and cell types that respond in a coordinated fashion to environmental challenges to protect the host against pathogens in addition to other important physiological functions (Rankin & Artis 2018). These cells include monocytes/macrophages, dendritic cells (DC), natural killer (NK) cells, and B and T lymphocytes among others. T cells are classically categorized into different subsets based on their respective function; cytotoxic T cells mediate cytolysis, helper T cells orchestrate humoral vs cell-mediated immunity, while regulatory T cells maintain tolerance (Kumar, Connors *et al.,* 2018). The cytokine environment instructs naïve $CD4^+$ T cells to differentiate into functionally distinct T helper (Th) subsets, with Th1 cells specializing in producing interleukin (IL)-2, interferon-gamma (IFN)-γ, and tumor-necrosis factor-alpha (TNF)-α while Th2 cells produce IL-4, IL-5, and IL-13 (Goldsby, Kindt *et al.,* 2003, Saravia, Chapman *et al.,* 2019, Yaqoob & Calder 2011). The immune system is regulated by many environmental and biological factors such as sex, age, diet, and the microbiome (Childs, Calder *et al.,* 2019, Klein & Flanagan 2016, Montecino-Rodriguez, Berent-Maoz *et al.,* 2013, Riscalla 1982, Wu & Wu 2012).

Obesity is characterized by an excessive accumulation of fat in adipose tissue (AT) increasing the size and number of adipocytes which can lead to hypoxia and adipocyte death (Gonzalez-Muniesa, Martinez-Gonzalez *et al.,* 2017). This in turn leads to an increased production of pro-inflammatory cytokines which attract immune cells into the AT that further contribute to the local pro-inflammatory environment. Obesity is also associated with chronic systemic low-grade inflammation, well demonstrated by numerous studies reporting elevated concentrations of circulating pro-inflammatory markers including C-reactive protein (CRP), IL-6, IL-18 and TNF-α among others, in individuals with obesity (Reilly & Saltiel 2017). Low-grade systemic inflammation is closely associated with an increased risk of type 2 diabetes (T2D) and cardiovascular diseases (Calder, Ahluwalia *et al.,* 2011).

Obesity has also been associated with impaired immune responses to infection and vaccination in both rodents and humans (Karlsson, Sheridan *et al.,* 2010, Milner & Beck 2012, Sheridan, Paich *et al.,* 2012). Mice fed a high-fat diet (HFD) have increased mortality upon influenza infection and decreased memory T cell function (Karlsson, Sheridan *et al.,* 2010). We had previously demonstrated that individuals with obesity and T2D have impaired T cell function compared to body mass index-matched normoglycemic individuals with obesity, which was characterized by a lower production of IL-2 (marker of cell proliferation), IL-6, and TNF-α by phytohemagglutinin-stimulated peripheral blood mononuclear cells (PBMCs) (Richard, Wadowski *et al.,* 2017). Similarly, Lamas et al. (Lamas, Martinez *et al.,* 2002) reported that Wistar rats fed a cafeteria diet (a diet-induced obesity (DIO) model) had a lower IL-2 production by splenocytes stimulated with Concanavalin A. Therefore, Wistar rats appears to be a suitable model to study some of the reported obesity-related immune dysfunction in humans.

Most studies using preclinical models so far have only included male and therefore, have not taken into consideration sex differences when assessing the immune system. Moreover, sex plays a role in the development of obesity with marked differences in the proportion of body lean mass compared to fat mass between male and females due to several distinctions in genetic factors, number of adipocyte precursor cells, fatty acid uptake by tissues, and gonadal hormones (extensively reviewed in ((Karastergiou & Fried 2017, Link & Reue 2017, Zore, Palafox *et al.,* 2018)). Biological sex is also an important determinant of immunological responses, and sex differences have been reported across a wide range of diseases and health scenarios in both mice and humans (Klein & Flanagan 2016). Generally, females orchestrate stronger innate and adaptive immune responses than males (Beagley & Gockel 2003). Females tend to generate greater protective responses and enhanced antibody production following influenza vaccination (Engler, Nelson *et al.,* 2008, Lorenzo, Hodgson *et al.,* 2011). Although this might be an advantage regarding the course of some infectious diseases, an overactivation of immune pathways has been linked to a higher prevalence of antibody-mediated autoimmune diseases in women (Laffont & Guery 2019). Specific underlying mechanisms for sex differences are still not fully understood but are likely attributed to X chromosomes, as well as variations in sex hormones, among others (Bouman, Heineman *et al.,* 2005, Libert, Dejager *et al.,* 2010). Furthermore, less is known regarding the role of biological sex in the context of specific health conditions, such as obesity-related immune dysfunction. Studying sex differences is an emerging topic of interest as highlighted by several research funding agencies to ensure that sex is accounted as a biological variable in an attempt to improve the interpretation, validation, and generalizability of research findings (Arnegard, Whitten *et al.,* 2020).

Therefore, the main objective of this study was to determine the sex differences in innate and adaptive immune function in response to a HFD compared to a control low-fat diet (LFD) in Wistar rats. Since age-matched female rats are known to weigh less than males and that obesity has been previously linked to immune impairments, we hypothesized that feeding a HFD would lead to greater immune dysfunction in male compared to female rodents.

Materials and methods

Animals, diet and experimental design

All animal procedures in this study were performed in accordance with the Canadian Council on Animal Care guidelines and approved by the Animal Care and Use Committee of the University of Alberta. Three-week-old male (n=12) and female (n=12) Wistar rats were obtained from Charles River Laboratories (Montreal, QC, Canada). Two rats per cage of same sex were housed together in a temperature- and humidity-controlled environment under a reversed 12:12-h light-dark cycle. Access to water was provided *ad libitum* throughout the study. During the one-week acclimatization period, rats were fed standard rat chow (Lab diet 5001; PMI Nutrition International, Brentwood, MO, USA). At four weeks of age, male and female rats were randomly assigned to one of two experimental diets with *ad libitum* access to either a LFD (3.9 kcal/g) or HFD (4.6 kcal/g) totalling four groups (n=6/group). Each group received their respective diet for nine weeks and rats were terminated at 13 weeks of age. The HFD was composed of 50% kcal% fat, 23 kcal% carbohydrate, and 27 kcal% protein whereas the LFD diet was composed of 20 kcal% fat, 53 kcal% carbohydrate, and 27 kcal% protein. The composition of the experimental diets is presented in **Table 1**. The fatty acids composition was matched closely using a mixture of oils (**Table 2**). Both diets met the essential fatty acid requirements of the rodent (Nutrition. 1995) and had similar ratios of polyunsaturated fatty acid (PUFA) to saturated fatty acids (SFA) and n-6 to n-3 PUFA. Diets were prepared weekly and stored at 4°C until used. Feed cups were weighed and replaced every two to three days to prevent lipid oxidation. Dietary intake and body weight were monitored three times a week throughout the intervention.

Table 1. Composition of experimental diets

Ingredient (g/kg diet)[1]	Low-fat diet	High-fat diet
Casein	261.9	261.9
Corn starch	273.9	123.9
Sucrose	200.0	200.0
Vitamin Mix[2]	19.0	19.0
Mineral Mix[3]	50.0	50.0
Calcium Phosphate Dibasic	3.4	3.4
Inositol	6.3	6.3
Cellulose	80.0	80.0
L-cystine	1.8	1.8
Choline bitartrate	3.7	3.7
Fat mixture ingredients		
Total fat mix	100.0	250.0
Flax Oil	10.0	25.0
Corn oil	40.0	100.0
Lard	50.0	125.0

[1] All ingredients were purchased from Harlan Teklad (Indianapolis, IN, USA), with the exception of the dietary oils that were all purchased from Safeway (Edmonton, AB, Canada).

[2] Vitamin Mix (AIN-93-VX) (1).

[3] Bernhart–Tomarelli Salt Mixture (2).

Table 2. Fatty acid composition of experimental diets.

	Low-fat diet	High-fat diet
Fatty acid[1]	**% of total fatty acids**	
14:0	0.72 ± 0.00	0.76 ± 0.00
16:0	20.30 ± 0.68	19.40 ± 0.83
16:1	1.14 ± 0.12	1.24 ± 0.16
18:0	10.50 ± 1.47	10.81 ± 1.54
18:1	33.30 ± 0.63	33.92 ± 0.41
18:2n-6	28.30 ± 1.90	28.40 ± 0.78
18:3n-3 (ALA)	5.64 ± 0.57	5.47 ± 0.65
Total SFA	31.60 ± 2.15	30.9 ± 0.71
Total MUFA	34.40 ± 0.74	35.2 ± 0.57
Total PUFA	33.90 ± 1.32	33.9 ± 0.13
n-6:n-3	5.06 ± 0.85	5.24 ± 0.76
PUFA:SFA	1.08 ± 0.12	1.09 ± 0.03

[1]Values are means ± SD. Analysis of the fatty acid composition of the two experimental diets collected weekly (n=2 batches). Abbreviations: ALA, α-linolenic acid; LA, linoleic acid; MUFA, monounsaturated fatty acids; n, omega; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

Lipid analysis

The fatty acid composition of the two experimental diets was measured by gas chromatography (GC). Briefly, total lipids from diets were extracted using the Folch method (Folch, Lees *et al.,* 1957), saponified, and then methylated with hexane and boron trifluoride. Samples were then dried down, resuspended in hexane, and read on the Agilent 7890 GC system (Agilent Technologies, Palo Alto, CA, USA).

Blood count and plasma metabolites measurement

At 12 weeks of age, non-fasting blood glucose was measured from the saphenous vein using a glucometer (Accu-chek, Roche Diagnostics, Basel, Switzerland). At termination, blood was collected by cardiac puncture in tubes containing EDTA, and dipeptidyl peptidase-4 inhibitor (EMD Millipore, Billerica, MA, USA). The complete blood count and automated differential were performed on whole blood using a Siemens Advia 2120 hematology analyzer (Siemens Healthcare Ltd., Oakville, ON, Canada). Whole blood was centrifuged at 1,800 x g for 10 min to obtain plasma. Plasma concentrations of adiponectin and leptin were measured by enzyme-linked immunosorbent assay (Athyros, Ganotakis *et al.,*) according to the instructions of the manufacturer (R&D Systems, Minneapolis, MN, USA). Adipokine concentrations were quantified using a microplate reader (SpectraMax 190, Molecular Devices, Sunnyvale, CA, USA). Plasma concentrations of cytokines were measured using an electrochemiluminescent multiplex immunoassay kit (V-Plex Proinflammatory Panel 1, Meso Scale Discovery, Rockville, MD, USA). The concentrations of IFN- γ, IL-10, IL-13, IL-1β, IL-4, IL-5, IL-6, TNF-α and chemokine KC/GRO (keratinocyte chemoattractant/human growth-regulated oncogene) were determined following the instructions of the manufacturer (R&D Systems, Minneapolis, MN, USA). Plates

were read on the Meso Scale Discovery Sector Imager 6000, Model1200 (Meso Scale Discovery, as above).

Tissue collection and immune cell isolation

At 13 weeks rats were weighed and euthanized by CO_2 asphyxiation in the morning hours. Tissues were collected aseptically, weighed, and splenocytes were isolated for further processing. Isolation of immune cells from the spleen has been previously described (Field, Wu *et al.,* 1990). Prior to *ex vivo* analyses, a haemocytometer was used to count live cells using trypan blue dye exclusion (Sigma-Aldrich, as above) to assess cell viability and it was > 90% for all treatment groups. All cell suspensions were then diluted to 1.25 x 10^6 cells/mL. Remaining cells were spun down, resuspended in 20% Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies, Burlington, ON, Canada) and 10% dimethyl sulfoxide (DMSO) and frozen in a Mr. Frosty for 2 h before transferring to liquid nitrogen for long-term storage.

Immune cell phenotype analysis

Multicolor flow cytometry panels were designed and splenocytes were stained with different antibodies as follow: T cell (CD3-allophycocyanin (APC)-Vio770, CD4-fluorescein isothiocyanate (FITC), CD8-brilliant ultra violet (BUV)395, CD25-peridinin chlorophyll protein (PerCP)-eFluor710, CD27-BUV737, CD28-phycoerythrin (PE)-Vio770, CD127-Alexa Fluor 594)); B cell (CD45RA- Alexa Fluor 40), CD80-PE, CD27-BUV737, CD74 (OX6)-PerCP; NK cell (CD3-APC-Vio770, CD74 (OX6)-PerCP, CD161-Alexa Fluor 647), iOX62-PE); and monocytes and macrophages (CD68-APC-Vio770, CD11bc-PE-Vio770). All panels contained viability dye (Zombie Yellow-V525). After incubation, cells were washed with IF buffer (5% FCS

in phosphate buffered saline (PBS), pH 7.4) and fixed in paraformaldehyde (PFA) (10 g/L; Thermo Fisher Scientific, as above) in PBS. All the samples were acquired within 72 h of preparation on a BD LSRFortessa X-20 SORP flow cytometer (Becton, Dickinson and Company, San Jose, CA, USA) and data was analyzed using FlowJo Software v10.8.0 (Becton, Dickinson and Company, Ashland, OR, USA).

Ex vivo cytokine production by mitogen-stimulated cells

The measurement of cytokine production by mitogen-stimulated splenocytes has been previously described (Blewett, Gerdung *et al.,* 2009). Briefly, freshly isolated cells (1.25 x 10^6 cells/mL) were cultured in 3 mL RMPI-1640 medium (as above) for 48 h at 37ºC and 5% CO_2 without mitogen (unstimulated) or with mitogens including phorbol 12-myristate 13-acetate plus ionomycin (PMA+I) (2 µg/mL; Fisher Scientific), lipopolysaccharide (LPS) (5 µg/mL; Fisher Scientific), and pokeweed (PWM) (55 µg/mL; Sigma-Aldrich Canada Ltd., as above). After incubation, cells were centrifuged for 10 min at 200 x g and supernatants were collected and stored at -80°C until analyses. Concentrations of IL-1β, IL-2, IL-6, IL-10, TNF-α, and IFN-γ were measured by commercial ELISA kits according to the instructions of the manufacturer and as previously described (Blewett, Gerdung *et al.,* 2009) The detection limits for all cytokines ranged from 15.6 to 4000 pg/mL (R&D Systems, Minneapolis, MN, USA). Cytokine concentrations were quantified using a microplate reader (SpectraMax 190, as above) and all measurements were conducted in duplicate, with an intra-assay coefficient of variation < 10%. IL-1β was measured in the supernatants of LPS and PWM stimulated cells and IL-2 was measured in the supernatants of PMA+I and PWM stimulated cells.

Ex vivo T cell proliferation assay

Ninety-six-well plates were coated with 1 μg/mL of CD3 and were incubated overnight at 4°C. Frozen splenocytes were warm thawed for 10 min at 37°C before being washed of DMSO in 10% RPMI 1640 medium (Life Technologies, as above). Afterwards, cells were transferred in 5mL 10% RPMI to 10mL culture tubes and incubated for 4 h at 37°C. After incubating, cells were washed and resuspended in 10% RPMI. A haemocytometer was used to count live cells using trypan blue dye exclusion (Sigma-Aldrich Canada Ltd., as above) to assess cell viability. The 96-well plates were set up to get 1.25 x 10^6 cells/mL in 200 μL. Samples were added in triplicate, with CD28 stimulation (0.3 μg/mL; BioLegend, San Diego, CA, USA) of coated CD3 (BioLegend, as above) wells, and an unstimulated treatment. Plates were then incubated at 37°C for 72 h. After incubation, cell numbers were fixed with 100 μL 4% PFA for 20 min, followed by a 20-min incubation with crystal violet (Acros Organics) solution (0.1%, w/v, with ethanol 2%, v/v in 0.5 M Tris-C1, pH 7.8; 100 μL per well) at room temperature. The stained cell layer was rinsed thoroughly with deionized water, vacuum aspirated and incubated with sodium dodecyl sulfate (SDS) solution (0.1%, w/v, with ethanol 50%, v/v, in 0.5 M Tris-C1, pH 7.8; 100 μL per well) for 30 min at room temperature. Meanwhile, crystal violet was completely released from the cells into the supernatant. The supernatant was scanned in a microplate reader (SpectraMax 190; Molecular Devices, Sunnyvale, CA, USA) and read at 590 nm. All samples were measured in triplicate. One male rat fed the LFD did not have enough viable cells to perform the assay and its data was excluded from the analysis due to lack of proliferation.

Statistical analysis

Data are reported as means ± standard error of the mean (SEM) unless otherwise indicated. The study was powered to assess significant differences in immune function (i.e., *ex vivo* cytokine production as the primary outcome) for the main effect of sex and diet using a two-way ANOVA. Data were analyzed using two-way ANOVA in SAS (v9.4, Cary, NC, USA) with sex and diet as the main effects and their interaction. In cases where a significant main effect of diet, sex or sex*diet interaction was found, *post hoc* analysis was performed using the DUNCAN adjustment to determine differences between groups. Pearson correlation analyses were performed between continuous variables. Ratios were calculated by dividing the absolute serum levels of pro-inflammatory cytokines by the anti-inflammatory cytokine. Variables that were not normally distributed were log-10 transformed prior to statistical analysis. Differences at $P \leq 0.05$ (two-sided) were considered significant.

Results

Anthropometric characteristics and daily food intake

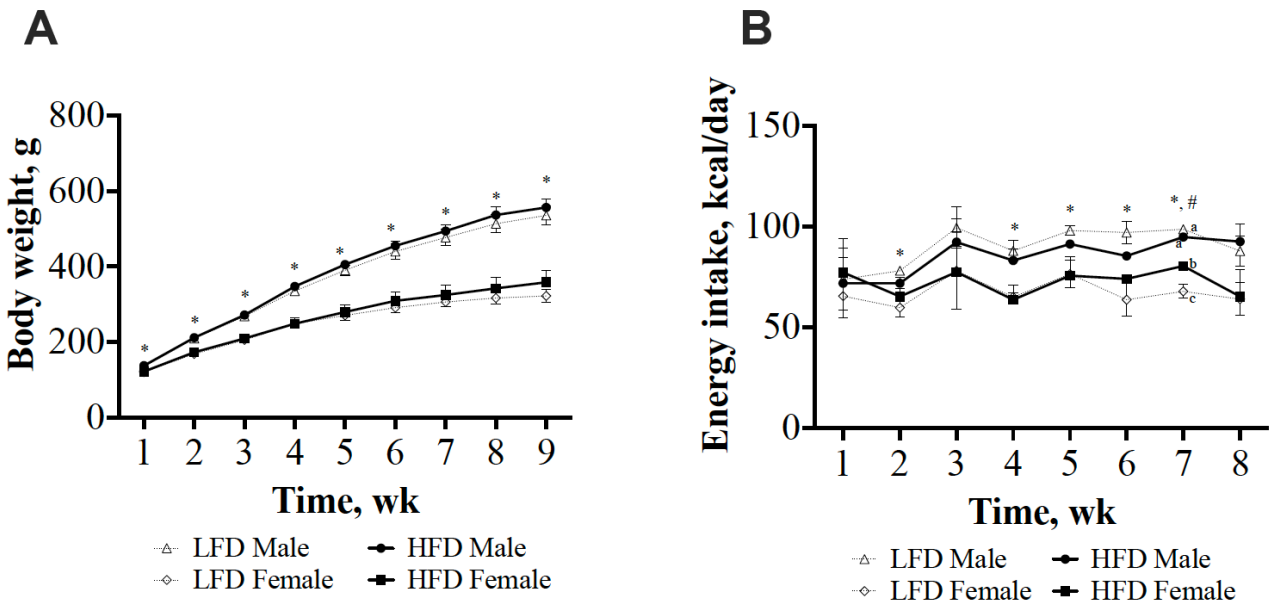
As expected, females had lower body weight, body length and liver weight compared to males independently of their respective diet (**Table 3**). No significant sex, diet or interaction effects were observed for the other anthropometric data, or in the number of splenocytes among groups (all $P > 0.05$, **Table 3**). As shown in **Figure 1**, males had significantly higher body weight compared to females throughout the duration of the study (**Figure 1A**). Males fed the LFD had higher calorie intake compared to females starting from week two and throughout most of the study (**Figure 1B)**. Animals fed the LFD consumed the same amount of calories compared to HFD, and therefore, no differences were observed in body weight between the LFD and HFD for each sex. Considering the 8-week feeding period, LFD-fed males consumed on average about 6.7 grams of diet more per week (i.e., approximately 1.0 g/day) compared to HFD-males (data not shown). Significant differences in food intake between LFD-fed and HFD-fed males were observed for week 2, 5, 6 and 7 (all $P < 0.05$) while no differences were observed for week 1, 3, 4 and 8. Female rats on the LFD and HFD had no differences in food intake throughout the study.

Table 3. Sex differences in anthropometric data and plasma concentration of metabolites of male and female Wistar rats fed a LFD or HFD for 9 wks.

Variable[1]	Male		Female				
	LFD (N=6)	HFD (N=6)	LFD (N=6)	HFD (N=6)	*P*-sex	*P*-diet	*P*-sex*diet
Body weight (g)	546±24.9[a]	555±25.6[a]	340±34.4[b]	352±31.3[b]	<0.001	0.706	0.945
Animal length (cm)	25.2±0.307[a]	25.0±0.365[a]	22.4±0.245[b]	22.7±0.494[b]	<0.001	0.896	0.571
Spleen weight (g)	1.36±0.122[ab]	1.49±0.0879[a]	0.982±0.137[b]	1.08±0.148[b]	0.005	0.379	0.895
Liver weight (g)	26.1±1.33[a]	25.7±1.12[a]	14.5±1.66[b]	15.3±0.764[b]	<0.001	0.894	0.617
Intestine length (cm)*	123.2±4.14	121±3.14	125±14.0	111±2.30	0.358	0.278	0.417
Cecum weight (g)	5.54±0.436	4.15±0.365	4.90±0.387	3.89±0.474	0.305	0.011	0.660
Splenocytes, n x 10^6/100 g tissue	19.1±4.68	11.2±3.58	13.5±2.79	16.5±2.49	0.959	0.498	0.142
Glucose (mmol/L)	6.10±0.500	6.40±0.400	6.10±0.400	6.10±0.400	0.741	0.727	0.770
Adiponectin (µg/mL)	3.78±0.368[a]	3.43±0.215[a]	5.61±0.679[b]	5.33±0.369[b]	<0.001	0.458	0.927
Leptin (µg/mL)	7.93±1.08	9.25±1.75	4.88±1.68	7.10±1.28	0.092	0.242	0.761
Adiponectin: Leptin*	0.533±0.093[a]	0.443±0.086[a]	1.56±0.370[b]	0.895±0.168[a]	<0.001	0.131	0.508
IL-13 (pg/mL)	1.82±0.312[a]	2.78±0.274[b]	2.84±0.277[b]	1.46±0.307[a]	0.618	0.503	0.001
IL-1β (pg/mL)*	32.4±4.28	38.5±8.89	35.4±3.67	38.2±9.50	0.808	0.830	0.767
IL-4 (pg/mL)	1.67±0.481	1.20±0.235	1.00±0.326	1.54±0.461	0.683	0.935	0.210
IL-5 (pg/mL)*	111±7.62	117±13.9	112±7.75	112±24.5	0.676	0.793	0.650
IL-6 (pg/mL)	58.3±9.38	60.0±11.2	68.8±3.04	63.0±7.84	0.461	0.817	0.678
TNF-α (pg/mL)	10.6±1.09	10.8±1.23	9.73±0.961	10.0±1.31	0.486	0.859	0.955
IL-10 (pg/mL)	16.1±0.604	12.7±2.53	15.6±2.10	16.4±4.40	0.600	0.668	0.493
KC/GRO (pg/mL)	65.4±19.8	54.6±16.8	82.3±12.5	79.6±18.8	0.254	0.709	0.821
IFN-γ (pg/mL)*	8.98±2.12[ab]	8.10±1.72[a]	11.5±1.60[ab]	19.9±4.89[b]	0.029	0.605	0.280
TNF-α:IL-10*	0.730±0.0584	0.994±0.211	0.692±0.139	0.815±0.242	0.340	0.450	0.682
TNF-α:IL-13*	7.34±2.08[a]	3.64±0.368[b]	3.52±0.392[b]	7.00±1.00[a]	0.935	0.744	0.003

[1]Values are means ± SEM. Labeled means in a row without a common letter differ based on the two-way ANOVA test with the Duncan adjustment for multiple comparisons test ($P < 0.05$).
*Analysis performed on log-transformed values.
HFD, high-fat diet; IFN-γ, interferon gamma; IL, interleukin; KC/GRO, keratinocyte chemoattractant/human growth-regulated oncogene; LFD, low-fat diet; TNF-α, tumor necrosis factor alpha.

Figure 1. (A) Body weight and (B) energy intake of male and female Wistar rats fed a LFD or HFD for 9 wks. Values are means ± SEM, n=6. * indicates a significant sex effect (P < 0.05); # indicates a significant diet*sex interaction (*P* = 0.01) based on the two-way ANOVA test with the Duncan adjustment for multiple comparisons performed at each time point.



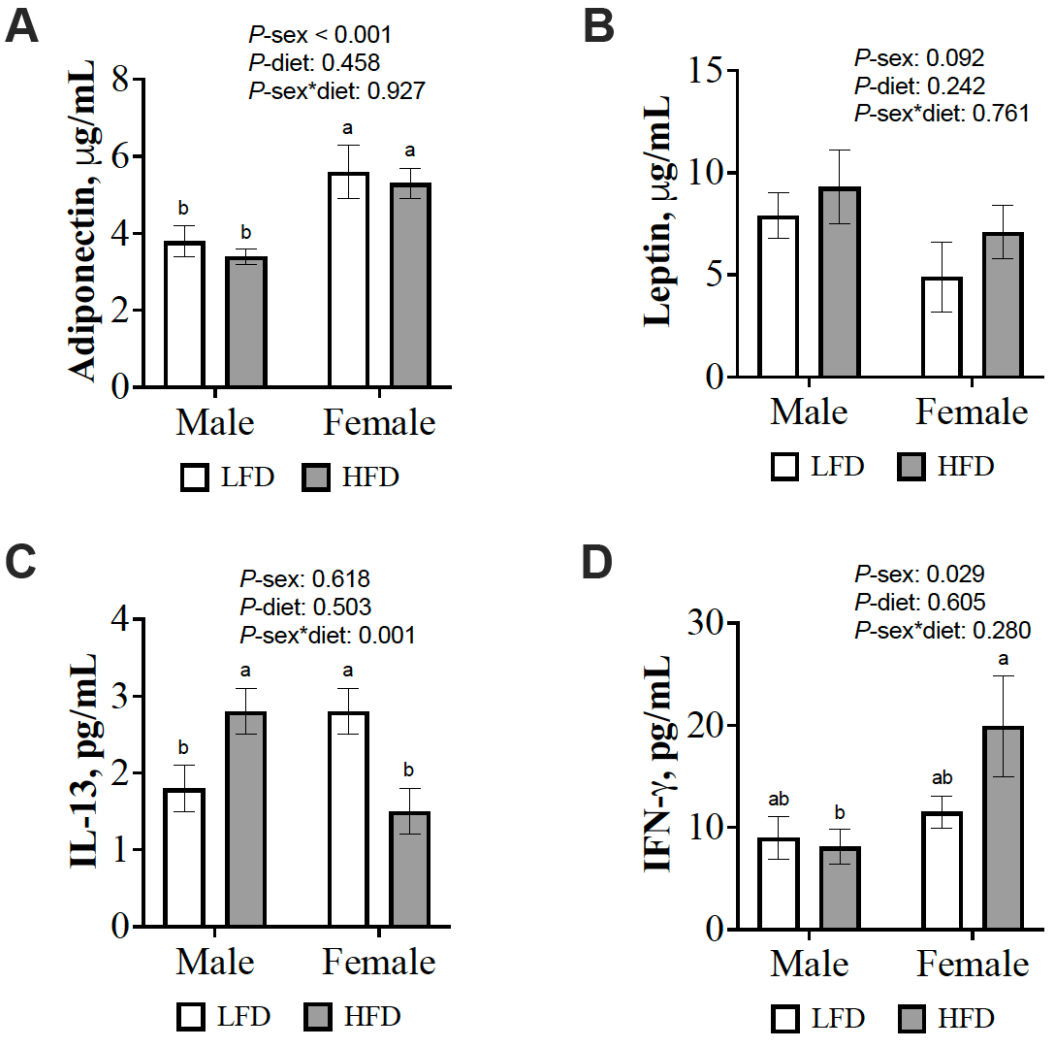
Abbreviations: HFD, high-fat diet; LFD, low-fat diet.

Plasma hormones, cytokines, and metabolites

There was no significant difference in plasma glucose levels amongst the four groups (**Table 3**). Females had higher plasma levels of adiponectin compared to males independent of diet (**Figure 2A**). Females also tended to have lower plasma leptin concentrations compared to males, although it did not reach statistical significance (*P*-sex = 0.09) **(Figure 2B**). Adiponectin and leptin were negatively and positively correlated with body weight at week nine, respectively (r= -0.702, *P* <0.01; *r* =0.604, *P* = 0.02). In addition, females fed the LFD showed better adipose tissue function as indicated by a higher adiponectin: leptin ratio compared to all other groups.

There was a significant sex*diet interaction for plasma IL-13 levels for which males and females fed the HFD had higher and lower IL-13 concentrations respectively, compared to their LFD counterpart (**Figure 2C**). The opposite was observed for the TNF-α:IL-13 ratio, a marker of Th1:Th2 cytokine response. Females fed the HFD had higher plasma levels of IFN-γ compared to males fed the HFD, whereas no sex differences were observed in rats fed the LFD (**Figure 2D**).

Figure 2. Plasma concentrations of (**A**) adiponectin, (**B**) leptin, (**C**) IL-13, and (**D**) IFN- γ of male and female Wistar rats fed a LFD or HFD for 9 wks. Values are means ± SEM, n=6. Labeled means without a common letter differ based on the two-way ANOVA test with the Duncan adjustment for multiple comparisons test ($P < 0.05$).



Abbreviations: HFD, high-fat diet; IL, interleukin; LFD, low-fat diet.

Complete blood cell count

Overall, there were some effects of sex, diet or their interaction on complete blood cell counts (**Table 4**). Males had a higher white blood cell count and lower proportion of eosinophils compared to females. There was a significant sex*diet interaction for hemoglobin (HGB), red cell distribution (Ordway, Henao-Tamayo *et al.,*), and reticulocytes. HFD lead to higher HGB concentration in male compared to females, whereas the opposite was observed for RDW and reticulocytes.

T cell proliferation assay and ex-vivo cytokine production by splenocytes after mitogen stimulation

Ex-vivo cytokine production by splenocytes isolated from males and females on LFD and HFD is presented in **Table 5**. After PMA+I stimulation, the HFD decreased the production of IL-2 (*P*-diet = 0.02) and increased the production of IL-10 (*P*-diet = 0.05), independently of sex. Specifically, splenocytes from HFD-fed males had lower production of IL-2 upon stimulation compared to all other groups. There was a significant sex*diet interaction for TNF-α and IL-6 production after PMA+I stimulation. Males fed the HFD tended to produce less TNF-α compared to males fed the LFD (*P*-diet = 0.08; *P*-sex*diet = 0.05), while diet had no effect on TNF-α production in females. Similarly, HFD-fed males had lower production of IL-6 compared to those fed the LFD, whereas the HFD tended to increase IL-6 production in females after PMA+I stimulation (*P*-sex*diet = 0.01) (**Figure 3B**). However, the Th1:Th2 ratio, as assessed by the TNF-α:IL-10 ratio, was lower in the HFD-fed groups, independently of sex. There was no significant effect of either sex or diet on anti-CD3/anti-CD28-stimulated T cell proliferation, as shown in **Figure 4**. However, males fed the HFD showed lower T cell

Table 4. Sex differences in complete blood cell count of male and female Wistar rats fed a LFD or HFD for 9 wks.

Variable[1]	Male		Female		*P*-sex	*P*-diet	*P*-sex*diet
	Low-fat diet (N=4)	High-fat diet (N=3)	Low-fat diet (N=3)	High-fat diet (N=4)			
WBC (× 109/L)*	8.79±0.788^{ab}	12.1±2.29^{a}	6.45±0.455^{b}	6.84±0.675^{b}	0.006	0.145	0.241
RBC (× 1012/L)*	8.74±0.0882	8.51±0.549	8.58±0.158	8.32±0.139	0.558	0.354	0.997
HGB (g/L)	151±0.750^{b}	160±3.84^{a}	154±4.04^{ab}	146±0.854^{b}	0.050	0.714	0.005
HCT	0.513±0.00992	0.501±0.0370	0.515±0.0140	0.499±0.0478	1.000	0.450	0.902
MCV (fL)	58.7±0.575	58.8±1.68	60.0±0.581	60.0±1.02	0.243	0.952	0.908
MCH (pg)*	17.3±0.119	19.0±1.68	17.9±0.273	17.6±0.342	0.694	0.364	0.189
MCHC (g/L)*	294±4.49	324±29.9	299±3.18	294±3.06	0.357	0.365	0.198
RDW (%)	14.9±0.349	14.4±0.384	14.0±0.20	15.0±0.189	0.633	0.473	0.029
Platelets (× 109/L)	722±80.7	921±163	736±102	805±26.4	0.603	0.188	0.508
Neutrophils (%)*	14.3±1.74	22.4±4.85	11.3±0.788	13.8±4.23	0.115	0.344	0.429
Lymphocytes (%)	79.5±2.29	70.4±4.62	80.6±0.463	78.3±4.75	0.249	0.152	0.384
Monocytes (%)	3.17±0.669	3.03±0.384	1.97±0.649	2.40±0.567	0.160	0.815	0.646
Eosinophils (%)*	1.00±0.0408	1.30±0.20	2.30±0.608	1.97±0.610	0.041	0.935	0.343
LUC (%)*	1.57±0.131	2.13±0.895	3.40±0.513	3.05±1.34	0.109	0.757	0.436
Basophils (%)*	0.525±0.0479	0.70±0.208	0.433±0.0333	0.50±0.122	0.248	0.595	0.694
Reticulocytes (%)	2.62±0.110^{ab}	2.31±0.133^{b}	2.423±0.281^{ab}	3.06±0.229^{a}	0.189	0.421	0.038

[1]Data are presented as means ± SEM. Labeled means in a row without a common letter differ based on the two-way ANOVA test with the Duncan adjustment for multiple comparisons test ($P < 0.05$).

*Analysis performed on log-transformed values.

HCT, hematocrit; HDW, hemoglobin distribution width, HGB, hemoglobin; LUC, large unstained cells; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; RBC, red blood cells; RDW, red cell distribution; WBC, white blood cells.

Table 5. Sex differences in *ex vivo* cytokine production by splenocytes after mitogen stimulation in male and female Wistar rats fed a LFD or HFD for 9 wks.a

Variable[1]	Male		Female				
	LFD (N=6)	HFD (N=6)	LFD (N=6)	HFD (N=6)	*P*-sex	*P*-diet	*P*-sex*diet
PMAI stimulation (T cell mitogen)							
IL-2 (pg/mL)*	2146±173[a]	883±223[b]	2820±787[a]	1949±502[a]	0.092	0.022	0.215
IL-10 (pg/mL)	167±37.9	208±24.6	128±20.4	212±15.3	0.265	0.046	0.461
TNF-α (pg/mL)	102±13.8[a]	61.2±8.55[b]	80.4±9.28[ab]	82.7±8.48[ab]	0.992	0.080	0.052
IFN-γ (pg/mL)*	80.7±52.6	26.4±12.5	42.4±30.3	106±40.9	0.309	0.925	0.077
IL-6 (pg/mL)	220±52.8[a]	54.1±18.7[b]	120±31.9[ab]	190±54.8[a]	0.669	0.269	0.013
TNF-α:IL-10	0.702±0.110[a]	0.311±0.0396[b]	0.679±0.0985[a]	0.417±0.0260[b]	0.645	0.002	0.473
LPS stimulation (Bacterial challenge)							
IL-1β (pg/mL)*	28.0±4.68	33.5±5.28	35.0±10.6	35.7±5.10	0.510	0.432	0.843
IL-10 (pg/mL)	488±110	570±75.6	609±87.3	644±69.6	0.278	0.510	0.493
TNF-α (pg/mL)	189±18.9	173±21.7	248±29.0	198±31.9	0.134	0.235	0.542
IL-6 (pg/mL)	519±57.1	566±82.6	465±82.8	540±122	0.661	0.503	0.876
TNF-α:IL-10*	0.448±0.139	0.330±0.0566	0.479±0.150	0.332±0.0654	0.837	0.272	0.745
PWM stimulation (T cell and APC mitogen)							
IL-1β (pg/mL)*	111±13.2	153±34.3	100±25.5	104±18.1	0.348	0.533	0.863
IL-2 (pg/mL)*	433±71.0	358±74.6	238±46.7	269±48.3	0.031	0.758	0.319
IL-10 (pg/mL)	385±52.8	527±61.6	455±63.2	402±28.1	0.608	0.409	0.083
TNF-α (pg/mL)*	312±45.8	268±31.9	407±86.4	279±46.2	0.395	0.107	0.437
IFN-γ (pg/mL)*	119±49.3	315±173	122±85.5	44.4±14.9	0.387	0.953	0.700
IL-6 (pg/mL)	1259±216[b]	2044±323[a]	1033±122[b]	887±91.4[b]	0.004	0.147	0.041
TNF-α:IL-10	0.886±0.163	0.546±0.0921	0.831±0.114	0.701±0.100	0.710	0.094	0.440

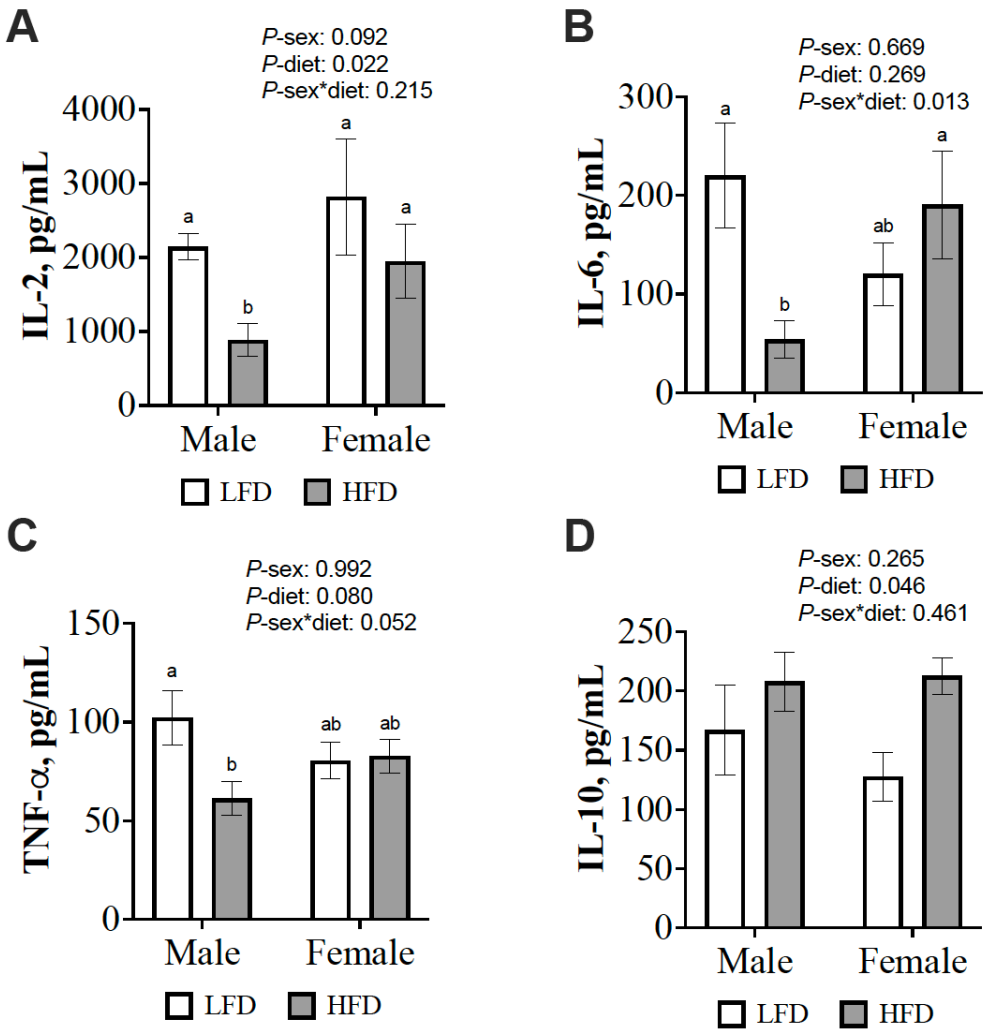
[1]Values are means ± SEM. Labeled means in a row without a common letter differ based on the two-way ANOVA test with the Duncan adjustment for multiple comparisons test ($P < 0.05$).

*Analysis performed on log-transformed values.

Abbreviations: HFD, high-fat diet; IFN-γ, interferon gamma; IL, interleukin; LFD, low-fat diet; TNF-α, tumor necrosis factor alpha.

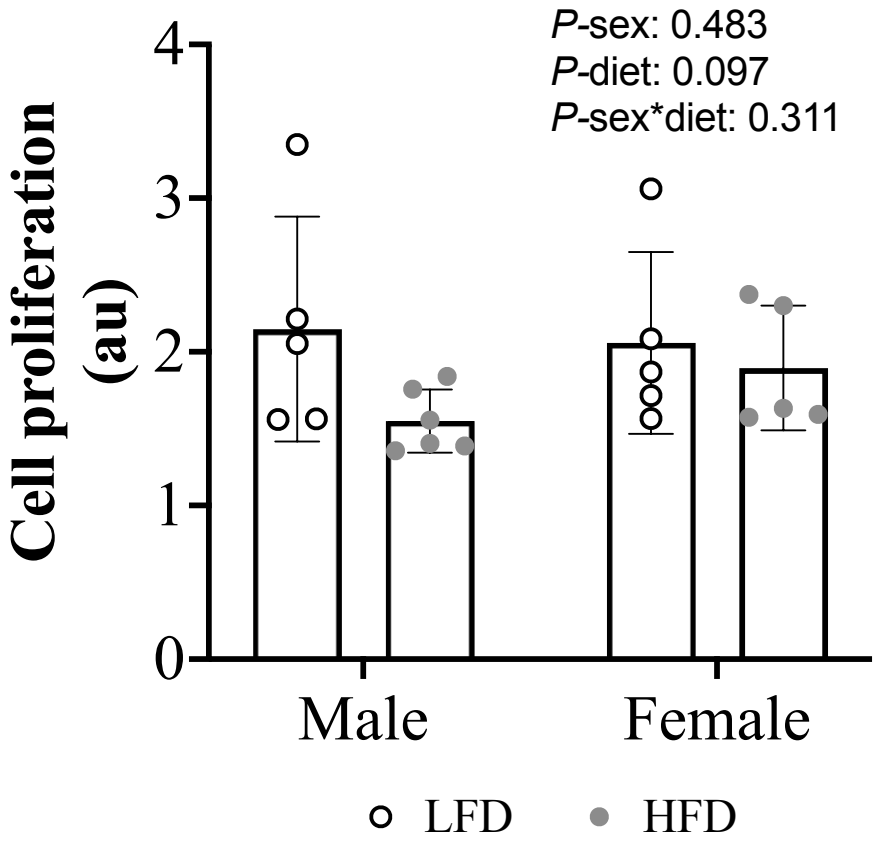
proliferation compared to all other groups, consistent with the lower IL-2 production following PMA+I-stimulation. When immune cells were stimulated with PWM, a sex effect was observed for IL-2 and IL-6 with a lower production of both cytokines in females compared to males following a HFD (**Figure 5A and 5B**). Similarly, the Th1:Th2 ratio after PWM stimulation tended to be lower in the HFD-fed groups (*P*-diet = 0.09). There was no effect of diet or sex on LPS-stimulated cytokine production by splenocytes (**Table 5**).

Figure 3. Ex-vivo production by splenocytes of (A) IL-2, (B) IL-6, (C) TNF-α, and (D) IL-10 after stimulation with PMA+I in male and female Wistar rats fed a LFD or HFD for 9 wks. Values are means ± SEM, n=6. Labeled means without a common letter differ based on the two-way ANOVA test with the Duncan adjustment for multiple comparisons test ($P < 0.05$).



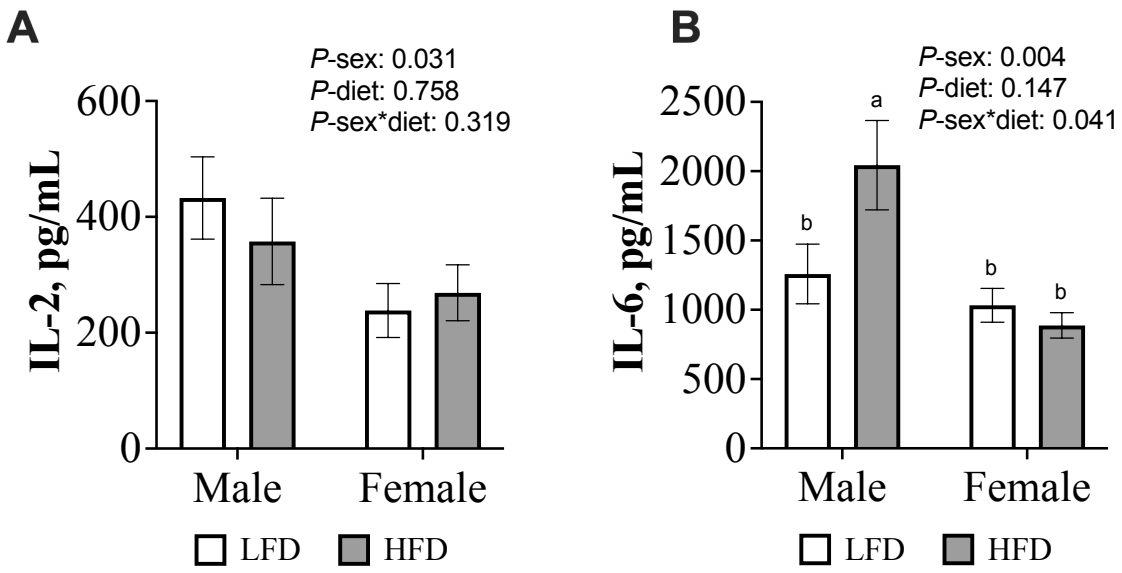
Abbreviations: HFD, high-fat diet; IL, interleukin; LFD, low-fat diet.

Figure 4. Effect of sex and diet on T cell proliferation following stimulation with CD3/CD28 in male and female Wistar rats fed a LFD or HFD for 9 wks. Values are means ± SEM, n=5-6. Two-way ANOVA test was used to estimate main effects and their interaction *(P* < 0.05).



Abbreviations: Au, arbitrary units; HFD, high-fat diet; LFD, low-fat diet.

Figure 5. *Ex-vivo* production by splenocytes of (**A**) IL-2 and (**B**) IL-6 after stimulation with pokeweed mitogen in male and female Wistar rats fed a LFD or HFD for 9 wks. Values are means ± SEM, n=6. Labeled means without a common letter differ based on the two-way ANOVA test with the Duncan adjustment for multiple comparisons test *(P* < 0.05).



Abbreviations: HFD, high-fat diet; IL, interleukin; LFD, low-fat diet.

Table 5. Sex differences in *ex vivo* cytokine production by splenocytes after mitogen stimulation in male and female Wistar rats fed a LFD or HFD for 9 wks.

Variable1	Male		Female				
	LFD (N=6)	HFD (N=6)	LFD (N=6)	HFD (N=6)	*P*-sex	*P*-diet	*P*-sex*diet
PMAI stimulation (T cell mitogen)							
IL-2 (pg/mL)*	2146±173[a]	883±223[b]	2820±787[a]	1949±502[a]	0.092	0.022	0.215
IL-10 (pg/mL)	167±37.9	208±24.6	128±20.4	212±15.3	0.265	0.046	0.461
TNF-α (pg/mL)	102±13.8[a]	61.2±8.55[b]	80.4±9.28[ab]	82.7±8.48[ab]	0.992	0.080	0.052
IFN-γ (pg/mL)*	80.7±52.6	26.4±12.5	42.4±30.3	106±40.9	0.309	0.925	0.077
IL-6 (pg/mL)	220±52.8[a]	54.1±18.7[b]	120±31.9[ab]	190±54.8[a]	0.669	0.269	0.013
TNF-α:IL-10	0.702±0.110[a]	0.311±0.0396[b]	0.679±0.0985[a]	0.417±0.0260[b]	0.645	0.002	0.473
LPS stimulation (Bacterial challenge)							
IL-1β (pg/mL)*	28.0±4.68	33.5±5.28	35.0±10.6	35.7±5.10	0.510	0.432	0.843
IL-10 (pg/mL)	488±110	570±75.6	609±87.3	644±69.6	0.278	0.510	0.493
TNF-α (pg/mL)	189±18.9	173±21.7	248±29.0	198±31.9	0.134	0.235	0.542
IL-6 (pg/mL)	519±57.1	566±82.6	465±82.8	540±122	0.661	0.503	0.876
TNF-α:IL-10*	0.448±0.139	0.330±0.0566	0.479±0.150	0.332±0.0654	0.837	0.272	0.745
PWM stimulation (T cell and APC mitogen)							
IL-1β (pg/mL)*	111±13.2	153±34.3	100±25.5	104±18.1	0.348	0.533	0.863
IL-2 (pg/mL)*	433±71.0	358±74.6	238±46.7	269±48.3	0.031	0.758	0.319
IL-10 (pg/mL)	385±52.8	527±61.6	455±63.2	402±28.1	0.608	0.409	0.083
TNF-α (pg/mL)*	312±45.8	268±31.9	407±86.4	279±46.2	0.395	0.107	0.437
IFN-γ (pg/mL)*	119±49.3	315±173	122±85.5	44.4±14.9	0.387	0.953	0.700
IL-6 (pg/mL)	1259±216[b]	2044±323[a]	1033±122[b]	887±91.4[b]	0.004	0.147	0.041
TNF-α:IL-10	0.886±0.163	0.546±0.0921	0.831±0.114	0.701±0.100	0.710	0.094	0.440

[1] Values are means ± SEM. Labeled means in a row without a common letter differ based on the two-way ANOVA test with the Duncan adjustment for multiple comparisons test ($P < 0.05$).

*Analysis performed on log-transformed values.

Abbreviations: HFD, high-fat diet; IFN-γ, interferon gamma; IL, interleukin; LFD, low-fat diet; TNF-α, tumor necrosis factor alpha.

Splenocyte phenotypes

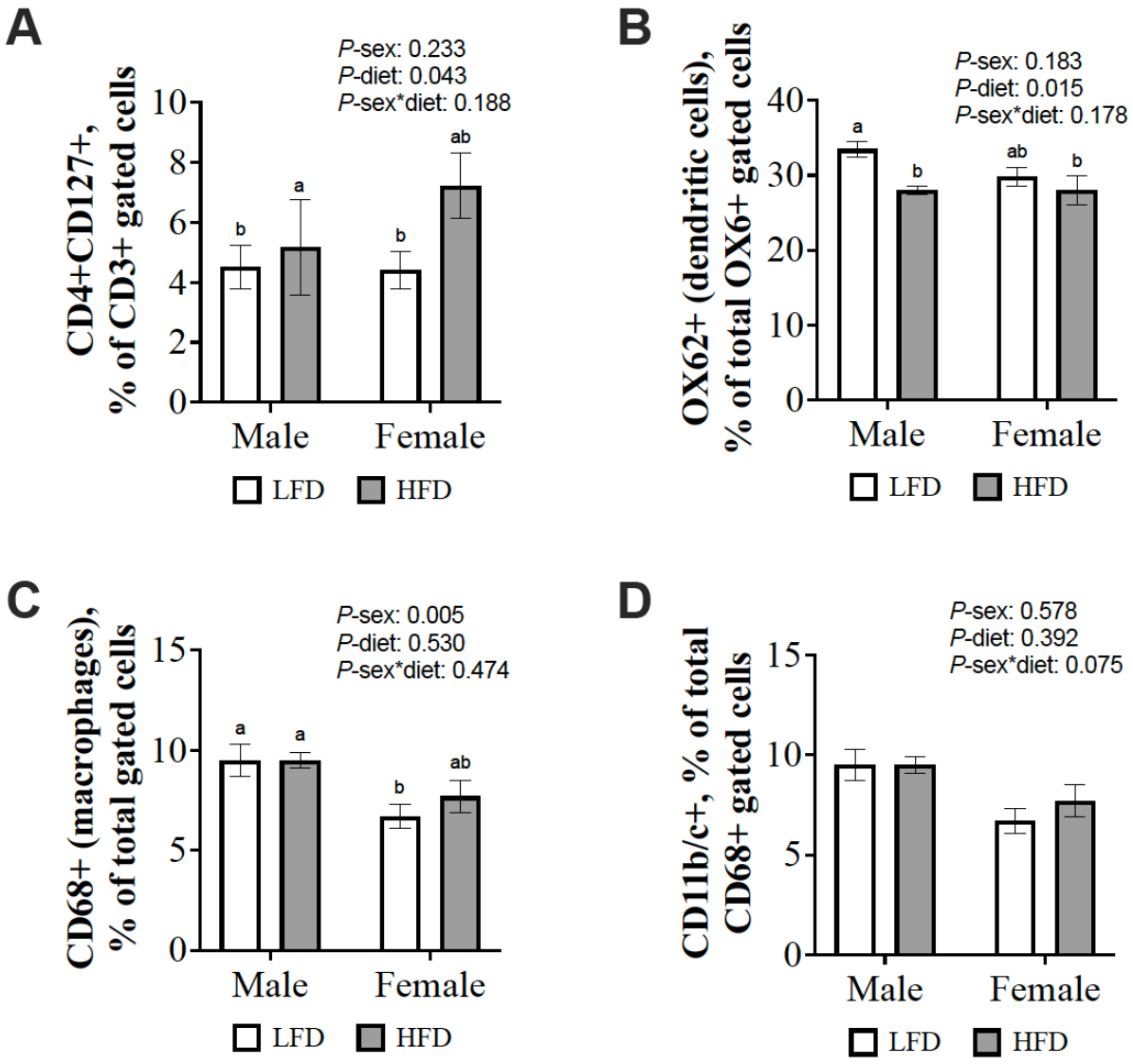
Immune cell phenotypes of male and female fed the two experimental diets are presented in **Table 5**. Sex and diet had little effect on the proportion of T cells and their subsets, including helper and cytotoxic T cells. No significant differences were observed among groups for total cells expressing CD25+, CD27+, CD28+ or CD127+ or in the CD4+:CD8+ ratio, but the proportion of helper T cells expressing CD127 (CD3+CD4+CD127+) was affected by diet and was higher in females fed the HFD (**Figure 6A**). There was a significant diet effect on the proportion of dendritic cells (OX6+OX62+), which was reduced in HFD-fed male rats compared to LFD-fed males, whereas diet had no effect in females (**Figure 6B**). There was a significant sex effect on the proportion of monocytes/macrophages where females had a lower proportion of cells expressing CD68+ compared to males (**Figure 6C**). There was also a trend for a sex*diet interaction for the proportion of macrophages expressing CD11b/c+ in which HFD-fed females tended to have lower proportion of CD68+CD11b/c+ cells compared to LFD-fed females (*P*-sex*diet = 0.07), whereas diet had no effect on the proportion of these cells in males (**Figure 6D**).

Table 6. Sex differences in immune cell phenotypes from splenocytes of male and female Wistar rats fed a LFD or HFD for 9 wks.

Phenotype[1]	Male		Female				
	LFD (N=6)	HFD (N=6)	LFD (N=6)	HFD (N=6)	*P*-sex	*P*-diet	*P*-sex*diet
	Antigen-presenting cell panel (% of total gated cells)						
Total CD45RA+ (B cells)	35.1±4.20	41.0±2.51	40.7±3.14	32.8±1.38	0.149	0.178	0.559
CD45RA+CD27+	27.4±2.94	29.9±1.85	28.0±2.30	30.3±1.88	0.974	0.974	0.767
CD45RA+CD80+	10.4±0.671	12.5±0.734	13.4±0.781	13.3±1.14	0.746	0.903	0.791
Total OX6+ (MHC class II+)*	38.5±3.95	41.9±1.73	37.9±4.04	41.6±2.73	0.100	0.913	0.636
OX6+OX62+ (dendritic cells)	33.5±0.996[a]	28.0±0.528[b]	29.9±1.32[ab]	28.0±1.90[b]	0.183	0.015	0.178
Total CD11b/c+	15.5±1.28	16.9±1.47	15.4±1.75	14.1±1.65	0.357	0.987	0.384
Total CD68+ (macrophages)	9.55±0.863[a]	9.48±0.417[a]	6.70±0.621[b]	7.69±0.815[ab]	0.005	0.530	0.474
CD68+CD11b/c+*	23.1±0.698	25.8±0.815	26.8±1.62	20.7±2.60	0.578	0.392	0.075
CD3-CD161+ (natural killer cells)	9.36±0.638	11.2±0.90	10.1±1.10	8.36±1.38	0.335	0.981	0.100
	T cell panel (% of total gated cells)						
Total CD3+ (T cells)	32.0±3.15	30.8±2.08	31.3±3.26	29.6±2.77	0.177	0.483	0.960
Total CD25+ (IL-2 receptor)	14.5±1.58	16.6±2.21	16.9±1.81	15.8±1.29	0.663	0.791	0.360
Total CD28+ (co-stimulatory molecule)	19.9±4.85	31.5±4.22	24.9±6.79	22.8±5.95	0.748	0.415	0.240
Total CD127+	4.03±0.306	4.36±0.412	4.48±0.765	4.48±0.755	0.628	0.784	0.779
	% of CD3+ gated cells						
CD3+CD4+ (helper T cells)*	65.3±1.47	63.9±1.35	67.0±4.19	67.0±1.71	0.309	0.763	0.770
CD3+CD4+CD127+	4.52±0.731[b]	5.16±1.59[ab]	4.41±0.630[b]	7.22±1.08[a]	0.233	0.043	0.188
CD3+CD4+CD25+	23.2±2.37	22.7±3.01	21.4±3.25	18.6±1.91	0.319	0.583	0.698
CD3+CD8+ (cytotoxic T cells)	34.9±1.31	35.4±1.50	33.1±3.73	32.4±1.55	0.256	0.971	0.786
CD3+CD8+CD127+	6.45±0.974	5.76±0.616	6.21±0.494	6.96±0.833	0.572	0.975	0.395
CD3+CD8+CD25+	16.6±1.42	18.4±1.33	17.5±1.73	17.6±2.32	0.982	0.582	0.641
CD3+CD4+:CD3+CD8+	1.89±0.114	1.83±0.117	2.19±0.366	2.10±0.163	0.165	0.719	0.954

[1] Values are means ± SEM. Labeled means in a row without a common letter differ based on the two-way ANOVA test with the Duncan adjustment for multiple comparisons test ($P < 0.05$).
*Analysis performed on log-transformed values.
Abbreviations: HFD, high-fat diet; LFD, low-fat diet.

Figure 6. Immune cell phenotypes from splenocytes of male and female Wistar rats a LFD or HFD for 9 wks. Values are means ± SEM, n=6. Labeled means without a common letter differ based on the two-way ANOVA test with the Duncan adjustment for multiple comparisons test ($P < 0.05$).



Abbreviations: HFD, high-fat diet; LFD, low-fat diet.

Discussion

In this study, we aimed to determine sex differences on immune function following a HFD compared to a LFD. Our data partially support our hypothesis that feeding a HFD impairs immune response to a greater extent in males compared to females in the Wistar rat model. We observed that females gain less weight than males independently of diet, consistent with other findings in the literature (Casimiro, Stull *et al.,* 2021, Grove, Fried *et al.,* 2010, Medrikova, Jilkova *et al.,* 2012). Female rats on the LFD and HFD also had similar food intake throughout the study. However, LFD-fed males tended to increase their food intake (g/week), and therefore led to similar body weight gain compared to those fed the HFD. Females are more resistant to HFD-induced metabolic changes compared to males (Estrany, Proenza *et al.,* 2013, Medrikova, Jilkova *et al.,* 2012), and therefore longer feeding periods are required for females to develop similar metabolic complications (Camporez, Lyu *et al.,* 2019). DIO is a widely used model, which best mimics human obesity by inducing metabolic changes when compared to genetically modified rodent models (Madsen, Hansen *et al.,* 2010). Yet, not all DIO models lead to important changes in body weight between animals fed a LFD or HFD (Foright, Johnson *et al.,* 2020, Miras, Seyfried *et al.,* , Storlien, James *et al.,* 1986). Moreover, similarly to humans, wild type rat models gradually develop obesity, therefore longer feeding periods would be required to achieve significant differences in metabolic changes (de Moura e Dias, dos Reis *et al.,* 2021, Wang & Liao 2012).

Metabolic changes induced by a high-fat diet in males and females

Plasma glucose concentrations were unaffected by sex and diet in our study, and this is partly attributable to the fact that glucose levels were measured in the non-fasting state and that the LFD have higher proportion of carbohydrates than the HFD. On the other hand, females had

higher plasma concentrations of adiponectin and tended to have lower plasma concentrations of leptin compared to males, independently of diet. Similar results were previously reported by others (Manieri, Herrera-Melle *et al.,* 2019, Raff, Hoeynck *et al.,* 2018, Ribot, Rodriguez *et al.,* 2008). Adiponectin levels correlate negatively while leptin levels correlate positively with visceral obesity (Cancello, Tounian *et al.,* 2004). In our study, similar correlations were observed between adiponectin/leptin and body weight. Therefore, these sex differences could be partly explained by the fact that females gain less body weight overall compared to males.

Sexual dimorphism in adiponectin concentrations and the role of sex hormones has been described in humans (Dupont, Bertoldo *et al.,* 2018). In men, adiponectin concentration decreases with sexual maturation, whereas in women it is unchanged (Böttner, Kratzsch *et al.,* 2004). Previous studies reported that castration of male mice led to similar plasma adiponectin concentrations to those measured in females (Gui, Silha *et al.,* 2004, Manieri, Herrera-Melle *et al.,* 2019), whereas ovariectomy does not seem to influence plasma adiponectin concentrations (Combs, Berg *et al.,* 2003, Gui, Silha *et al.,* 2004). Therefore, the sex difference in adiponectin could be at least partially explained by differences in sex hormones (Cnop, Havel *et al.,* 2003). The HFD in our study, independently of sex, tended to increase leptin levels. The adiponectin to leptin ratio has been considered as an indicator of AT dysfunction as it has been associated with increased cardiometabolic risk factors (Frühbeck, Catalán *et al.,* 2018). Indeed, our findings showed that both LFD- and HFD-fed males had similar adipose tissue dysfunction (i.e., low adiponectin: leptin ratio) compared to HFD-fed females, whereas LFD-fed females had higher adipose tissue function compared to all other groups. Altogether, this data suggests that males have an enhanced "obesity phenotype" as assessed by adipokines plasma levels, and that feeding a HFD can exacerbate this phenotype.

Sex differences in systemic inflammatory profile

Females fed the HFD had higher plasma concentrations of IFN-γ compared to both LFD- and HFD-fed males. Similar sex differences were observed for *ex vivo* cytokine production after PMA+I stimulation. Females fed the HFD produced approximately four times more IFN-γ than HFD-fed males, although this did not reach statistical significance. IFN-γ is an important Th1 cytokine involved in antigen presentation, activation of innate immune responses, cellular proliferation, and apoptosis (Tau & Rothman 1999). It has been previously reported that females have a stronger Th1 immune response compared to males, including increased IFN-γ during parasitic and fungal infections, or upon stimulation with anti-CD3 and anti-CD28 (Pinzan, Ruas *et al.,* 2010, Roberts, Walker *et al.,* 2001, Zhang, Rego *et al.,* 2012). Naïve Th cells from both female Swiss mice and healthy women produce more IFN-γ in response to proteolipid protein p-139-151 (immunizing agent) and anti-CD3/anti-CD28 stimulation (Zhang, Rego *et al.,* 2012). Interestingly, peroxisome proliferator-activated receptor (PPAR)-α mRNA was highly expressed in male compared to female T cells in both mice (Dunn, Ousman *et al.,* 2007) and humans (Zhang, Rego *et al.,* 2012), and it has been reported that PPAR-α exclusively limits Th1 cytokine production in males (Zhang, Ahn *et al.,* 2015). Although we did not measure PPAR-α expression, this may explain the differences observed in IFN-γ plasma concentrations and *ex vivo* cytokine production in our study.

A significant sex and diet interaction was observed for plasma IL-13 concentrations. Males fed the LFD had lower IL-13, which was increased on the HFD-fed group. The opposite was seen in females where they had higher levels of IL-13 when fed the LFD and decreased when fed the HFD. IL-13 is a Th2 cytokine involved in humoral immunity by stimulating B cells to produce

immunoglobulins (Spellberg & Edwards 2001). Interestingly, a link between IL-13 and sex hormones has been previously reported. Estrogen induces humoral responses by the activation of B cells, increasing the production of IL-10, and therefore is associated with a Th2 skewed immune response (Salem 2004). Similarly, progesterone stimulates the secretion of antibodies and stimulate the production of IL-10 by antigen presenting cells (APCs). Progesterone and estradiol have been shown to increase the *in vitro* production of IL-13 by human PBMCs isolated from patients with allergic rhinitis (Hamano, Terada *et al.,* 1998), and progesterone only was shown to regulate IL-13 production by monocyte-derived DCs from human PBMCs of healthy donors (Kyurkchiev, Ivanova-Todorova *et al.,* 2007). Accordingly, after stimulating splenocytes with PWM, we observed similar diet*sex interactions for IL-6 ($P = 0.04$) and IL-10 ($P = 0.08$), in which the production was higher in HFD-fed male rats, whereas the opposite was observed for females. We observed a similar diet*sex interaction for IL-6 production after PMA+I stimulation, a T cell mitogen, but not for IL-10 production. This suggests that the sex difference in the IL-10 response may have been primarily driven by antigen-presenting cells, including macrophages, whereas the IL-6 response is most likely primarily driven by T cells.

A similar sex and diet interaction was observed for the ratio of plasma Th1:Th2 cytokines as assessed by the TNF-α:IL-13 ratio. Th1:Th2 cytokine profile is skewed towards Th2 in women, whereas in men it is skewed towards Th1 in the general population (Giron-Gonzalez, Moral *et al.,* 2000, Pellegrini, Contasta *et al.,* 2011). Consistently, we demonstrated that LFD-fed males have a skewed plasma Th1 cytokine profile compared to females fed the LFD. However, in the context of an obesogenic diet, females shift the balance towards an enhanced Th1 cytokine profile whereas males exhibit a skewed Th2 cytokine profile. Corroborating the results seen in females, Gong et al. (Gong, Shi *et al.,* 2018) recently reported that women with polycystic ovary syndrome had a

higher Th1:Th2 ratio compared to controls, and this was positively correlated with abdominal obesity (Gong, Shi *et al.,* 2018).

Sex differences on immune function

We demonstrated that feeding a HFD impaired T cell responses to a greater extent in males compared to females in our rodent model. We observed a decrease in the production of IL-2 and Th1 pro-inflammatory cytokines (TNF-α and IL-6) in splenocytes in response to PMA+I stimulation. No sex or diet effects were noted on most T cell subsets investigated. However, there was a diet effect on the proportion of helper T cells expressing CD127 which was increased with the HFD. CD127, also known as the IL-7 receptor α chain, is important for the development and regulation of naïve and memory T cells (Surh & Sprent 2008). Although we did not measure plasma IL-7 levels, this cytokine has been shown to induce the production of pro-inflammatory cytokines (e.g., IL-1β, IL-6, TNF-α) in human PBMCs and has been implicated in the pathogenesis of inflammatory diseases, such as rheumatoid arthritis (Kim, Hwang *et al.,* 2008).

The lower production of Th1 cytokines could be responsible for the lower T cell proliferation rate since they are known to increase IL-2 production (Hayden & Ghosh 2014, Kasahara, Hooks *et al.,* 1983). Yet, the lower T cell proliferation could also explain the lower amount of Th1 cytokines produced. We also confirmed in a proliferation assay, that HFD-fed males had greater reduction in T cell proliferation in response to anti-CD3/anti-CD28 stimulation compared to all other groups, although this did not reach statistical significance (P-diet = 0.097). This suggests that feeding a HFD in males impairs T cell receptor (TCR) responsiveness. Interestingly, the pronounced effect of the HFD on T cell function was not observed in females. Splenocytes from females fed the LFD showed higher production of IL-2 when compared with all

other groups. Although the HFD tended to lower its production, in HFD-fed females IL-2 production was still at the same level as in the LFD-fed males. This could be explained, at least in part, by how differently Th1 cytokines respond to an HFD in females. Indeed, TNF-α production remained stable while IL-6 production tended to increase in females fed the HFD, whereas they both decreased significantly in males fed the HFD. Hence, females likely maintain higher T cell function with a HFD partly because they can maintain a better Th1 cytokine response. This is also consistent with the circulating inflammatory profile of cytokines observed in females in response to a HFD.

We have also demonstrated that feeding a HFD, independently of sex, increases IL-10 production by splenocytes after PMA+I stimulation compared to a LFD. Although we did not observe significant differences between groups, the magnitude of increase was greater in females (66%) compared to males (24%). IL-10 is an important immunomodulatory cytokine involved in the resolution phase of inflammation and signals through signal transducer and activator of transcription 3 to inhibit inflammatory responses, such as the production of TNF-α and IFN-γ (Schottelius, Mayo *et al.,* 1999). The increase in IL-10 could be a compensatory mechanism in trying to control the excess inflammation in the context of HFD. Of note, although IL-6 is often considered a Th1 cytokine, it has a dual role and can act as both a pro- or anti-inflammatory cytokine. Therefore, the increase in IL-6 production after PMA+I stimulation in females fed the HFD could also mean a higher Th2 response. Together, feeding a HFD in male Wistar rats increases Th2 (IL-10) and reduces Th1 (TNF-α) leading to an overall lower Th1:Th2 ratio. In females, while there is an increase in Th2 response, there are no changes (or even an increase) in Th1 response following a HFD. This is consistent to some degree with studies reporting that females are less susceptible to a wide range of infections and present a better prognosis than males

(vom Steeg & Klein 2016), which has also been shown regarding coronavirus disease 2019 (COVID-19) mortality (Peckham, de Gruijter *et al.,* 2020).

Although most of the immune phenotypic changes were observed in APCs, there were little changes in *ex vivo* cytokine production after LPS and PWM stimulation. Males tended to have a higher proportion of APCs in general. Males fed the LFD presented a higher proportion of DC compared to rats fed the HFD and a higher proportion of monocytes in both diets compared to females. Moreover, the proportion of activated monocytes (i.e., CD68+ expressing CD11b/c+) tended to be lower in females fed the HFD. It has been previously reported that macrophages from male mice produce more C-X-C motif chemokine ligand 10, IL1-β, and IL-6 following LPS stimulation than macrophages from females (Marriott, Bost *et al.,* 2006). Altogether, this could explain to some extent the better response (i.e., higher IL-2 and IL-6 production) in males after PWM stimulation. However, it is unclear how these minor changes in immune cell subsets can help explain the important changes we observed in T cell function. Finally, females fed the HFD had a lower proportion of activated macrophages suggesting development of a milder "obesity phenotype" compared to males, which is consistent with the adiponectin and leptin results. Leptin has been demonstrated to upregulate CD11b and CD11c in human monocytes in a similar manner to that produced by LPS and to promote proliferation and cytokine production (Santos-Alvarez, Goberna *et al.,* 1999).

Our study has several strengths and limitations. We had a small sample size to conduct *post hoc* analysis and could mainly assess main sex and diet effect as per designed *at priori*. Although our sample size was enough to see differences in our primary outcome (i.e., *ex vivo* cytokine production), we were underpowered for secondary analysis with higher inter-individual variations (e.g., leptin). Other potential mechanisms to explain sex differences on immune function were not

explored such as the effect of sex hormones, insulin, and diet-induced alterations in the gut microbiome. Our animals were not pair-fed, which would have avoided differences in food intake between males. Specifically, HFD-males consumed less essential nutrients on week 2, 5, 6, and 7 compared to LFD-fed males. Moreover, we did not evaluate body composition and although we did not see differences in body weight, alterations in different adipose sites based on sex may contribute to immune response in this HFD rodent model. Female rats could have reached puberty earlier since they were housed in the same room as the male rats for the duration of the experiment, therefore this could have influenced to some extent our cytokine results. Finally, although *ex vivo* cytokine production from splenocytes is a useful method to understand how different immune cell populations respond together to challenges, it does not allow to understand the changes in cytokines from specific cell populations and residing in specific tissues (e.g., peritoneal macrophages). It is important to note that most studies investigating the effect of DIO on immune function do not match for the proportion of different fatty acids between the LFD and the HFD. This is an important consideration when designing nutritional rodent studies since fatty acids are known to influence immune function and systemic inflammation.

Sex differences are also present in the fatty acid content of tissues. For examples, females generally have higher plasma DHA concentrations than males (Childs, Romeu-Nadal *et al.,* 2008, Lohner, Fekete *et al.,* 2013). Females also have lower PPAR-α receptors expression compared to males (Jalouli, Carlsson *et al.,* 2003), which affects lipid metabolism and hepatic β-oxidation (Yoon 2010). Therefore, matching the proportion of fatty acids when assessing the effect of a HFD vs LFD is an important strength of our study. Yet, fatty acids are known to influence inflammation (Nakatani, Kim *et al.,* 2003), therefore the balanced lipid profile of our diet might have attenuated

some of the well-known metabolic effects of high saturated fat diet (Buettner, Parhofer *et al.,* 2006, Todoric, Loffler *et al.,* 2006).

Conclusion

In summary, our findings suggest that feeding a HFD impaired T cell responses to a greater extent in male Wistar rats compared to female rats by decreasing T cell proliferation and Th1 cytokine production. Females gained less weight compared to their male counterparts and developed a milder "obesity phenotype", which could be responsible for their overall enhanced T cell function compared to males. Our study demonstrates that sex plays an important role in modulating immune function in Wistar rats in the context of obesity. Future studies should explore other potential mechanisms to explain sex differences in immune function.

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