# The immunomodulatory effect of dietary phosphatidylcholine within the context of obesity

by

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**Abstract**

Obesity is a worldwide epidemic that is associated with immune dysfunction. In human and rodent models, obesity-induced immune dysfunction has been characterized by lower T cell proliferation and interleukin (IL)-2 production. While the mechanisms responsible for the observed reduction in T cell function is unclear, it has been suggested that lipotoxicity and increased intestinal permeability leading to bacterial translocation may play a role. Phosphatidylcholine (PC) is a lipid-soluble form of the essential nutrient choline that can be found in both animal and plant foods (i.e., eggs and soy). PC is an integral phospholipid in cell membranes and contributes to the hydrophobicity of the gastrointestinal mucosal layer. Our lab has shown that a diet containing 100% egg-PC enhances both peripheral and gut-associated T cell responses early in life when compared to a diet containing only free choline (FC), a water-soluble form of choline. Soy-derived PC has also been shown to support T cell response (albeit to a slightly lesser degree) and villi length. Further, we have shown that providing 100% egg-PC in a high-fat diet (HFD) improved T cell response when compared to a HFD containing only FC. However, no human diet contains choline solely in the form of PC. Therefore, the objective of this thesis was to determine the impact of feeding different doses and dietary forms of PC on immune function in a rodent model of obesity.

In our first study, we examined the effect of different doses of PC in the context of a HFD. Male Wistar rats four weeks of age were randomized to consume one of six diets for 12 weeks containing the same amount of total choline but differing in the forms of choline: 1- Control Low-fat (CLF, 20% fat, 100% free choline (FC)); 2- Control High-fat (CHF, 50% fat, 100% FC); 3- 100% PC (100PC, 50% fat, 100% egg-PC); 4- 75% PC (75PC, 50% fat, 75% egg-

PC+25% FC); 5- 50% PC (50PC, 50% fat, 50% egg-PC+50% FC); 6- 25% PC (25PC; 50% fat, 25% egg-PC+75% FC). We observed that feeding the CHF diet increased intestinal permeability compared to the CLF diet, and doses of PC 50% of greater returned permeability to levels similar to that of the CLF diet. Feeding the CHF diet lowered splenocyte production of IL-1β, IL-2, IL-10, and tumor necrosis factor-alpha (TNF-α), and mesenteric lymph node (MLN) lymphocyte production of IL-2 compared to the CLF group. The 50PC diet most consistently significantly improved cytokine levels (IL-2, IL-10, TNF-α) compared to the CHF diet.

In our second study, we examined the differential effect on immune function of egg- and soy-PC in the context of a HFD. Male Wistar rats were randomized to consume one of 4 diets for 12 weeks, all containing 1.5g of total choline/kg of diet but differing in choline forms: 1- CLF (100% FC); 2- CHF (100% FC); 3- High-fat Egg-derived PC (EPC, 100% Egg-PC); 4- High-fat Soy-derived PC (SPC, 100% Soy-PC). After T cell mitogen stimulation, the CHF-fed rats had a lower production of IL-2 compared to the CLF group which was normalized by feeding EPC and SPC. After T and antigen presenting cell (APC) mitogen stimulation, the CHF-fed rats had a lower production of IL-1B, IL-10 and TNF-α vs. the CLF group and feeding EPC normalized the production of IL-10 and TNF-α while SPC did not.

Overall, we concluded that a dose of 50% of total choline derived from egg-PC can ameliorate HFD induced intestinal permeability and immune cell dysfunction. In addition, while both egg- and soy-PC have similar effects on intestinal permeability, egg-derived PC attenuates obesity-induced T cell dysfunction to a greater extent than soy derived PC.

**Preface**

This thesis is an original work by Tianna Rusnak. 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 "High-fat diet and T cell function" with protocol number AUP00002859, August 2018. The contributions made by the candidate, Tianna Rusnak, and the co-authors to the completion of this work are described here.

**Chapter 4** of this thesis has been submitted for publication as Rusnak T, Azarcoya-Barrera J, Wollin B, Makarowski A, Nelson R, Field CJ, Jacobs RL, Richard C. A physiologically relevant dose of 50% egg-phosphatidylcholine is sufficient in improving gut permeability while attenuating immune cell dysfunction induced by a high-fat diet in male Wistar rats to The Journal of Nutrition (2023). CR and RLJ designed and obtained funding for this study. AM, TR, JAB, BW, and RN conducted research and analyzed data. TR performed the statistical analysis and wrote the manuscript under the supervision of CR, RLJ and CJF. CR has primary responsibility for final content.

**Dedication**

I want to thank my parents, Deb and Craig, my sister, Sydney, and my grandfather, Tony, for their love, words of encouragement, and continuous support. This achievement is as much yours as it is mine.

**Acknowledgements**

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**List of Abbreviations**

AI - adequate intake

ALA - α-linoleic acid

APC - antigen presenting cells

ARA - arachidonic acid

CD - cluster of differentiation

CRP - C-reactive protein

CTL - cytotoxic T cells

DC - dendritic cells

DHA - docosahexaenoic acid

EPA - eicosapentaenoic acid

ER - endoplasmic reticulum

FA - fatty acid

FC - free choline

GALT - gut-associated lymphoid tissue

GPC - glycerophosphocholine

HFD - high-fat diet

IFN-γ - interferon-gamma

IEC – intestinal epithelial cells

Ig - immunoglobulins

IL - interleukin

LA - linoleic acid

LP - lamina propria

LPS - lipopolysaccharide

Lyso-PC - lysophosphatidylcholine

MHC - major histocompatibility complex

MLN - mesenteric lymph nodes

MFGM - milk fat globule membrane

MUFA - monounsaturated fatty acid

n - omega

NF-κB - nuclear factor kappa B

NK - natural killer

PC - phosphatidylcholine

Pcho - phosphocholine

PE - phosphatidylethanolamine

PEMT - phosphatidylethanolamine N-methyltransferase

PMA+I - phorbol 12-myristate 13-acetate plus ionomycin

PP - Peyer's patches

PWM - pokeweed mitogen

PUFA - polyunsaturated fatty acid

SEM - standard error of the mean

SFA - saturated fatty acid

SM - sphingomyelin

T2D - type 2 diabetes

TCR - T cell receptor

Tfh - T follicular helper cells

Th - T helper

TGF-β - transforming growth factor beta

TLR-4 - toll-like receptor-4

TNF-α - tumor necrosis factor-alpha

Treg - T regulatory cells

UC - ulcerative colitis

VLDL - very low-density lipoprotein

# CHAPTER 1: Introduction and literature review

## 1.1 Overview of phosphatidylcholine

Phosphatidylcholine (PC) is a lipid-soluble form of the essential nutrient choline. PC is one of the most abundant phospholipids of all mammalian cells and subcellular organelles, and thus plays a major role in the structure and function of those membranes (van der Veen, Kennelly et al. 2017). Further, PC comprises a major component of the lipids involved in the mucosal layer of the epithelium (Ehehalt, Wagenblast et al. 2004). PC can be acquired through *de novo* synthesis, but dietary intake of choline is also required to satisfy physiological needs (Zeisel and da Costa 2009).

### 1.1.1 Dietary sources

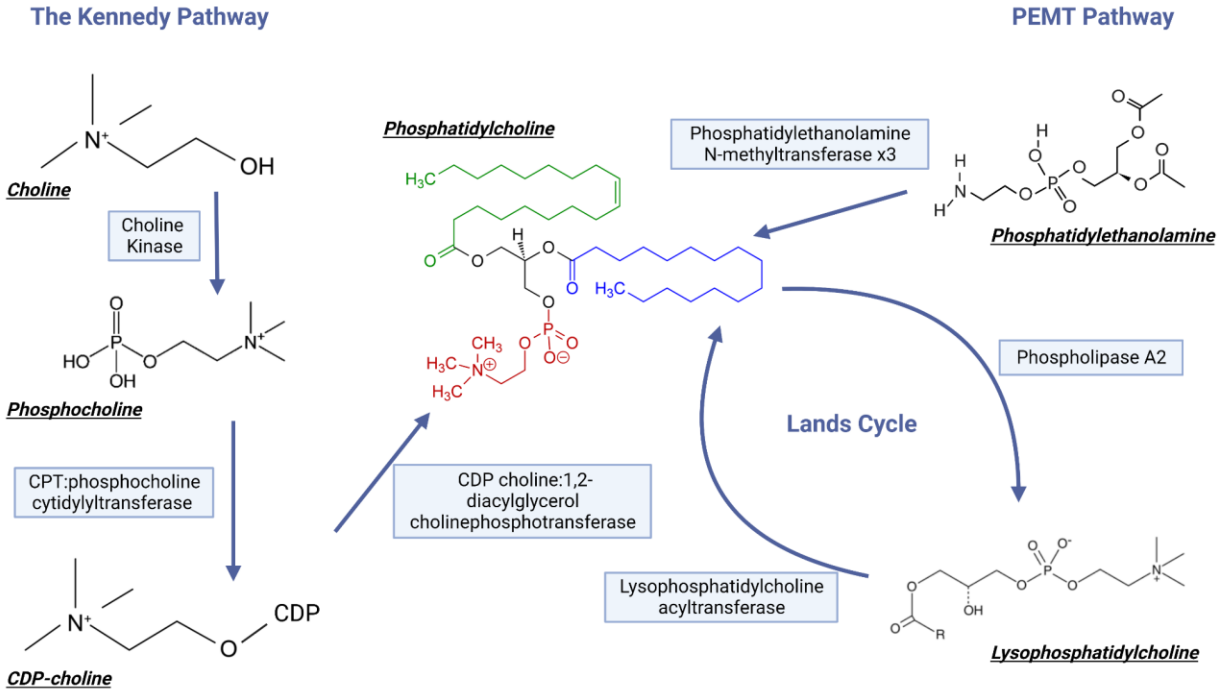
While there are dietary recommendations for choline, set as adequate intake (AI), no recommendations currently exist for the specific forms of choline. Dietary choline is found mainly in water-soluble forms (free choline [FC], phosphocholine [Pcho], glycerophosphocholine [GPC]), or lipid-soluble forms (PC, sphingomyelin [SM]). Across several world-wide cohorts, PC has been reported as the major form of choline consumed, with proportions ranging between 45-55% of total choline (Lewis, Subhan et al. 2014; Detopoulou, Panagiotakos et al. 2008, Wiedeman, Barr et al. 2018). Dietary patterns can highly influence the amount of PC consumed. PC is rich in animal products, such as dairy, meat, and especially eggs (Lewis, Field et al. 2015). In the Alberta Pregnancy Outcomes and Nutrition (APrON) cohort, the top reported individual dietary sources of PC were eggs and milk (Lewis, Subhan et al. 2014). A major source of PC in milk comes from milk fat globule membranes (MFGM). MFGM

comprises approximately 30% PC, as well as several other phospholipids (Singh 2006), and many studies have shown the immunoprotective role of MFGMs in infant formula (Zanabria, Tellez et al. 2014).

### 1.1.2 *De novo* synthesis of PC

A simplified overview of the pathways of *de novo* PC synthesis is summarized in **Figure 1-1**. In most eukaryotic cells, PC is synthesized from choline through the Kennedy Pathway primarily. The branch of the Kennedy Pathway that forms PC, the CDP-choline pathway, consists of 3 enzymatic steps: 1 - Choline kinase catalyzes the ATP dependent phosphorylation of choline to form phosphocholine; 2 - CTP:phosphocholine cytidylyltransferase uses phosphocholine and CTP to form CDP-choline (rate limiting step); 3 - CDP-choline phosphotransferase catalyzes the formation of PC by adding the choline headgroup of CDP-choline to a lipid anchor such as diacylglycerol (Gibellini & Smith 2010). In another pathway, the Lands cycle, lysophosphatidylcholine (lyso-PC) is reacylated by adding a fatty acid (FA) at the sn-2 position to yield PC (O'Donnell 2022). Finally, PC can be synthesized through the phosphatidylethanolamine *N*-methyltransferase (PEMT) pathway, which produces PC through 3 successive methylations of phosphatidylethanolamine (PE) catalyzed by the PEMT enzyme (Verkade, Havinga et al. 2007). Of note, this last pathway primarily occurs in the liver.

**Figure 1-1: An overview of the Kennedy Pathway, PEMT Pathway, and Lands Cycle for PC synthesis (Adapted from Armitage, Barnes et al. 2021)[1]**




[1] Abbreviations: CDP, Cytidine-5′-diphosphocholine CTP, Cytidine-5′-triphosphocholine; PEMT, phosphatidylethanolamine N-methyltransferase. Created with Biorender.com

### 1.1.3 Consequences of PC deficiency

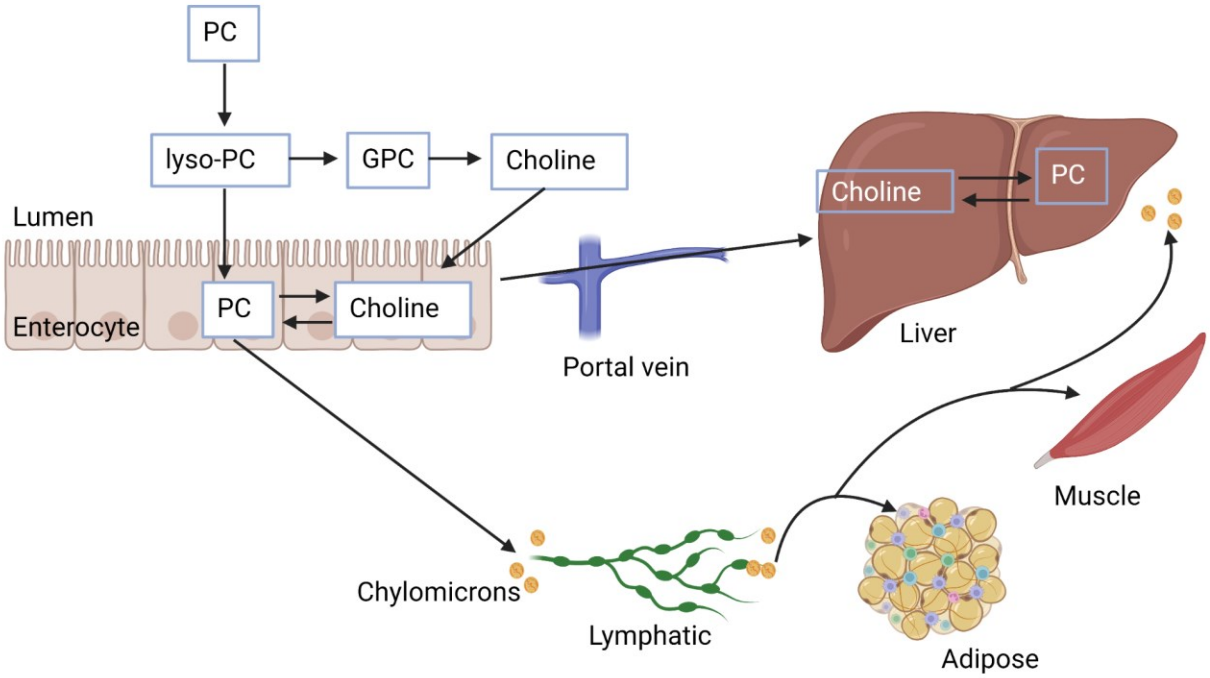
Consequences of choline deficiency have been well documented, including development of hepatosteatosis and subclinical organ dysfunction (fatty liver or muscle damage), apoptosis of lymphocytes, and DNA damage (da Costa, Badea et al. 2004, da Costa, Niculescu et al. 2006). Several studies have examined PC deficiency in mice by knocking out genes involved in PC synthesis from the PEMT and CDP-choline pathways (van der Veen, Kennelly et al. 2017). Several disease states were induced, such as muscular dystrophy, metabolic dysfunction-associated steatotic liver disease, bone deformation, and embryonic lethality (van der Veen, Kennelly et al. 2017, Rinella, Lazarus et al. 2023). Less is known about dietary PC deficiency, but our lab consistently has shown that feeding a diet consisting either of a mixture of choline

forms (including PC, SM, GPC, and FC) or solely PC improves immune function in maternal and developmental periods, as well as under the context of obesity when compared to a diet containing only FC (Azarcoya-Barrera, Field et al. 2021, Azarcoya-Barrera, Wollin et al. 2022, Dellschaft, Richard et al., 2018 Lewis, Richard et al. 2017). These findings suggest that regardless of the total amount of choline in the diet, providing choline as PC leads to beneficial effects on the immune system. Yet, it is unknown what is the ideal proportion of the different choline forms for optimal immune function. This will be discussed further in section 1.3.

### 1.1.4 Absorption and metabolism of dietary PC

PC is composed of two FAs covalently linked to a glycerol moiety by ester bonds in the sn-1 and sn-2 positions (**Figure 1-1**). Due to this structure, PC cannot be absorbed directly into the jejunum, and thus must be hydrolyzed first by phospholipase A2 to form lyso-PC in order to be absorbed by the enterocyte (Parthasarathy Subbaiah et al. 1974) (**Figure 1-2**). Lyso-PC is then either acetylated back to PC or further broken down to form GPC, then FC. As a lipid soluble form of choline, PC is secreted into chylomicrons in the enterocyte and enters the bloodstream through the thoracic duct (Scow, Stein et al. 1967, Le Kim and Betzing 1976). Thus, PC is directly delivered to peripheral organs (adipose tissue, muscle) on route to the liver. Both dietary and endogenously synthesized sources of PC can then be utilized in the formation of lipoproteins, bile, and various signaling molecules such as phosphatidic acid and arachidonic acid (ARA).

**Figure 1-2: Intestinal absorption and metabolism of dietary PC in humans (Adapted from Lewis, Field et al. 2015, Murota 2020)[1]**




[1] Abbreviations: GPC, glycerophosphocholine; PC, phosphatidylcholine. Created with Biorender.com

### 1.1.5 Major functions of PC

#### 1.1.5.1 Phospholipid metabolism and lipoprotein secretion

PC plays an important role in formation and stability of lipoproteins. PC comprises an estimated 60-80% of total phospholipids in lipoproteins containing apo, such as apoB100 found in the liver and apoB48 in the intestine, both of which are important for the assembly of very low-density lipoproteins (VLDLs) and chylomicrons, respectively (Yao & Vance 1988). It has been shown that low PC levels in the membranes of the endoplasmic reticulum, the site of lipidation of apoB, will diminish VLDL secretion (van der Veen, Kennelly et al. 2017). Further,

mice lacking the enzymes CTalpha and PEMT have impaired VLDL secretion leading to increased lipid accumulation in hepatocytes (Jacobs, Devlin et al. 2004, Jacobs, Lingrell et al. 2008, Noga, Zhao et al. 2002).

#### 1.1.5.2 Cell membrane structure and signaling roles

PC contributes to 40-60% of total phospholipids in cell membranes and thus plays an integral role in cell structure and function. As part of the cell membrane, PC plays a role in both intracellular and extracellular signals, and can modulate cell growth, enzymatic activity, phagocytosis, and apoptosis, among other important physiological functions (Chakraborty & Jiang 2013, Exton 1994). PC is also cleaved to form lyso-PC, which is another membrane phospholipid, and is required for T cell activation, cell lysis, and chemotaxis (Nishizuka 1992). Since PC contains two FA covalently bonded to the sn-1 and sn-2 positions of the glycerol backbone, the FA present can have different signaling roles. For example, ARA can be released from the sn-2 position of cellular PC to form eicosanoids that go on to regulate inflammatory processes (Kanno, Wu et al. 2007). Docosahexaenoic acid (DHA) is another FA found in some PC molecules and can go on to regulate development, neuroprotection, and anti-inflammatory processes in the body (Kim, Huang et al. 2022, Wang, Guo et al. 2019).

#### 1.1.5.3 Mucosal barrier formation and integrity

PC plays a role in intestinal barrier formation and function by interacting with heavily glycosylated proteins called mucins to contribute to the hydrophobicity of the mucosal layer. PC and lyso-PC comprise approximately 90% of the phospholipids in the intestinal mucus (Korytowski, Abuillan et al. 2017). Several studies have shown that PC is diminished in the gut mucosa under the context of intestinal disorders such as Crohn's disease and ulcerative colitis

(UC) (Ehehalt, Wagenblast et al. 2004, Braun, Treede et al. 2009). Further, it is well documented that the gut mucosa of animal models of obesity is disrupted, suggesting that there may be perturbations in PC levels under the context of other metabolic disorders such as obesity (Paone & Cani 2020).

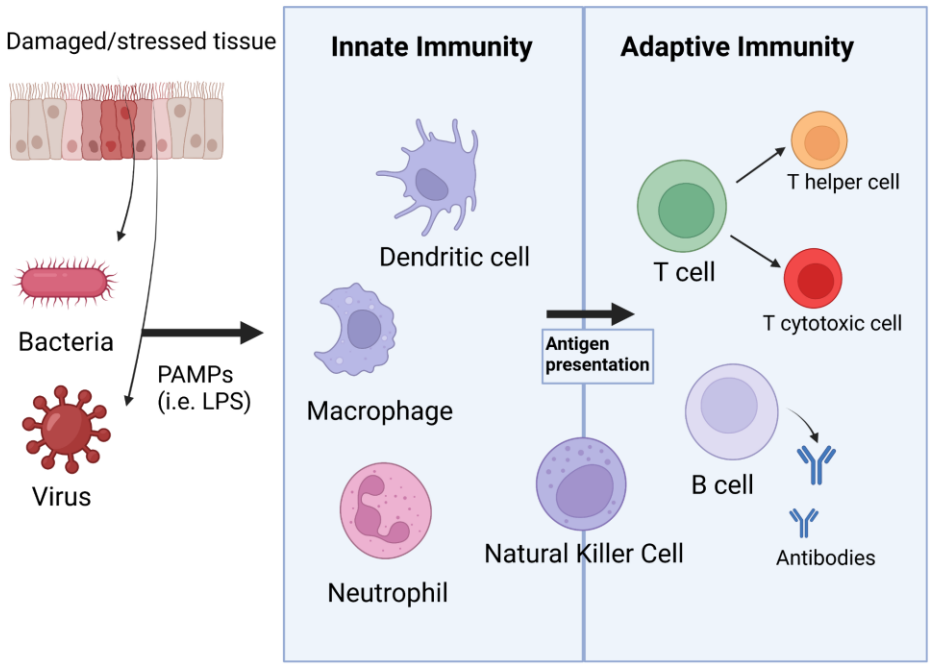
## 1.2 Overview of the immune system

The immune system plays an integral role in the body by providing protection from infectious agents through the action of several effector immune cells and molecules (Calder 2007). These actions are done by differentiating between "self" and "non-self" antigens to suppress or enhance responses respectively. The immune system is divided into two components: the innate and acquired (or adaptive) immune systems that coordinate the action of several subsets of immune cells, anatomical, and chemical barrier (Yaqoob & Calder 2011).

### 1.2.1 Peripheral immune function

Outside of the central nervous system the immune system can be divided into the peripheral and gut-associated immune systems. Both systems have innate and adaptive cells originating from and interacting with different lymphoid tissue and organs. The spleen is the major lymphatic organ of the peripheral immune system. The following two sections elaborate on the innate and adaptive cells. A general overview of the innate and adaptive immune cells is summarized in **Figure 1-3**.

**Figure 1-3: Overview of innate and adaptive immune cells[1]**




[1] Abbreviations: LPS, lipopolysaccharides; PAMPs, pathogen associated molecular patterns. Created with Biorender.com

### 1.2.1.1 Innate immunity

Innate immune response is characterized by a rapid response to a pathogen by phagocytic cells that can recognize conserved features on pathogens (Alberts, Jonson et al. 2002). The innate immune system is composed of cells that do not possess the ability to recognize specific molecules on invading pathogens called antigens. Another hallmark of innate immunity is the lack of ability to form immunologic memory. The innate immune system in vertebrates begins with the skin and other epithelial surfaces that provide a protective physical barrier between the inside of the body and the outside world. Interior epithelial surfaces are lined with a mucosal layer that both provides physical protection through hydrophobicity as well as containing

substances, such as antimicrobial peptides called defensins, that kill or deter pathogens (Alberts, Johnson et al. 2002). If a pathogen manages to enter the epithelial barrier, it can be recognized by innate cells to stimulate an inflammatory response, such as through NF-κB and inflammasome formation, and a phagocytic response by cells such as neutrophils and macrophages. This activation of macrophages stimulates the release of cytokines and other chemoattractants (chemokines) to attract other immune cells, such as neutrophils, monocytes and dendritic cells (DCs) to the site of infection (Alberts, Johnson et al. 2002). DCs will retrieve antigens from the invading pathogen and transport them to lymph nodes, where they will present the antigens to lymphocytes to initiate the adaptive immune response. In addition, natural killer (NK) cells express neither T cell nor B-cell antigen receptors, and thus are viewed as an intermediate cell between the innate and adaptive immune systems. NK cells express major histocompatibility complex (MHC) class 1-specific receptors and will destroy MHC class 1-deficient hematopoietic cells (Vivier, Tomasello et al. 2008). Healthy cells in steady-state conditions heavily express MHC class 1 molecules, and thus inhibitory MHC class 1 receptors present on NK cells ensure tolerance to self. Both NK cells and lymphocytes of the adaptive immune system can kill infected or stressed cells by inducing them to undergo apoptosis (Alberts, Johnston et al. 2002).

#### 1.2.1.2 Adaptive immunity

Adaptive immunity is characterized by an antigen-specific response (Alberts, Johnson et al. 2002). Due to this specificity, the adaptive immune response is delayed. However adaptive immune cells have the capacity for memory so that a subsequent exposure to a specific antigen will mount a more efficient immune response. Recognition of an antigen occurs by presentation of antigens by antigen presenting cells (APC) that express MHC I or II molecules, such as DCs (Goldsby 2003). Adaptive immune cells called lymphocytes are produced in the bone marrow by

a process called hematopoiesis. Once outside the bone marrow, lymphocytes circulate in the bloodstream and lymphatic system. They also reside in lymphatic organs such as the spleen (Goldstein & White 1971). B and T lymphocytes are two major lymphocyte populations responsible for immune response coordination.

#### 1.2.1.2.1 B cells

B cells arise and maturate in the bone marrow and represent approximately 5-15% of total circulating lymphocytes. Mature B cells express a unique antigen-binding receptor called B cell receptor, which upon binding with a specific antigen allows them to produce antibodies called immunoglobulins (Ig) (Cano & Lopera 2013). These antibodies include IgG, IgA, IgM, IgD, and IgE, and can neutralize a pathogen, tag it for phagocytosis, or activate complement proteins to lyse pathogens or infected cells. Polymeric antigens, such as lipopolysaccharide (LPS), can activate B cell responses directly, but this activation does not result in the development of B cell memory. In order to activate memory development, T cell co-stimulation must occur (Hoffman, Lakkis et al. 2016). B cells can serve as APC for T cells, which results in T cells co-stimulating B cells through secretion of cytokines. B cells that are specific to that antigen proliferate, becoming plasma cells or memory B cells. The plasma B cells secrete antibodies and memory B cells can respond to the recognized antigen upon future immune penetration. This form of immune response involving B cells and Ig response is known as humoral immunity.

#### 1.2.1.2.2 T cells

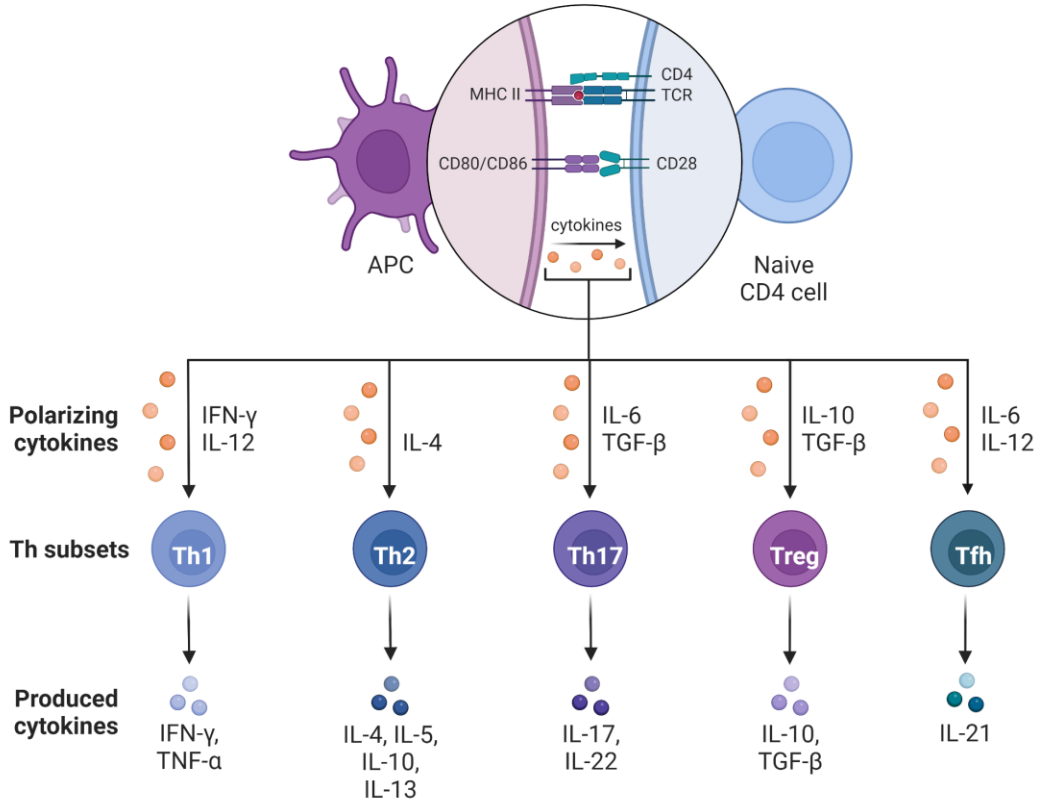
T cells, or T lymphocytes, arise in the bone marrow and migrate to the thymus for further maturation. In circulation T cells represent the highest proportion of lymphocytes as well as in

many lymph nodes including spleen and mesenteric lymph nodes (MLN) (Cano & Lopera 2013). Like B cells, mature T cells express a T cell receptor (TCR) on their surface that is antigen specific. Antigens must be presented to T cells by APC expressing MHC molecules for recognition to occur (Calder, Krauss-Etschmann et al. 2006). T cell activation through TCR binding stimulates the secretion of interleukin (IL)-2 that promotes further cell proliferation and differentiation. Membrane phospholipid hydrolysis occurs early in the process of lymphocyte activation. Interestingly, the FA composition of phospholipids from lymphocyte membranes have been shown to be affected by dietary manipulation thus resulting in changes in immune function (Moussa, Tkaczuk et al. 1999). Increased levels of linoleic acid (LA) in spleen lymphocytes correlated negatively with IL-2 receptor α chain (CD25) expression and the cell proliferation index. Activated T cells can go on to regulate innate, humoral, and other T cell activity as well as directly destroy infected cells. The immune response involved with T cells is termed cell-mediated immunity.

T cells can differentiate into distinct populations, CD4+ T helper (Th) and T regulatory (Treg) cells, and CD8+ T cytotoxic cells (CTL) (Cano & Lopera 2013). Th cells recognize MHC class II molecules that are expressed on APC (**Figure 1-4**). Th cells influence adaptive immune response by releasing different cytokines and chemokines that activate other effector cells and promote cell-mediated immunity. Th cells can be further subdivided based on the cytokines that stimulate proliferation and the subsequent cytokines the Th cell releases. Th1 cells typically develop in the presence of IL-12 and will secrete IL-2, interferon-gamma (IFN-γ), and tumor necrosis-alpha (TNF-α) (Kaiko, Horvat et al. 2008). Th1 cells and the cytokines they produce are important for the clearance of intracellular pathogens, as well the activation of other immune

cells such as macrophages and DCs. Th2 cells will proliferate in the presence of IL-4 and will go on to secrete cytokines including IL-4, IL-5, IL-10, and IL-13. These cytokines promote a humoral response by activating B cells to respond to extracellular pathogens and can down regulate the Th1 response. Some of these cytokines can also exert anti-inflammatory effects such as for IL-10. Th17 cells differentiate in the presence of a wide range of cytokines and/or other environmental factors, and are another subset involved more specifically in anti-fungal and anti-bacterial defenses. Th17 cell produce IL-17, IL-22, and TNF-α which result in a proinflammatory response recruiting other immune cells to the site and subsequent clearing of the pathogen (Mills 2008). Treg are another subset of Th that are involved in immune and oral tolerance and resolving inflammatory processes. Treg are characterized by the expression of CD4+ and CD25+ along with FoxP3 intracellularly (Josefowicz, Lu et al. 2012). Treg secrete 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 smaller specialized CD4+ subset that produce cytokines such as IL-4 and IL-21 and help with B cell activation and differentiation. CD8+ CTL are involved in cell-mediated immune responses by exhibiting a cytotoxic or "killing" effect on virus-infected and tumor cells. CD8+ cells recognize MHC class I molecules expressed on all cell types. CTL does not secrete many cytokines; IFN-$\gamma$ and TNF-α are the primary cytokines secreted by CTL.

**Figure 1-4. T helper cell activation and differentiation.**[1]



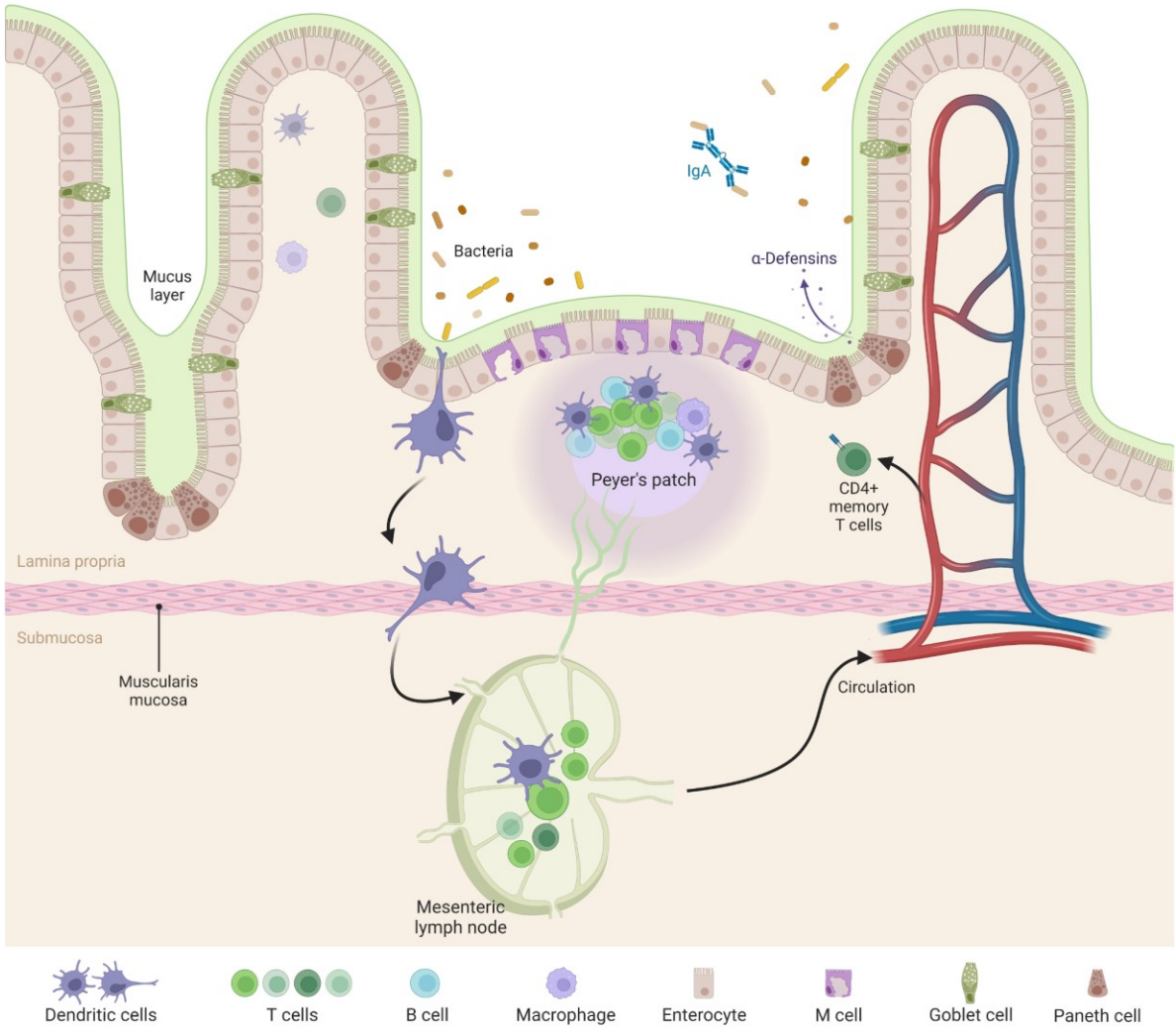

[1] Abbreviations: IFN-γ, interferon gamma; IL, interleukin; TGF-β, transforming growth factor-beta, TNF-α, tumor necrosis factor-alpha. Created with Biorender.com

### 1.2.2 Gut-associated immune function

An overview of the gut-associated immune system anatomy is presented in **Figure 1-5**. It is estimated that 70-80% of immune cells reside in the intestinal tract. A single layer of epithelial cells separates the inside of the body from the bacteria outside (Murch 2011). There are both well organized and diffuse lymphoid tissue in the gut. Well-organized immune compartments can be divided into two inductive sites, which are the MLN and gut-associated lymphoid tissue (GALT). In these areas innate immune cells will undergo initial priming and differentiation (Forchielli & Walker 2005). The intestinal lamina propria (LP) and epithelium can act as effector sites for the adaptive immune system that assist in maintaining gut barrier integrity and

protection (Morbe, Jorgensen et al. 2021). T cells are the predominant immune cell population in gut-associated adaptive immunity. The GALT contains Peyer's patches (PP) and isolated lymphoid follicles within the intestinal wall. PP and MLN are connected through lymphatic vessels and allow for antigen presentation to T and B cells (Morbe, Jorgensen et al. 2021). One of the primary functions of the intestinal immune system is to balance immunological response between protecting against pathogens and providing tolerance toward self-antigens and commensal bacteria within the gut microbial community. Perturbations in this balance can lead to intestinal diseases such as intestinal bowel disease, Crohn's disease, and UC (Koboziev, Karlsson et al. 2010). Further, it has been shown that high-fat diet (HFD) feeding leading to obesity can alter the gut microbiota leading to degradation of the intestinal barrier and subsequent increase in systemic inflammation (Cani, Bibloni et al. 2008).

**Figure 1-5: Overview of the gut-associated immune system[1]**



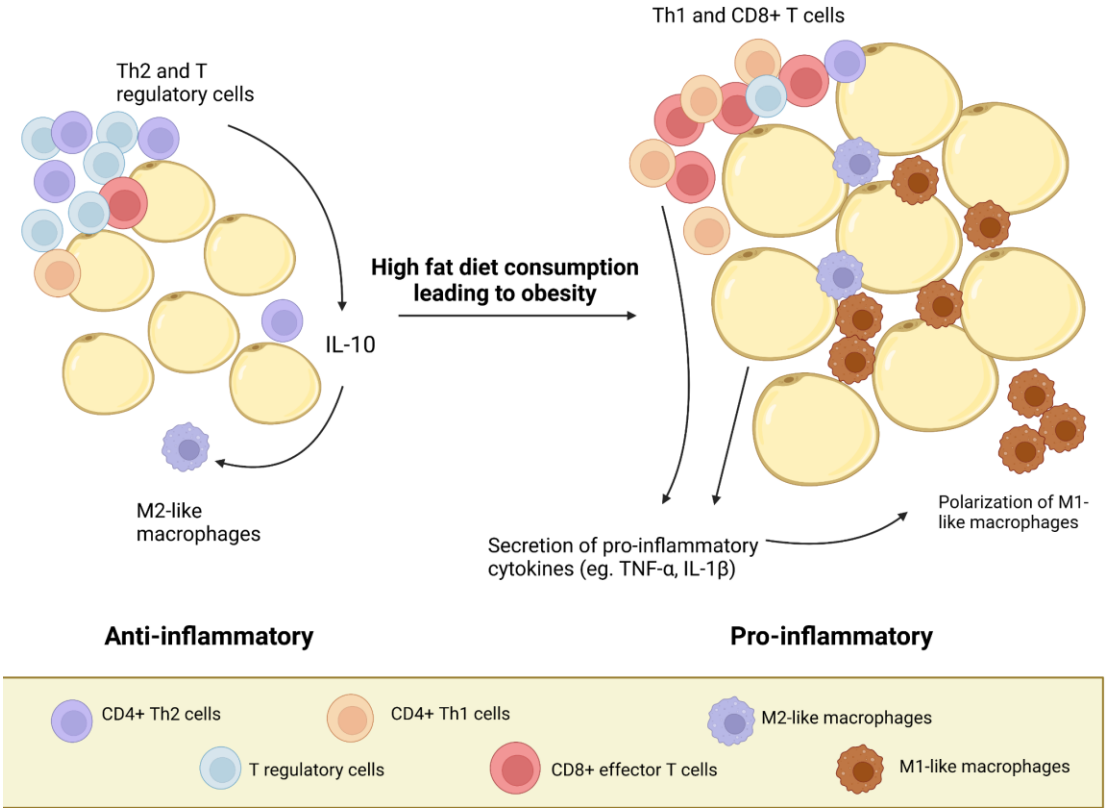

[1] Abbreviations: IgA, Immunoglobulin A. Created with Biorender.com

## 1.3 Effect of a HFD and obesity on immune function

Clinical and epidemiologic studies have shown that obesity is a risk factor for the development and severity of illnesses related to immune dysfunction (Nieman 1999). As such, obesity has been implicated as a factor in infection, poor wound healing, and poor vaccine response. Macrophages play a major role in obesity-associated inflammation through their infiltration of adipose tissue. Adipose tissue macrophages (ATMs) can comprise up to 40% of

cells in adipose tissue. It is postulated that the increase in size and number of adipose tissue results in intracellular stress, which stimulates adipocytes to secrete chemokines and cytokines and therefore perpetuating inflammatory response and macrophage infiltration (**Figure 1-6**; Reilly & Saltiel 2017). ATMs can be further subdivided into M1-like macrophages, which secrete primarily proinflammatory cytokines, and M2-like macrophages, which secrete anti-inflammatory cytokines. M1-like macrophages can accumulate lipids, which result in a foamy appearance in adipose tissue. M1-like ATMs are the predominant macrophage subset in obesity (Guzik, Skiba et al. 2017). Both T and B lymphocytes also play a role in adipose tissue inflammation. The exact timeline of lymphocyte recruitment in adipose tissue is still unclear, but Th1 cells begin to predominate and secrete proinflammatory cytokines, while the number of anti-inflammatory Th2 and Treg cells decreases in obesity (SantaCruz, Bharath et al. 2022). Further, CTL numbers increase and promote further recruitment and activation of ATMs. The number of B cells will also increase in adipose tissue and produce IgG antibodies which can further activate T cells and M1-like macrophage polarization.

**Figure 1-6: Immune cell alterations under the context of increasing adipose tissue[1] (Adapted from Osborn & Olefsky 2012)[1]**




[1] Abbreviations: IL-1β, interleukin-1beta; Th, T helper; TNF-α, tumor necrosis factor-alpha

### 1.3.1 Effects of HFDs and obesity on peripheral immune function

The cytokines that are secreted from adipose tissue will exert local paracrine effects, but also lead to a spillover into systemic circulation if levels become excessive (McNelis & Olefsky 2014). Further, adipose tissue inflammation mobilizes free FAs, which go on to deposit in the liver and muscle and interfere with metabolic signaling promoting local inflammation in other tissues. Prolonged metabolic dysfunction in these tissues will perpetuate further immune dysfunction systemically. This state is characterized by elevated circulating levels of inflammatory cytokines, such as TNF-α, IL-6, and C-reactive protein (CRP) (de Herdia 2012). This has implications for type II diabetes (T2D) risk as high levels of TNF-α can impair insulin-

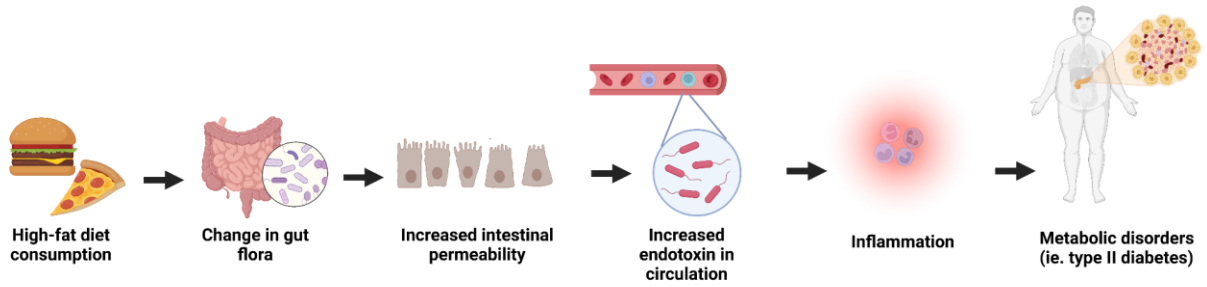
mediated glucose uptake (Guilherme A, Virbasius JV et al. 2008). Fat deposition will also occur in primary lymphoid tissues (Cortez, Carmo et al. 2013, Yang, Youm et al. 2009). This disruption will go on to adversely affect immune cell function in peripheral secondary lymphoid tissue, such as in the spleen (Kanneganti & Dixit 2012). HFD feeding has been shown to increase effector/memory T cells and decrease TCR diversity in male C57BL/6 mice when compared to a standard chow diet. Furthermore, Lamas *et al.,* (2002) showed that HFD feeding and obesity result in a lower T cell proliferation rate and decreased IL-2 production in mitogen-stimulated splenocytes in Wistar rats. This phenomenon is also observed in the clinical setting. While circulating levels of cytokines such as TNF-α and IL-6 are elevated in individuals with obesity and T2D, both conditions, especially T2D, are associated with poorer infectious disease outcomes (Kanneganti & Dixit 2012). Chandra *et al*., (1980) showed that 38% of obese children and adolescents had decreased lymphoproliferative responses to mitogens and a decreased killing capacity of intracellular bacteria by peripheral blood mononuclear cells (PBMCs). Our lab showed that the PBMCs of individuals with obesity and T2D produce significantly less IL-2, IL-6, and TNF-α after mitogen stimulation when compared to healthy individuals with obesity, further confirming the link between insulin resistance and immune dysfunction (Richard, Wadowski et al. 2017).

### 1.3.2 Effects of a HFD and obesity on gut-associated immune function

The intestines also display changes in microbial composition and the type of immune cell present in obesity. A potential hypothesis as to how HFD feeding may lead to systemic inflammation through the gut is presented in **Figure 1-7.** In both animal and human studies, obesity has been associated with changes in specific gut microbial populations with an overall decreased diversity of species. These alterations have been shown to be associated with intestinal

permeability that precedes inflammation (Cani, Bibiloni et al. 2008). This suggests that the changes in the gut microbiota are at least partially responsible for the degradation of the mucosal layer and tight junction proteins responsible for keeping bacterial content out of circulation. Indeed, obesity has been associated with an increase in circulating bacterial content (i.e., LPS) called endotoxins. In circulation, endotoxins bind with toll-like receptor 4 (TLR-4), which triggers a downstream signaling cascade resulting in the activation of the NF-κB pathway leading to an inflammatory response (Shi, Kokoeve, et al., 2006). These changes to intestinal barrier function result in increases in Th1 cells and CTLs secreting proinflammatory IFN-γ and decreases in Treg cells secreting anti-inflammatory IL-10 in the lamina propria (Khan, Luck et al. 2021). Diet-induced obesity has also been associated with a reduction in secretory IgA from B cells, which is involved in immune tolerance.

**Figure 1-7: Potential pathway by which HFDs induce inflammation related diseases (Adapted from Cani, Biblioni et al. 2008)**



### 1.4 PC and inflammatory-related diseases

#### 1.4.1 Cell culture studies

A full summary of the cell culture studies examining the role of PC in regulating immune dysfunction is presented in **Table 1-1**. Treede *et al.,* (2007) demonstrated that exogenously administered PC is integrated into intestinal epithelial cells (IECs) and significantly inhibits TNF-α induced inflammatory responses. Adding 10 μM of different PC species to latex bead phagosomes inhibited an early reaction in inflammation, membrane catalyzed actin assembly, in vitro. Further, pretreatment of cells with 200 μM of PC for 1 hour inhibited TNF-α induced NF-κB activation. PC significantly delayed TNF-α up-regulation of inflammatory genes in Caco-2 cells treated with PC (1,2-dipalmitoyl glycero-3-PC) for an hour. Altogether, Treede *et al.*, (2007) showed that PC is integrated into cell membranes to inhibit one of the first mechanisms of the inflammatory response, actin assembly, and goes on to impair TNF-α induced NF-κB signaling, and expression of proinflammatory genes.

Olson *et al.,* (2014) examined the potential for PC to protect the intestinal barrier against *Clostridium difficile* (*C. diff*) toxin using mucus-producing (HT29-MTX) and non-mucus-producing (HT29) intestinal epithelial monolayers. For both mucus- and non-mucus-producing cells, PC lowered *C.* diff stimulated TNF-α to levels similar to the IECs control group. PC also lowered IL-6 production in both cell lines, but IL-6 levels were still significantly higher than the baseline control. Permeability of IECs after *C. diff* challenge was highest in the non-mucus producing strain, but still elevated in the mucus producing strain. Exogenous PC administration reduced intestinal permeability to baseline or below baseline levels in the non-mucus-producing and mucus-producing cells, respectively.

Zanabria *et al.,* (2014) investigated the immunomodulatory effect of MGFM isolate on LPS stimulated splenocytes. Incubating cells with both LPS and Concanavalin A (ConA) lead to a significant increase in splenocyte proliferation, while both a 50 µg/mL and 100 µg/mL dose of MGFM reduced proliferation to baseline levels. Further, MFGM reduced IFN-$\gamma$ production by 65%, TNF-α by 38%, and IL-4 by 52% after ConA stimulation, a T cell mitogen. IFN-$\gamma$ was inhibited by 25%, and TNF-α by 34% after stimulation by LPS. This suggests that the immunomodulatory effect of MFGM may depend on the type of stimulation, and that cell-mediated response through Th1 cells may be most impacted by MFGM. However, while MFGM is rich in PC, it should be noted that other phospholipid molecules present in MFGM could also be contributing to these results, as the authors did not specifically state the composition of the MFGM used, nor did they control for the presence of other phospholipids.

Collectively, these studies show that PC exerts an anti-inflammatory effect by influencing cell membrane interactions, which reduce TNF-α secretion and NF-κB pathway signaling. PC also influenced IECs and lowered inflammatory signals through improving intestinal barrier integrity and protecting against endotoxin challenge. However, while Treede *et al.,* examined different species of PC in some of their assays, none of the studies controlled for FA composition in order to investigate the effect of the PC molecule on immune response. It is well documented that FAs can influence immune function. For example, saturated FAs (SFAs) seem to lower immune function while long chain polyunsaturated FAs (LCPUFAs) seem to enhance immune function (Fritsche 2015, Calder 2014). Further study should examine the effect of PC on immune function independently of FA to understand the immunomodulatory effect of PC *per se*.

**Table 1-1: Evidence from cell culture studies for the effect of PC on immune function[1]**

| Reference | Cell type | PC concentrations used | Incubation length | Immune parameters measured | Results |
|---|---|---|---|---|---|
| Treede et al. 2007 | Human Caco-2 cells | 10 or 200 µM PC | 1 hour pretreatment with PC<br><br>1-10 days incubation | Actin assembly<br><br>Cell survival rate of Mycobacteria tuberculosis macrophages<br><br>NF-κB activation<br><br>mRNA of proinflammatory genes | ↓ actin assembly<br><br>↑cell survival of M. tuberculosis<br><br>↓ NF-κB activation<br><br>↓ TNF-α upregulation of proinflammatory genes |
| Olson et al. 2014 | HT29 non mucus producing intestinal epithelial cells and HT29-MTX mucus producing intestinal epithelial cells. | 200 µM PC | 1 hour pretreatment with PC<br>6-hour co-culture with 50 µg/mL *C. difficile* toxin A | Intestinal permeability<br><br>Cytokine secretion | PC ↑ TNF-α production in both HT29 and HT29-MTX strains compared to HT29+C. Diff<br><br>PC ↓ IL-6 production in both HT29 and HT29-MTX strains compared to HT29+C. Diff<br><br>PC↓ intestinal permeability to baseline or below baseline levels in the non-mucus producing and mucus producing HT29 strains, respectively |

| | | | | | |
|---|---|---|---|---|---|
| Zanabria et al. 2014 | Splenocytes of female BALB/c mice | 50 or 100 µg/mL MFGM | 48 hours | Splenocyte proliferation by LPS and ConA stimulation<br><br>Cytokine production by LPS and ConA stimulation | MFGM ↓ Splenocytes proliferation to baseline levels<br><br>MFGM ↓ production of IFN-$\gamma$ by 65%, TNF-α by 38% and IL-4 by 52% after ConA stimulation<br><br>MFGM ↓ production of IFN-$\gamma$ by 25% and TNF-α by 34% after LPS stimulation |

[1] Abbreviations: ↑ increased; ↓ decreased; ↔ no change; ConA, concanavalin A; IFN-$\gamma$, interferon-gamma; LPS, lipopolysaccharide; MFGM, milk fat globule membrane; NF-κB, nuclear factor kappa B; PC, phosphatidylcholine; TNF-α, tumor necrosis factor-alpha;

### 1.4.2 Rodent studies

Several studies have investigated the effect of PC on immune function under the context of inflammatory conditions in rodent models, such as obesity, arthritis, neuroinflammation, diabetes, and colitis. A full summary of the animal studies examining the role of PC in regulating immune dysfunction is presented in **Table 1-2**. Hartmann *et al.,* (2009) examined the effect of PC on mitigating early signs of inflammation in arthritis induced male Wistar rats. Animals were given 150 mg/kg of a PC solution (1,2-diacylglycerol-3-phosphocholine in 5% glucose) 4 hours before and 4 hours after receiving an injection of 2% λ-carrageen-4% kaolin (C/K) in saline in one knee, and saline in the other knee. This was compared with providing an oral dose of a non-steroidal anti-inflammatory drug (NSAID) of 0.5mg/kg of diclofenac sodium or a control of saline. Oral treatments continued for 3 days total. Cross sections of the knee joint indicated about 35% percent more swelling in the C/K injected knee than the saline injection, and both PC and diclofenac were found to reduce swelling by ~20%. PC was also able to reduce leukocyte adherence (sticking) to the endothelial layer of venules in the C/K injected knee while diclofenac did not. Oral PC also reduced the accumulation of neutrophils in knee tissue and intercellular adhesion molecule (ICAM)-1 immunoreactivity in the venules. This indicates that PC may be beneficial in reducing inflammation in arthritis through the reduction of neutrophil leukocyte-mediated microcirculatory inflammatory reactions.

Kovacs *et al.,* (2012) aimed to investigate the anti-inflammatory effects of PC as it relates to colitis. Sprague Dawley rats were given either a 2,4,6-trinitrobenzene sulfonic acid (TNBS) enema (colitis group) or only the ethanol solvent for TNBS (sham-operated controls) and fed either standard laboratory chow or laboratory chow containing 2% PC (1,2-diacylglycerol-3-

phosphocholine) for either 1 or 6 days. Mucosal XOR, which is activated during inflammation processes, was decreased in the colitis PC group compared to the non-PC colitis group. Large bowel tissue leukocyte accumulation, as measured by MPO activity, was decreased in the colitis PC group compared to the non-PC colitis group. Plasma TNF-α was also decreased by treatment with PC in the colitis group. Interestingly the PC treated group exhibited elevated IL-6 levels on day 1 after colitis induction, but by day 6 the PC diet significantly reduced IL-6 levels relative to the non-PC colitis group. This study suggests that dietary PC decreases cytokine mediated inflammation and structurally preserves the microvascular of the colon.

Azarcoya-Barrera *et al*., (2022) examined the effect of PC in the context of HFD induced immune dysfunction. Male Wistar rats were fed one of the following three diets providing the same amount of total choline (1.5g/kg diet) differing in form: control low-fat (CLF) with 100% FC, control high-fat (CHF) with 100% FC, or PC high-fat (PCHF) with 100% of choline derived from egg-PC. After 9 weeks of feeding the authors found that the CHF diet significantly reduced IL-2 and TNF-α levels produced by splenocytes stimulated with the T cell mitogen PMA+I. This suggests that a HFD providing choline only in the form of water-soluble FC may impair T cells from responding adequately to an immune challenge. Adversely, providing PC instead of FC in a HFD returned both IL-2 and TNF-α to levels similar to the CLF diet. Interestingly, the total proportion of CD3+ cells of the PCHF group was lower than both control groups, suggesting that PC may attenuate obesity-related T cell dysfunction by supporting a more efficient T cell response. However, no human diet would contain choline solely in the form of PC. Thus, a study investigating differing doses of PC on immune cell function is required to establish the optimal proportion of PC relevant to human consumption.

Soy lecithin, which is composed largely of PC, was also examined under the context of diabetes and immune function (Miranda, Batista et al. 2008). Male Wistar rats were separated into a control diet non-diabetic group, a soy lecithin supplemented non-diabetic group, a control diet diabetic group, and a diabetic and soy lecithin supplemented group. The soy lecithin provided contained approximately 31.7% PC along with several other phospholipids, and the dose provided to the soy lecithin groups was 2g/kg of body weight (bw). Soy lecithin was provided 4 days after alloxan injection to induce diabetes and continued for 7 days. Providing soy lecithin significantly increased macrophage phagocytic activity in non-diabetic rats but had no effect on the reduction observed in the diabetic groups. PC decreased proliferative response after ConA stimulation in the non-diabetic group compared to the control, but significantly increased proliferative response compared to the diabetic group. This suggests that soy lecithin may have a beneficial effect under normoglycemic conditions but may not be able to ameliorate immune dysfunction present in diabetes. This study did not control phospholipid composition external to PC, which may influence immune function. It is not possible to conclusively state that PC modulates macrophage or lymphocyte function when PC comprises only 31.7% of the total dietary intervention. It should also be noted that the total duration of the diet was 7 days, which is not long enough to determine how PC may help modulate the chronic state of inflammation in diabetes.

Dial *et al.,* (2008) aimed to provide exogenous PC to overcome the degradation of PC in the gastrointestinal lumen induced by endotoxemia. The authors provided an oral gavage to male Sprague-Dawley rats of either water or 100 mg/kg PC (Phospholipon 90G), then injected rats 1 hour later with either saline or LPS (*Escherichia coli [E. coli*] 0111:B4). LPS injected rats with

no pretreatment of PC had significantly higher gastric and ileal permeability, while pretreatment with PC for 1 hour prior to injection reduced permeability similar to the saline injected group. However, while the level of TNF-α in the serum of non-PC LPS injected rats was significantly higher than saline injected rats, pretreatment with PC had no effect on the LPS-induced increase in TNF-α. While this study gives insight into the acute effect of PC on gut permeability, it would be worthwhile to examine the effect of PC in managing long term endotoxemia as induced by HFD and obesity. Outside of the gut, Tokés *et al.,* examined how PC administration before an LPS challenge may be protective in the central nervous system against excessive proinflammatory peripheral signaling. Sprague-Dawley rats were given either a standard laboratory chow or a laboratory chow diet containing 1% PC (1,2-diacylglycero-3-phosphocholine) for 5 days, then injected with either a 2mg/kg of bw dose of LPS (*E. coli* 055:B5) or saline, and then continued the diet for 7 days after. LPS injection resulted in a higher number of microglia in the hippocampus and providing a pretreatment of PC lowered microglia accumulation to levels similar to the saline control. Although not significant, both the MPO activity and XOR levels in the colon were higher in the LPS group, while the PC pretreated groups remained at the control level, suggesting lower inflammation in the PC pretreated group. Plasma TNF-α levels in the LPS group were higher 3 hours after injection, and the PC diet significantly reduced plasma TNF-α levels. However, while IL-6 was also higher in the LPS group compared to the control group, there was no significant difference between the LPS and PC pretreated LPS group.

Collectively these findings suggest that PC has systemic immunomodulatory effects. However, only 1 study examined the effect of PC alone by controlling for FA composition and

providing an otherwise nutritionally complete diet (Azarcoya-Barrera, Wollin et al. 2022). This study showed that PC can beneficially modulate T cell response under the context of a HFD. In another study of metabolic disease, soy lecithin composed of 30% PC was found to improve T cell proliferation in non-diabetic rats. Additionally, it has been shown that PC can have local effects in the gastrointestinal tract by aiding in intestinal barrier function and thus lowering endotoxemia. Kovacs *et al.*, (2012) showed that providing PC in the context of experimentally induced colitis lowered tissue damage leading to lower circulating TNF-α levels. This phenomenon was also observed in a model of neuroinflammation induced by LPS injection; oral PC supplementation prevented remote neuroinflammatory signs of endotoxemia and lower plasma TNF-α levels. It should be noted that many of the studies had significant limitations. Many studies focused on acute toxicity protection of PC, rather than long term dietary treatment under the context of inflammatory conditions. Especially in the case of chronic conditions, such as obesity, colitis, T2D, and arthritis, long term dietary treatment is required to better understand the role of PC in health and disease. Further, no studies examined the effect of PC in female rodents. It has been documented that males and females exhibit different immune phenotypes, and different metabolic complications have differential effects. For instance, female Wistar rats develop a milder obesity phenotype and maintain enhanced cytokine production for a longer period than male Wistar rats when fed a HFD (Tibaes, Azarcoya-Barrera et al., 2022). Thus, sex differences in response to PC should be investigated in order to provide recommendations to the general population.

**Table 1-2: Evidence from animal studies for the effect of PC on immune function[1]**

| Reference | Animal and disease model | PC dose used | Intervention details | Immune parameters measured | Results |
|---|---|---|---|---|---|
| Hartmann et al. 2009 | Male Wistar rats, 300+/-20 g<br><br>Arthritis | 150mg/kg | PC dose administered 4 h before and 4 h after arthritis induction | Joint inflammation<br><br>Neutrophil leukocyte infiltration<br><br>ICAM-1 expression | PC ↓ knee joint swelling by 20%<br><br>PC ↓ neutrophil infiltration and ICAM-1 expression |
| Kovacs et al. 2012 | Male Sprague Dawley rats (280-320g)<br><br>Colitis | 2% PC in diet | Series 1: 7 days (6 days of feeding before TNBS enema, anesthetized 1 day after enema)<br>Series 2: 12 days (6 days of feeding before TNBS enema, anesthetized 6 days after enema) | Mucosal XOR activity (tissue inflammation)<br><br>Colon tissue MPO activity (leukocyte infiltration)<br><br>Plasma TNF-α and IL-6 | PC ↓ XOR activity both 1 and 6 days after enema<br><br>MPO activity ↓ in PC group on both days 1 and 6<br><br>PC ↓ TNF-α levels on both day 1 and 6<br><br>PC ↑<br>+IL-6 levels on day 1 after colitis, and ↓ levels on day 6. |
| Azarcoya-Barrera et al. 2022 | Male Wistar rats<br><br>Obesity induced immune dysfunction | 1.5 g/kg diet of egg-PC (PCHF) | 9 weeks of feeding one of the three diets: PCHF control low-fat (CLF) | Plasma cytokines<br><br>Splenocyte cytokines<br><br>T cell proliferation | ↔ plasma cytokines<br><br>PCHF ↑ IL-2 production after PMA+I stimulation compared to CHF<br><br>↔ T cell proliferation |

| | | | | | |
|---|---|---|---|---|---|
| | | | Control high-fat (CHF)<br><br>(control diets contained 1.5 g/kg diet of free choline) | from splenocytes<br><br>Immune cell phenotype | PCHF ↓ CD3+ cells compared to CLF and CHF |
| Dial et al. 2008 | Male Sprague Dawley rats (150-250g)<br><br>LPS induced gastrointestinal inflammation | 100mg/kg PC (phospholipon 90G) | Given an oral supplement of either water or PC<br><br>Rats injected 1 h later with either saline or LPS | Intestinal permeability<br><br>Serum TNF-α | PC ↓ gastric and ileal permeability induced by LPS<br><br>PC ↔ on increase in serum TNF-α after LPS treatment. |
| Tokes et al. 2011 | Male Sprague-Dawley rats (180-230g)<br><br>LPS induced neuroinflammation | Diet containing 1%PC | Rats fed either standard laboratory chow and injected with saline (control), standard laboratory chow and injected with LPS, PC diet and injected with LPS<br><br>Rats fed diet for 5 days before injection, and | MPO and XOR activity in colon, ileum, and hippocampus<br><br>Plasma TNF-α and IL-6 concentrations | ↔ in XOR and MPO activity between PC diet and control group.<br><br>PC pretreatment ↓ LPS induced TNF-α level at 3 h, ↔ between groups at later time points<br><br>↔ in IL-6 between PC-pretreated and LPS group. |

| | | | | | |
|---|---|---|---|---|---|
| | | | fed for another 7 days after injection | | |
| Miranda et al. 2008 | Male Wistar rats (180-220g)<br><br>Alloxan-induced diabetes | 2g/kg body weight soy lecithin (31.7%PC) | Rats were injected either with saline or alloxan.<br>3 diet groups:<br>Standard research diet (C)<br>Diet +Soy lecithin<br>Diet +Diabetic soy lecithin | Macrophage phagocytosis<br><br>Lymphocyte number | PC ↑ macrophage phagocytosis in non-diabetic rats, but ↓ in diabetic rats<br><br>PC ↓ lymphocyte number in non-diabetic rats<br><br>PC ↑ proliferative capacity in diabetic rats |

[1] Abbreviations: ↑ increased; ↓ decreased; ↔ no change; CHF, control high-fat; CLF, control low-fat; ICAM-1, intercellular adhesion molecule -1; IL, interleukin; LPS, lipopolysaccharide; MFGM, milk fat globule membrane; MPO, myeloperoxidase; PC, phosphatidylcholine; PCHF, phosphatidylcholine high-fat; PMA+i, phorbol myristate acetate plus ionomycin; TNBS, trinitrobenzene sulfonic acid; TNF-α, tumor necrosis factor-alpha; XOR, Xanthine oxidoreductase

### 1.4.3 Human studies

Three studies have examined the effect of PC or PC rich MFGM in the context of inflammatory conditions in humans. Two of the studies had a study population of UC patients, and the other used MFGM to potentially mitigate inflammation caused by diarrheagenic *E. coli* in healthy adults. A full summary of the clinical trials examining the role of PC in regulating immune dysfunction is presented in **Table 1-3**.

Bruggencate *et al.,* (2016) hypothesized that MFGM isolates may protect healthy adults from diarrheagenic *E. coli* infection in humans due to the documented antiviral and antibacterial activities of MFGM in animals and in vitro. Participants were randomized to consume 10g of either MFGM or a control powder in 125 mL of soy milk twice daily for four weeks. Two days after supplement consumption began, participants were challenged with the diarrheagenic *E. coli.* The authors used fecal calprotectin as a measure for inflammation in the bowels, which increased in both groups on day 1, but the MFGM group increased significantly less than the control group. Both groups returned to baseline by day 14. The authors found no significant difference in serum CFAII-specific IgG between the two diet groups, but participants in the MFGM group reported less stool frequency and gastrointestinal complaints than the control group. This led the authors to conclude that MFGM may partially affect in vivo resistance to diarrheagenic *E. coli*. The authors did not measure circulating cytokine levels, nor did they measure intestinal permeability. It is thus not clear if MFGM is providing immunological benefit, or how exactly gastrointestinal symptoms of diarrheagenic *E. coli* were reduced.

The other two clinical trials examined the effect of a modified release PC product in UC patients. UC is an inflammatory condition of the colon (Ungaro, Mehandru et al. 2016). Stremmel *et al.,* (2005) provided UC patients with either a PC rich phospholipid mixture (30% PC) or a placebo. The administered dose was 1.5g/dose 4 times daily after meals for 3 months. The study's primary outcome of clinical remission was attained by 16 of the 30 patients in the PC group and only 3 of the 30 in the placebo group. The group examined inflammation status using endoscopic activity index (EAI), a reported inflammatory bowel syndrome score, and histological appearance of the colon. The EAI improved in 11 of the 29 evaluated PC patients compared to none of the 29 placebo patients. An improved histology score was also noted in 13 of the 25 evaluated patients in the PC group compared to 3 of the 23 of the placebo group. Nearly 10 years later, the same research group conducted a similar study examining the efficacy of a novel PC treatment called LT-02, which was composed of 94% soy lecithin, on improving inflammation-related symptoms of UC patients (Karner, Kocjan et al. 2014). UC patients were randomized to consume a placebo treatment, or either 0.8g, 1.6g, or 3.2g of LT-02 per day for 3 months. A disease activity score (Simple Clinical Colitis Activity Index) indicated that there was a 33% reduction in disease activity in the placebo group compared to a 44.3%, 40.7%, and 51.7% reduction in disease activity in the 0.8g, 1.6g, and 3.2g LT-02 groups respectively. Mucosal healing was achieved in 32.5% of the placebo group compared to 47.4% of the pooled LT-02 groups with a drop in histological scores of 20% compared to 40.5% in the placebo and LT-02 groups respectively.

Overall, the limited number of human studies suggests that PC may provide benefit to gastrointestinal-related inflammatory conditions by improving colon tissue damage and mucosal

healing. However, a major limitation of all three studies is the lack of measurement of immune cell function, either by measuring circulating cytokine levels, or stimulating PBMCs with mitogens to measure cytokine response. Further, given the extensive research in rodents showing preclinical benefit of PC under the context of several chronic inflammatory conditions, it is pertinent to examine PC when provided as part of a diet for mitigating inflammation.

Collectively, several studies conducted in cell, rodent, and human models suggest that dietary PC intake beneficially modulates immune function under the context of inflammatory conditions. However, there has been a lack of control for other dietary factors that may influence immune function, such as FA composition of the PC molecules. Further, the mechanisms involved in how PC modulates peripheral and gut-associated immune function have not fully been elucidated. Given the growing literature of PC on immune function, well controlled studies focusing on mechanistic pathways of PC on immune function should be further explored.

**Table 1-3: Evidence from human studies for the effect of PC on immune function[1]**

| Reference | Study design | Study population | PC dose used | Immune parameters measured | Results |
|---|---|---|---|---|---|
| Bruggencate et al. 2016 | Randomized clinical trial | Healthy adults n=58, 18-55y | Participants were randomized to consume either:<br><br>20 g of Lacprodan PL-20 (MFGM)<br><br>20 g of Miprodan 30 powder (control) | Gastrointestinal neutrophil infiltration via fecal calprotectin excretion<br><br>Serum IgG | MFGM ↓ fecal calprotectin excretion 1 day after E. coli challenge<br><br>↔ in IgG between diet groups |
| Karner et al. 2014 | Randomized clinical trial | UC patients | Patients were randomized to consume one of:<br><br>Placebo<br><br>0.8g LT-02 (94% PC concentrated soy lecithin)<br><br>1.6g LT-02 | UC disease activity | All LT-02 groups ↓ disease activity vs placebo<br><br>The remission rate for the 3.2 g LT-02 was twice as high as the placebo group<br><br>Mucosal and histological healing ↑ in PC groups |

| | | | | | |
|---|---|---|---|---|---|
| | | | 3.2g LT-02 | | |
| Stremmel et al. 2005 | Randomized clinical trial | UC patients, n=36 male and n=24 female, 24-58 y | Patients were randomized to consume one of:<br><br>6g/day PC rich phospholipid mixture (30% PC)<br><br>Placebo | UC disease activity<br><br>Histological appearance for inflammation | Clinical remission observed in 16 of PC group patients, only 3 placebo patients<br><br>>50% improvement in disease activity in 27 of 30 PC patients, only 3 placebo patients<br><br>Improved histological score of inflammation in 13 of 25 PC patients, only 3 of 23 placebo patients<br><br>19 of 30 PC group patients reduced length of affected area, no chance in placebo patients |

[1] Abbreviations: ↑ increased; ↓ decreased; ↔ no change; IgG, immunoglobulin G; MFGM, milk fat globule membrane; PC, phosphatidylcholine; UC, ulcerative colitis

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## CHAPTER 2: Research plan

### 2.1 Rationale

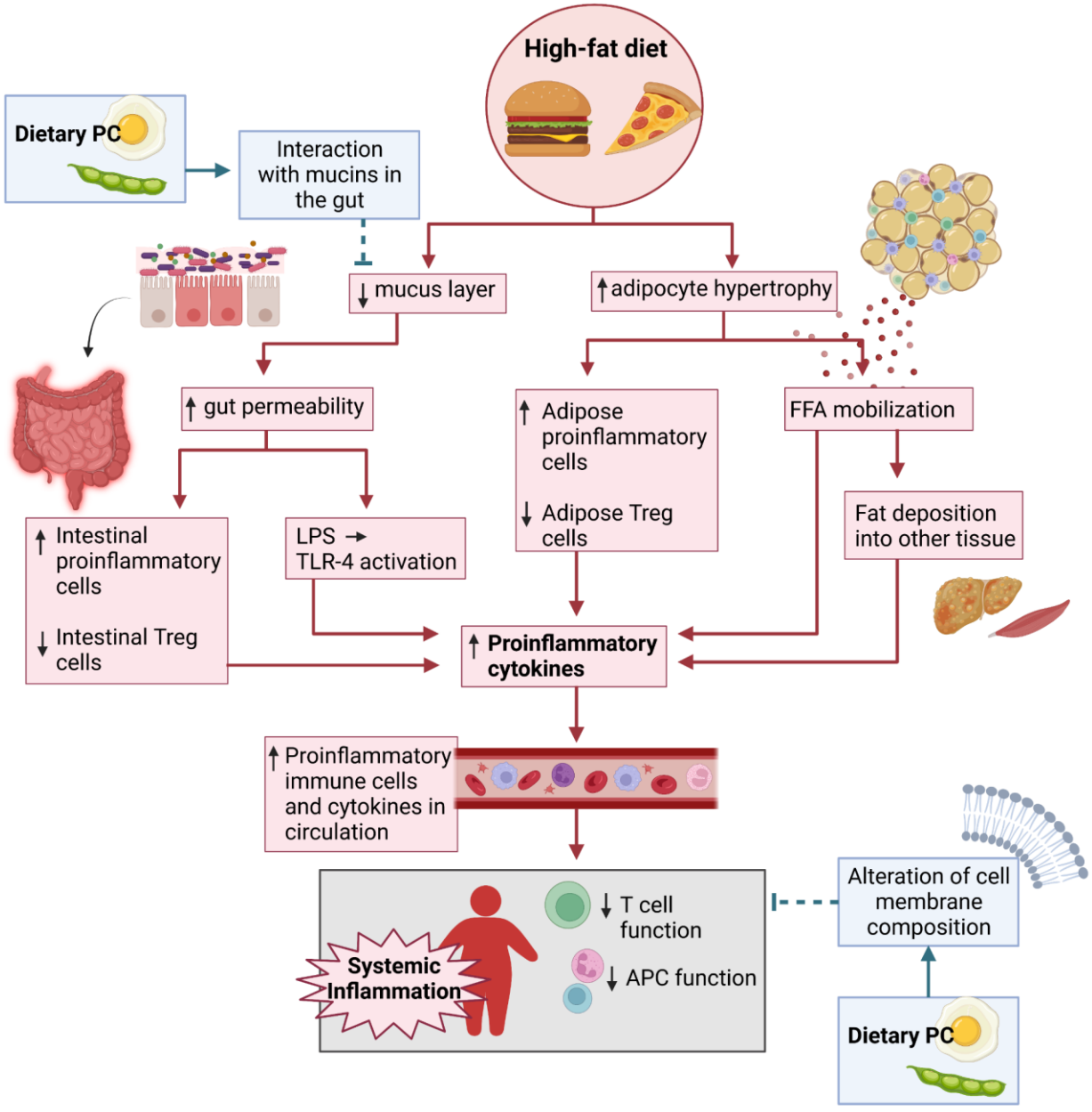
Obesity is a worldwide epidemic that increases the risk of several chronic inflammatory conditions, such as T2D, atherosclerosis, and inflammatory bowel disease (Santa-Cruz, Bharath et al. 2022, Guzik, Skiba et al. 2017, Moayyedi, Ford et al. 2010). Rodent studies have shown that increasing dietary fat intake as a model for obesity is associated with changes in the gut microbiota composition, which can degrade intestinal barrier integrity (Cani, Bibiloni et al. 2008, Teixeira, Collado et al. 2012). The resulting increase in intestinal permeability leads to increased translocation of bacterial content, such as LPS, from the gut to the bloodstream (Moreira, Teixeira et al. 2012). LPS in circulation can bind and activate TLR-4 present on peripheral immune cells, which increases the expression of several pro-inflammatory cytokines (TNF-α, IL-6, IL-1β, IFN-γ) through the activation of transcription factors such as NF-κB (Hoshino, Takeuchi et al. 1999). HFDs and obesity also lead to an increase in the number and size of adipocytes, which can result in a spillover of FAs into the bloodstream and ectopic lipid deposition into other tissues (Reilly & Saltiel 2017). This can promote local inflammation by impairing metabolic pathways and inducing endoplasmic reticulum stress and activation of the NF-κB pathway, which leads to increase in pro-inflammatory cytokines and chemokines (Liu, Zhang et al. 2017). This state of systemic inflammation has been shown to impede immune response by leading to a lower proliferation rate and cytokine production by splenocytes (representing peripheral immune function) (Mito, Hosoda et al. 2000, Tanaka, Isoda et al. 1998). Further, our lab has shown that individuals with obesity and T2D have additional immune dysfunction as shown by a lower production of IL-2, IL-6, and TNF-α following T cell

stimulation compared to individuals with obesity that are normoglycemic (Richard, Wadowski et al. 2017).

Our working hypothesis of how PC may improve immune function under the context of a HFD is summarized in **Figure 2-1**. PC is a lipid-soluble form of the essential nutrient choline that is an integral phospholipid in mammalian cell membranes and contributes to the hydrophobicity of the intestinal mucus barrier (Li & Vance 2008, Korytowski, Abuillan et al. 2017). Thus, PC may directly contribute to immune function by supporting cell proliferation and structure, as well as indirectly by maintaining intestinal barrier integrity and subsequently reducing endotoxin translocation. Indeed, PC has been shown to inhibit TNF-α induced activation of NF-κB and improve intestinal barrier defense against *C. diff* toxin A (Olson, Diebel et al. 2014). PC is found in several dietary sources, such as dairy, beef, and soybeans, but is richest in egg yolks (Van Parys, Karlsson et al. 2021). Our lab has previously shown that feeding a maternal diet that contains choline 100% in the form of egg-derived PC compared to 100% FC (the standard form of choline in rodent research diets) in Sprague-Dawley rats enhanced peripheral and intestinal T cell function in lactating dams (Dellschaft, Richard et al. 2018, Lewis, Richard et al. 2017), and their offspring (Lewis, Richard et al. 2016, Richard, Lewis et al. 2017). We also demonstrated that feeding a mixture of choline forms containing 50% soy-PC compared to 100% FC in the maternal diet had no effect on IL-2 production, but still led to an overall more efficient immune response (Richard, Lewis et al. 2017). Further, we showed that providing a HFD to male Wistar rats containing 100% PC compared to 100% FC increased TNF-α, IL-2, and IL-6 production (Azarcoya-Barrera, Wollin et al. 2022), suggesting that PC can also be beneficial in the context of obesity induced immune dysfunction. However, a 100% PC diet is of

little physiological relevance as no human diet would contain choline solely in the form of PC. So far, no study has investigated the dose response of dietary PC on immune function in the context of obesity.

**Figure 2-1**: **Working hypothesis of how PC may improve immune function under the context of a HFD**[1]




[1] Abbreviations: APC, antigen presenting cells; FFA, free fatty acid; LPS, lipopolysaccharide; PC, phosphatidylcholine; TLR-4, toll-like receptor-4

## 2.2 Objectives and hypotheses

The overall aim of this research is to investigate the impact of feeding different doses and dietary forms of PC on immune function under the context of obesity. To address this overall aim, two specific objectives and their hypotheses were established:

1. The first objective of this research was to determine the effect of feeding a HFD with differing doses of PC on A) peripheral immune function (spleen) and B) gut-associated immune function (MLN). For both areas of the immune system, we hypothesized that 1) a HFD leading to obesity reduces T cell function but can be mitigated by egg-PC and 2) a dose of 50-75% egg-PC reflecting typical human consumption of choline will provide the most beneficial effect on T cell function compared to only FC. We also proposed two further hypotheses specific to either peripheral or gut-associated immune function:
    a. Feeding a HFD with egg-PC compared to FC will alter splenocyte membrane phospholipids composition where providing more PC in the diet will increase PC in immune cell membrane.
    b. Feeding a HFD with egg-PC compared to FC will improve intestinal barrier integrity, thus indirectly supporting immune function by reducing risk of endotoxemia.
2. The second objective of this research was to determine the differential effect of feeding a HFD containing egg- or soy-derived PC on both peripheral and gut-associated immune function. We hypothesized that egg-PC will improve T cell function to a greater extent than soy-PC.

### 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. Chapter 4 has been submitted for publication as an individual manuscript.

**Chapter 3** provides an overview of all methodology used to test the objectives and hypotheses. There is extensive overlap in the methods used in chapter 4-5, thus for conciseness, the methods have been compiled into one chapter.

**Chapter 4** examines the impact of feeding a HFD with varying doses of egg-derived PC on both peripheral and gut-associated immune responses in Wistar rats. Objective 1 was addressed in this chapter. In summary, feeding rats with a HFD impaired peripheral T cell responses by decreasing T cell proliferation, IL-2, and IL-10 production. We also found that feeding rats with a HFD increased intestinal permeability and subsequently lowered IL-2 production in MLN cells. Providing egg-PC decreased intestinal permeability starting at a 50% dose, and ameliorated T cell responses to some extent in all doses, but the 50% dose conferred the greatest immune benefits.

**Chapter 5** examines the impact of feeding a HFD with either egg- or soy-derived PC on immune responses in Wistar rats. Objective 2 was addressed in this chapter. We found that both egg- and soy-PC provided similar benefits for gut barrier integrity and splenocyte phospholipid composition. Both PC diets had a beneficial effect on *ex vivo* cytokine production. Interestingly,

egg-PC seemed to improve Th2 cytokines, and soy-PC seemed to improve cytokines involved in the Th1 response and T cell proliferation to a greater extent.

**Chapter 6** provides an overall discussion that includes the results from each objective and hypotheses as well as directions for future research.

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## CHAPTER 3: Methodology

### 3.1 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 (AUP 00002859). Three-week-old male Wistar rats (n=60) were obtained from Charles River Laboratories (Montreal, Quebec, Canada). The rats were housed in pairs in a temperature and humidity-controlled environment with a 12hr/12hr reversed light cycle. A standard rat chow was fed to the rats for one week to allow the rats to acclimatize to the environment (Lab diet 5001; PMI Nutrition International, Brentwood, MO, USA). At 4 weeks of age, rats were randomized to one of seven nutritionally complete experimental diets: Control-low-fat (CLF, 100% FC, n=10), Control-high-fat (CHF, 100% FC, n=10), 25% Egg-PC high-fat (25PC, 25% Egg-PC, 75% FC, n=10), 50% Egg-PC high-fat (50PC, 50% Egg-PC, 50% FC, n=9), 75% Egg-PC high-fat (75PC, 75% Egg-PC, 25% FC, n=10), 100% Egg-PC high-fat (100PC, 100% PC, n=10), 100% Soy-PC high-fat (SPC, 100%PC, n=10). In Chapter 4, data from the animals consuming the CLF, CHF, 25PC, 50PC, 75PC, and 100PC diets was analyzed and compared. In Chapter 5, data from the animals consuming the CLF, CHF, 100PC and SPC diet was analyzed and compared. In Chapter 5, the 100PC diet is labeled as EPC. All diets contained 1.5 g of total choline/kg of diet differing in the forms of choline provided. The rats were fed *ad libitum* for the total 12 weeks of the study. One of the rats on the 50PC diet died from complications unrelated to the study protocol prior to the study conclusion.

The 5 HFDs were matched for macronutrient content (50% fat, 23% carbohydrates, and 27% protein), and the CLF diet was 20% fat, 53% carbohydrates, and 27% protein. The nutrient

composition of the experimental diets is presented in **Table 3-1**. The nutrient composition of the CLF, CHF, and 100PC diet has been previously described (Azarcoya-Barrera, Wollin et al. 2022). Regardless of total fat content in diets, the FA composition of all the diets were closely matched using a mixture of oils (**Table 3-2**). The oil mixture consisted of flax oil, lard, and corn oil. The PC diets also contained PC extracted from eggs or soy (Lipoid E 80 and Bluebonnet Nutrition, respectively). Lipoid E 80 is a phospholipid extract derived from eggs that contains about 85% PC+ lyso-PC, 9.5% PE and 3% SM (Lipoid GmBH, Frigenstr. 4 D-67065 Ludwigshafen, Germany). Soy lecithin granules contain about 42% PC, 36% PE, and 22% phosphatidylinositol (PI) (Bluebonnet Nutrition). Since the PC molecules contain different FAs depending on the source, the diets were analyzed using gas chromatography and the fat mix was adjusted accordingly. FA requirements were satisfied in all the diets. The SFA:polyunsaturated fatty acid (PUFA)/ and omega (n)-6:n-3 PUFA ratios were matched across all diets. Diets were prepared on a weekly basis and stored at 4°C until needed. Feed cups were weighed and replaced every 2 to 3 days to prevent oxidation. Body weight was measured at the beginning and end of each week throughout the intervention.

### 2.2 Assessment of Intestinal Permeability

During the last week of the diet period, rats were fasted for 12 hours and weighed. Blood samples were collected (T0) and then rats were given a 420 µL oral gavage of 500 mg/mL 4-kDa dextran conjugated with fluorescein isothiocyanate (FITC–dextran; FD4; Sigma) diluted in distilled water. Blood samples were collected from the saphenous vein 2 hours after administration (T120). The blood was centrifuged at 2000 g for 5 minutes at 4ºC to isolate the plasma. Twenty-five µL of plasma was diluted in 175 µL dd$H_20$ for a total of 200 µL per well, and fluorescence was measured using a fluorescence spectrophotometer (Synergy H4. Biotek)

with an excitation wavelength of 485 nanometres (nm) and an emission wavelength of 528 nm. Samples were measured against a prepared standard curve of 15.6 ng/mL to 1000 ng/mL (200 µL per well), and samples above the curve were further diluted. Samples from rats where we were unable to gavage the complete FITC–dextran dose or collect enough blood during the procedure were not analyzed resulting in 7-9 rats per group. The appearance of the nondigestible FITC–dextran in plasma after oral administration is a measure of paracellular permeability.

### 3.3 Tissue collection and plasma metabolites measurement

After 12 weeks of feeding, the rats were weighed and euthanized by $CO_2$ asphyxiation in the morning hours. Livers were collected aseptically and weighed. Spleens and MLNs were collected aseptically, weighed, and immune cells were isolated for further processing (see immune cell isolation section below). The intestinal tract was removed from the rats aseptically and the length of the intestine was recorded. The first 10 cm were removed as the duodenum, and the remaining was cut in half to represent the jejunum and ileum. The jejunum section was rinsed first with cold, sterile phosphate buffered saline (PBS), and then rinsed with PBS with protease inhibitor (1mL/L, Sigma P8340, Ontario, Canada). The first few cm of jejunum tissue was removed, and a small section was cut and placed into a cassette with formalin for histological analysis (see H&E staining section below). The remaining section of the jejunum was opened longitudinally, placed on a glass plate over ice, and scraped with a glass slide. Jejunal scrapings were frozen at -80°C until gene expression analysis (See mRNA extraction section below). Blood was collected by cardiac puncture in K2 EDTA vacutainers (Fisher Scientific, Canada) and centrifuged at 1800 g for 10 min at 22°C to obtain plasma, which was frozen at -80°C until further assays were performed. Plasma cytokines and chemokines concentration were measured using a Proinflammatory Panel 1 V-PLEX electrochemiluminescent multiplex cytokine kit

(MesoScale Discovery, USA) to determine circulating concentrations of IFN-γ, IL-1β, IL-4, IL-5, IL-6, IL-10, IL-13 and TNF-α following the manufacturer's instructions. Plasma concentrations were measured in duplicate with a CV below 15%.

### 3.4 Lipid analysis

Total lipids from the diets were extracted using the Folch method (Folch, Lees et al. 1957) with a modified separation phase time of one hour. Lipids were then saponified in methanolic KOH for 1h at 110°C and methylated with 1.5 mL hexane and 1.5 mL boron trifluoride (BF3) at 110°C for 1 hour. After cooling, 1 mL of deionized water was added, and samples were stored overnight at 4°C. The top layer was then collected, dried under nitrogen, reconstituted with 100 µL hexane and then stored at −80°C until further analysis. FA composition of the liver was also analyzed post-mortem. The modified Folch method was used to extract total lipids from liver tissue as previously described (Layne, Goh et al. 1996, Field, Ryan et al. 1988). Briefly, 50-100 mg of liver tissue was homogenized in 1 mL of 0.1M KCl using a tissue processor (MP Biomedicals FastPrep-24, USA) and 1.00 mm diameter glass beads (BioSpec, USA) at 6 m/s for 40 seconds, resting for 5 minutes, and repeating for another 40 seconds. Eight mLs of a 2:1 solution of chloroform:methanol and 1 mL of 0.1M KCl were added to the tissue homogenate and vortexed. After overnight incubation at 4°C, samples were spun at 450 g for 5 minutes before removing the bottom layer of the lipid phase and drying under nitrogen gas at 2 psi and 37°C. (ReactiTherm; Thermo Fisher Scientific, Canada). FAs were methylated with 1.5 ml $BF_3$ and 1.5 ml hexane at 110°C for 1 hour. After cooling, 1 mL of deionized water was added and samples were stored overnight at 4°C. Samples were then spun at 450 g for 5 minutes and the top layer was collected, dried under nitrogen, reconstituted with

100 µL hexane and then stored at −80°C until further analysis. Gas chromatography (Agilent 8890; GC) equipped with a flame ionization detector was used to measure the FA composition of the 6 experimental diets and the extracted liver lipids. FA methyl esters were separated and identified using GLC standard 502 on a 100-m CPSIL 88 fused capillary column (100 m x 0.25 mm; Agilent 8890 GC with a 7693A Autosampler; Agilent Technologies) as described previously (Cruz-Hernandez, Deng et al. 2004).

### 3.5 Immune cell isolation

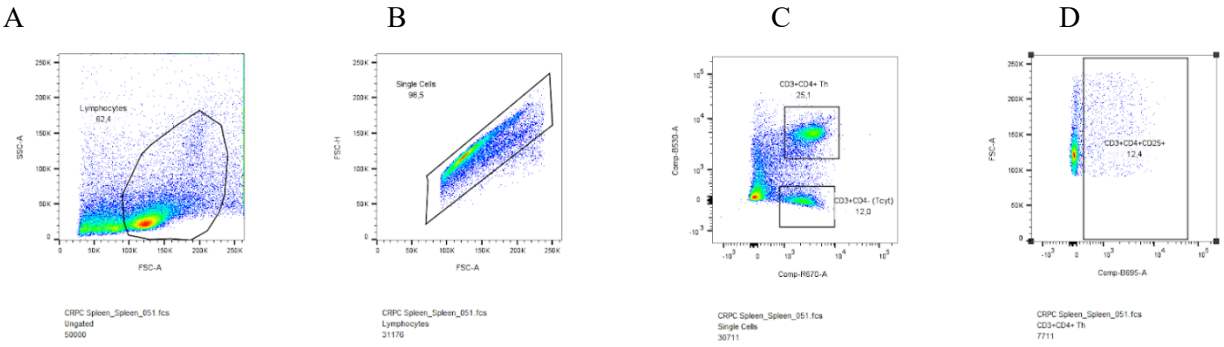
Single cell suspensions were obtained by trimming spleen or mesenteric tissue of excess fat and connective tissue, then pushing spleen or mesenteric tissue through a 100 µM nylon mesh screen in sterile Krebs-Ringer HEPES (KRH) buffer with bovine serum albumin (BSA) (pH: 7.2-7.4; 5 g/L; Sigma-Aldrich Canada Ltd., Oakville, ON, Canada). Volume was adjusted to 30mL with KRH buffer, and samples were spun at 450 g for 5 min at 21°C. Erythrocytes were lysed by incubating in 5 mL of ammonium chloride lysis buffer for 5-10 minutes (155 mM NH 4 Cl, 0.1 mM EDTA, 10mM KHCO3; Fisher Scientific, Edmonton, AB, Canada). Splenocytes or MLN lymphocytes were spun at 450 g for 5 min at 21°C, washed, filtered through a 40 µm sterile filter, and re-suspended in 25 mL of complete culture medium (10% RPMI 1640 media; Life Technologies, Burlington, ON, Canada), supplemented with 5% (v/v) heat-inactivated fetal calf serum (FCS), 25 mM HEPES, 2.5 µM 2-mercaptoethanol and 1% antibiotic (AB)/antimycotic (AM) (pH 7.4; Fisher Scientific). Live splenocytes or MLN lymphocytes were counted using trypan blue exclusion (Sigma-Aldrich) to assess cell viability and was > 90% for all treatment groups. Cell suspensions were used at 1.25 x $10^6$ cells/mL for *ex vivo* cytokine mitogen stimulation. After mitogen set up, the remaining splenocytes were spun at 450 g for 5 min at

21°C, supernatant was discarded and the pellet was resuspended in 3mL of complete culture media (10% RPMI 1640 media; Life Technologies, Burlington, ON, Canada). Dimethyl sulfoxide (DMSO) was added to two 1 mL aliquots of splenocyte cell suspension (10%v/v) and frozen at -80°C in a Mr. Frosty overnight before transferring to liquid nitrogen for long term storage. The remaining 1 mL of splenocyte cell suspension was added to an Eppendorf tube and frozen at -80°C for phospholipid quantification.

### 3.6 Immune cell phenotype analysis

Immune cell subsets present in freshly isolated splenocytes, and MLNs were identified by direct immunofluorescence assay, as previously described (Tibaes, Azarcoya-Barrera et al. 2022). Briefly, cells were stained with 20 µL antibody mix with four multicolor flow cytometry panels to identify the following combinations of surface molecules in splenocytes (**Table 3-3**) The proportion of CD3+CD4- cells were considered as CD3+CD8+ since CD8 BUV395 did not stain properly. After incubation, cells were washed with 200 µL IF buffer (5% in phosphate-buffered saline (PBS) with 1%AB/AM), spun at 450g for 2min at 10°C, and supernatant aspirated to remove excess antibodies. This wash step was repeated once more before splenocytes, or MLN lymphocytes were fixed in 200 µL paraformaldehyde (PFA) (10 g/L; Thermo Fisher Scientific) in PBS. All samples were acquired using bead compensations within 72 h of preparation on a BD LSRFortessa X20 SORP flow cytometer and data was analyzed using FlowJo v10 (USA). An example gating strategy is presented in **Figure 3-1**.

**Figure 3-1.** Gating strategy. **(A)** Splenocyte lymphocyte population based on forward scatter area versus side scatter area; **(B)** splenocyte single cells selected, doublets excluded; **(C)** splenocyte T cells population identified as CD3+CD4+ (T helper cells) and CD3+CD4- (identified as CD3+CD8+ [T cytotoxic cells]) within the lymphocytes population; **(D)** T helper cells expressing the IL-2 receptor were gated within the CD3+CD4+ population and identified as CD25+ cells.[1]




[1]Abbreviations: SSC-A, side scatter area; FSC-A, forward scatter area; FSC-H, forward scatter height.

### 3.7 *Ex vivo* cytokine production by mitogen-stimulated cells

The measurement of cytokine production by mitogen-stimulated splenocytes or MLN lymphocytes has been previously described (Blewett, Gerdung et al. 2009). Briefly, cells (1.25 x $10^6$ cells/mL) were incubated for 48h at 37°C with 5% CO 2 without mitogen (unstimulated) or with one of the following mitogens: phorbol 12-myristate 13-acetate plus ionomycin (PMA+I; 2 µg/mL) for both splenocytes and MLN lymphocytes; lipopolysaccharide (LPS; 5 µg/mL) for both splenocytes and MLN lymphocytes; pokeweed mitogen (PWM; 55 µg/mL) for only splenocytes. PMA+I activates T cells, LPS activates primarily APC, and PWM activates T cells and APC. Cells were then centrifuged for 5 minutes at 450 g and supernatants collected and stored at -80°C. Commercial ELISA kits were used to measure concentrations of IL-1β, IL-2, IL-6, IL-10, TNF-α, and IFN-γ for splenocytes, and IL-2, IL-10, and TNF-α for MLN lymphocytes 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 duplicate, with CV<15%.

### 3.8 *Ex vivo* T cell proliferation assay

Ninety-six well plates were coated with 100 µL of 1 µg /mL of anti-CD3 (Biolegend, USA) and were incubated overnight at 4°C. Frozen splenocytes were thawed for 10 minutes in a water bath at 37°C before being washed twice by rinsing with 10 mL of pre-warmed (37°C) 10% RPMI 1640 medium to remove DMSO and spun at 450 g for 5 min at 21°C with the supernatant discarded after. Afterwards, cells were transferred in 5mL of 10% RPMI to 10 mL sterile culture tubes to rest for 4 hours at 37°C in an incubator with 5% $CO_2$ . After incubating, cells were washed and resuspended in 10 mL of 10% RPMI. A haemocytometer was used to count live cells using trypan blue exclusion (Sigma-Aldrich) to assess cell viability. The 96-well plates were set up to get $1.25x10^6$ cells/mL in 200 µL. Samples were added in triplicate, with anti-CD28 stimulation (Biolegend, USA) (0.33 µg/mL) of previously coated anti-CD3 wells along with unstimulated cells (control). Plates were then incubated at 37°C for 72 hours. After incubation, cells were spun at 450 g for 2 minutes at 10°C, vacuum aspirated, and then cells were fixed with 100 µL 4% PFA for 20 minutes. Cells were then spun at 450 g for 2 minutes at 10°C, vacuum aspirated, then resuspended in 100µL crystal violet (Acros Organics, USA) solution (0.1%, w/v, with ethanol 2%, v/v in 0.5 M Tris-C1, pH 7.8) for 20 minutes. The stained cell layer was spun at 450 g for 2 minutes at 10°C, rinsed thoroughly with 200 µL deionized water, spun at 450 g for 2 minutes at 10°C, and vacuum aspirated. This wash was repeated, and cells were then incubated with 100 µL sodium dodecyl sulfate (SDS) solution (0.1%, w/v, with ethanol 50%, v/v, in 0.5 M Tris-C1, pH 7.8) 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. Data is presented as a fold increase from unstimulated cells to CD3/CD28 stimulated cells. One male rat fed the CLF diet did not have enough viable cells to perform the assay and its data was excluded from the analysis.

### 3.9 Assessment of phospholipid content of immune cell membranes

High performance liquid chromatography (HPLC) was carried out on an Agilent 1100 instrument (Santa Clara, USA) using a modified version of the method of Abreu, Solgadi et al. (2017). Briefly, 1 mL of splenocyte suspension was homogenized as described in section 2.3. Fifty µL of thawed splenocyte suspension was assayed for protein content using a BCA assay as per manufacturer instructions (Thermo Fisher Scientific). Homogenate equivalent to 2 mg protein was extracted using a modification of the method of Folch, Lees et al. (1957). Four mLs of a 2:1 solution of chloroform:methanol, 1 mL of 0.1M KCl, and the internal standards PDME and batyl alcohol were added (1 µg/µL and 4 µg/µL respectively) to the tissue homogenate and vortexed. After overnight incubation at 4°C, samples were spun at 450 g for 5 minutes before removing the bottom layer of the lipid phase and drying under nitrogen gas at 2 psi and 37°C, and resuspended in 100 µL chloroform:isooctane (1:1). Ten µL of the suspension was injected onto the column. Lipids were separated using a quaternary solvent system on a 4.6 x 50 mm Kinetex hydrophilic interaction liquid chromatography (HILIC) column (Phenomenex, Torrance, USA). The total content of PC and PE were quantified based on the internal standards PDME and batyl alcohol and the PC:PE ratio was calculated.

### 3.10 H&E staining and microscopy

For jejunum morphology, we examined the following subset of the study group for intestinal morphology changes: CLF, CHF, 50PC, 100PC (n=6 for each group). Jejunum samples (3-4 sections per rat) were taken and fixed in formalin, processed with successive washes of reagent alcohol and xylene, blocked in paraffin, and cooled for half an hour. See **Table 3-4** for a detailed description of the blocking protocol. After cooling, slides were made by slicing the blocks of sample in paraffin into 5 μM cross sections onto glass slides using a microtome (Leica HistoCore AUTOCUT, Ontario, Canada). The slides were then deparaffinized using xylene, rehydrated using lowering concentrations of alcohol, dyed with hematoxylin, differentiated using 1% acid alcohol (HCL in 70% ethanol), blued using 0.1% sodium bicarbonate in distilled water, and counterstained with eosin dye. Slides were then dehydrated using increasing concentrations of alcohol, cleared using xylene, and mounted with coverslips using Permount. For a detailed description of the staining technique, see **Table 3-5**. Slides were loaded onto the slide reader, imaged using light microscopy at a 4X magnification. (LionHeartLX, Agilent Technologies, Santa Clara, CA). Images were then scanned and stitched together to create a high-resolution image. Villus height and crypt depth were measured using Image using a known scale. The villus height was measured from the tip of the villus to the crypt-villus junction and the crypt depth was measured from the crypt-villus junction to the base of the crypt.

### 3.11 mRNA Isolation and Quantitation by RT-qPCR

Similar to the jejunum morphology, we examined the same subset of the study group for gene expression changes: CLF, CHF, 50PC, 100PC (n=6 for each group). Total RNA was isolated from frozen jejunum tissue in Trizol (Invitrogen, Carlsbad, CA) with polytron

disruption, and ran through RNEasy Mini (Qiagen, Hilden, Germany) columns. RNA integrity was assessed by Agilent Bioanalyzer chip analysis—all samples had RIN numbers greater than 8.3. RNA was incubated with amplification grade DNAse I (Invitrogen) and reversed transcribed using Superscript II (Invitrogen) with oligo dT and random hexamer priming (Invitrogen). Real time qPCR (RT-qPCR) with PowerSybr master mix (Thermo Scientific, Waltham, MA) was performed in triplicate in an Applied Biosystems StepOne Plus machine (Applied Biosystems, Waltham, MA). The standard curves method was used for quantification, with mRNA expression normalized to the reference gene *Rplp0*. Primer sequences and gene names are listed in **Table 3-6**.

### 3.12 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 the main effect. Data points were removed as outliers if they fell outside of the mean±3 times the standard deviation. 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.

### 3.12 References


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**Table 3-1: Composition of experimental diets[1]**

| Ingredient (g/Kg diet) | CLF diet | CHF diet | 25PC diet | 50PC diet | 75PC diet | 100PC diet[6] | SPC diet |
|---|---|---|---|---|---|---|---|
| **Casein** | 262 | 262 | 262 | 262 | 262 | 262 | 262 |
| **Corn starch** | 274 | 124 | 126 | 126 | 126 | 126 | 126 |
| **Sucrose** | 200 | 200 | 200 | 200 | 200 | 200 | 200 |
| **Vitamin Mix (AIN-93-Vx)[2]** | 19 | 19 | 19 | 19 | 19 | 19 | 19 |
| **Mineral Mix[3]** | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| **Calcium Phosphate Dibasic** | 3.4 | 3.4 | 3.4 | 3.4 | 3.4 | 3.4 | 3.4 |
| **Inositol** | 6.3 | 6.3 | 6.3 | 6.3 | 6.3 | 6.3 | 6.3 |
| **Cellulose** | 80 | 80 | 80 | 80 | 80 | 80 | 80 |
| **L-cystine** | 1.8 | 1.8 | 1.8 | 1.8 | 1.8 | 1.8 | 1.8 |
| **Choline bitartrate** | 3.7 | 3.7 | 2.78 | 1.85 | 0.93 | 0 | 0 |
| **Egg lecithin (PC)[4]** | 0 | 0 | 3.5 | 7 | 10.5 | 14 | 0 |
| **Soy lecithin (PC)** | 0 | 0 | 0 | 0 | 0 | 0 | 44.4 |
| **Fat Mixture** | | | | | | | |
| **Total fat mix** | 100 | 250 | 250 | 250 | 250 | 250 | 250 |
| **Flax Oil** | 10 | 25 | 25 | 25 | 25 | 25 | 23 |
| **Corn Oil** | 40 | 100 | 99 | 99 | 99 | 99 | 77 |
| **Lard** | 50 | 125 | 114 | 117 | 120 | 123 | 118 |
| **Olive Oil** | 0 | 0 | 0 | 0 | 0 | 0 | 8 |

[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 (54);
[3]Bernhart–Tomarelli salt mixture (55).

[4]Egg-PC was purchased from Lipoid GmbH.
[5]Obtained from Bluebonnet Nutrition Corporation. 12915 Dairy Ashford
[6]100PC diet is labeled as the EPC diet in Chapter 5.
CLF, control low-fat; CHF, control high-fat; 25PC, 25% egg-PC high-fat; 50PC, 50% egg-PC high-fat; 75PC, 75% egg-PC high-fat; 100PC, 100% egg-PC high-fat; EPC, egg-phosphatidylcholine; PC, phosphatidylcholine SPC, soy-phosphatidylcholine.

**Table 3-2: Fatty acid composition of experimental diets[1]**

| Fatty Acid | CLF diet | CHF diet | 25PC diet | 50PC diet | 75PC diet | 100PCdiet[2] | SPC |
|---|---|---|---|---|---|---|---|
| | (% of total fatty acids) | | | | | | |
| **14:0** | 0.72 ± 0.06 | 0.72 ± 0.03 | 0.66 ± 0.01 | 0.65 ± 0.0 | 0.61 ± 0.01 | 0.63 ± 0.02 | 0.72 ± 0.03 |
| **16:0** | 18.0 ± 0.74 | 18.1 ± 0.58 | 17.9 ± 0.15 | 17.8 ± 0.12 | 17.6 ± 0.41 | 18.0 ± 0.12 | 18.1 ± 0.19 |
| **16:1n9** | 0.15 ± 0.02 | 0.18 ± 0.03 | 0.15 ± 0.01 | 0.14 ± 0.01 | 0.14 ± 0.02 | 0.18 ± 0.02 | 0.16 ± 0.02 |
| **16:1n7** | 0.97 ± 0.09 | 0.96 ± 0.07 | 0.93 ± 0.01 | 0.92 ± 0.01 | 0.88 ± 0.04 | 0.89 ± 0.01 | 0.98 ± 0.02 |
| **18:0** | 10.1 ± 1.00 | 10.0 ± 0.59 | 9.87 ± 0.10 | 9.82 ± 0.09 | 9.52 ± 0.34 | 9.77 ± 0.16 | 10.1 ± 0.30 |
| **18:1n9** | 32.0 ± 0.23 | 31.8 ± 0.52 | 31.6 ± 0.36 | 31.4 ± 0.01 | 31.4 ± 0.29 | 31.3 ± 0.39 | 33.7 ± 0.46 |
| **18:1t11** | 1.00 ± 0.29 | 1.22 ± 0.18 | 1.07 ± 0.35 | 1.26 ± 0.04 | 1.12 ± 0.38 | 1.17 ± 0.26 | 1.15 ± 0.19 |
| **C18:2n-6** | 30.9 ± 0.33 | 29.7 ± 1.25 | 30.5 ± 0.27 | 30.6 ± 0.25 | 31.4 ± 0.29 | 30.5 ± 0.07 | 27.7 ± 0.49 |
| **20:0** | 0.31 ± 0.02 | 0.31 ± 0.0 | 0.31 ± 0.01 | 0.31 ± 0.0 | 0.31 ± 0.01 | 0.30 ± 0.0 | 0.30 ± 0.01 |
| **18:3n-3 (ALA)** | 7.00 ± 0.56 | 6.35 ± 0.35 | 6.38 ± 0.02 | 6.50 ± 0.01 | 6.40 ± 0.14 | 6.54 ± 0.03 | 6.53 ± 0.25 |
| **20:2n-6** | 0.31 ± 0.02 | 0.30 ± 0.02 | 0.28 ± 0.0 | 0.28 ± 0.0 | 0.26 ± 0.01 | 0.27 ± 0.0 | 0.30 ± 0.0 |
| **20:3n-6** | 0.07 ± 0.01 | 0.07 ± 0.01 | 0.07 ± 0.0 | 0.07 ± 0.0 | 0.08 ± 0.0 | 0.08 ± 0.0 | 0.08 ± 0.0 |
| **20:4n-6** | 0.80 ± 0.01 | 0.07 ± 0.0 | 0.14 ± 0.06 | 0.14 ± 0.04 | 0.18 ± 0.04 | 0.26 ± 0.0 | 0.08 ± 0.0 |
| **20:5n-6** | 0.07 ± 0.01 | 0.07 ± 0.01 | 0.07 ± 0.0 | 0.07 ± 0.0 | 0.07 ± 0.0 | 0.07 ± 0.0 | 0.07 ± 0.0 |
| **22:6n-3 (DHA)** | 0.01 ± 0.01 | 0.09 ± 0.10 | 0.03 ± 0.02 | 0.03 ± 0.02 | 0.03 ± 0.0 | 0.08 ± 0.02 | 0.00 ± 0.0 |
| **Total SFA** | 29.2 ± 1.76 | 29.2 ± 1.18 | 28.8 ± 0.24 | 28.6 ± 0.21 | 28.1 ± 0.75 | 28.7 ± 0.26 | 29.2 ± 0.45 |
| **Total MUFA** | 34.1 ± 0.61 | 34.2 ± 0.48 | 33.7 ± 0.02 | 33.7 ± 0.04 | 33.5 ± 0.11 | 33.5 ± 0.14 | 36.0 ± 0.30 |
| **Total PUFA** | 38.4 ± 0.44 | 36.6 ± 1.66 | 37.5 ± 0.21 | 37.7 ± 0.17 | 38.4 ± 0.87 | 37.8 ± 0.12 | 34.8 ± 0.70 |
| **n-6:n-3** | 4.44 ± 0.02 | 4.62 ± 0.12 | 4.79 ± 0.03 | 4.71 ± 0.06 | 4.90 ± 0.03 | 4.65 ± 0.02 | 4.27 ± 0.12 |
| **SFA:PUFA** | 0.86 ± 0.04 | 0.85 ± 0.02 | 0.85 ± 0.01 | 0.85 ± 0.01 | 0.84 ± 0.02 | 0.86 ± 0.01 | 0.81 ± 0.01 |

[1]Analysis of the fatty acid composition of the seven experimental diets collected weekly (n=2 batches for experimental diets; n=3 for control diets).
[2]100PC diet is labeled as the EPC diet in Chapter 5.
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; 25PC, 25% egg-PC high-fat; 50PC, 50% egg-PC high-fat; 75PC, 75% egg-PC high-fat; 100PC, 100% egg-PC high-fat; PC, phosphatidylcholine.

**Table 3-3: Spleen and MLN immune cell phenotype panels**

| Antibody | Colour | Filter | Dilution |
|---|---|---|---|
| T Cell | | | |
| CD3 | APC | R670 | 1 in 20 |
| CD4 | FITC | B530 | 1 in 40 |
| CD8[1] | BUV395 | U379 | 1 in 50 |
| CD25 | PerCP-eFluor 710 | B695 | 1 in 10 |
| B Cell | | | |
| CD45A | AlexaFluor 405 | V450 | 1 in 50 |
| CD74 (OX6) | PerCP | B695 | 1 in 60 |
| CD27 | BUV737 | U740 | 1 in 50 |
| CD80 | PE | Y586 | 1 in 100 |
| NK Cell | | | |
| CD161 | Alexa 647 | R670 | 1 in 40 |
| CD3 | FITC | B530 | 1 in 20 |
| CD74 (OX6) | PerCP | B695 | 1 in 60 |
| Monocytes and Macrophages | | | |
| CD68 | APC-Vio770 | R780 | 1 in 10 |
| TLR4 | Alexa 647 | R670 | 1 in 50 |
| T Reg Cell | | | |
| CD4 | FITC | B530 | 1 in 40 |
| CD3 | APC | R670 | 1 in 20 |
| FoxP3 | PE | Y586 | 1 in 20 |

[1] CD8 (BUV395) did not stain properly; CD3+CD4- was used as a surrogate marker for CD3+CD8+ (T cytotoxic cells)

**Table 3-4: Protocol for blocking jejunum sections in paraffin[1]**

| Step | Reagent | Duration (hour:minute) | Temperature (°C) |
|---|---|---|---|
| 1 | Formalin | 1:00 | 37 |
| 2 | Processing Water | 0.02 | - |
| 3 | Ethanol 70% | 0:40 | 45 |
| 4 | Ethanol 80% | 0:40 | 45 |
| 5 | Ethanol 95% | 0:40 | 45 |
| 6 | Ethanol 100% | 1:00 | 45 |
| 7 | Ethanol 100% | 1:00 | 45 |
| 8 | Ethanol 100% | 1:00 | 45 |
| 9 | Xylene | 1:00 | 45 |
| 10 | Xylene | 1:00 | 45 |
| 11 | Xylene | 1:00 | 45 |
| 12 | Paraffin | 1:00 | 61 |
| 13 | Paraffin | 1:00 | 61 |
| 14 | Paraffin | 1:30 | 61 |

[1] Performed using Leica Biosystems HistoCore PEARL, Ontario, Canada

**Table 3-5: H&E staining protocol**

| Step | Reagent/Procedure | Duration (minutes:seconds) |
|---|---|---|
| 1 | Xylene | 10:00 |
| 2 | Xylene | 10:00 |
| 3 | 100% Ethanol | 5:00 |
| 4 | 100% Ethanol | 5:00 |
| 5 | 95% Ethanol | 2:00 |
| 6 | 70% Ethanol | 2:00 |
| 7 | Wash in distilled water | 1:00 |
| 8 | Add Harris hematoxylin dropwise | - |
| 9 | Hold in humidity chamber (made by placing wet paper towels in a plastic box with a lid) | 5:00 |
| 10 | Wash in running tap water | 5:00 |
| 11 | Differentiate in 1% acid alcohol (HCL in 70% ethanol) | 0:30 |
| 12 | Wash in running tap water | 1:00 |
| 13 | Blue in 0.1% Sodium Bicarbonate in distilled water solution | 1:00 |

| | | |
|---|---|---|
| 14 | Wash in running tap water | 5:00 |
| 15 | Counterstain by adding Eosin dropwise to slides | 0:30 - 1:00 |
| 16 | Dip in and out of water bath | 3 x 1:00 cycles |
| 17 | 95% Ethanol | 5:00 |
| 18 | 100% Ethanol | 5:00 |
| 19 | 100% Ethanol | 5:00 |
| 20 | Xylene | 5:00 |
| 21 | Xylene | 5:00 |
| 22 | Mount with Permount | - |

**Table 3-6: Quantitative PCR primer sets**

| Gene Symbol | Gene name | Forward primer sequence | Reverse primer sequence |
|---|---|---|---|
| *Muc2* | Mucin 2 | ATGTGTGGGACCGGACAATG | GTTGCCCCTAGCACACTTC |
| *Tff3* | Trefoil factor 3 | AGCCTTCTGGATAACCCTGC | TTGCCGGGACCATACATTGG |
| *Gfi1* | Growth factor independent 1 transcriptional repressor | TCCACACTGTCCACACATCTAC | AGCTCTGACTGAAGGCTTTGC |
| *Spdef* | SAM pointed domain containing ETS transcription factor | TGCTCACCTGGACATCTGGA | ACGAATCCACCTCACCATCC |
| *Cldn 4* | Claudin 4 | CGAGAAAGAACAGCTCCGTTT | TTCGCTTGTCTTTTGGCTGC |
| *Casp4* | Caspase 4 | CCTGCTTCCGGATACATGCC | TCTTGTCAAGGTTGCCCGAT |
| *Pcyt1a* | Phosphate cytidylyltransferase 1, choline α isoform | TGGAATTCCTTCCAAAGTGC | TCACCCTGACATAGGGCTTACT |
| *Rplp0* | Ribosomal protein lateral stalk subunit P0 | CCTGCACACTCGCTTCCTA | TGATGGAGTGAGGCACTGAG |

# CHAPTER 4: A physiologically relevant dose of 50% egg-phosphatidylcholine is sufficient in improving gut permeability while attenuating immune cell dysfunction induced by a high-fat diet in male Wistar rats

A version of this chapter has been submitted for publication in the Journal of Nutrition as: Rusnak T, Azarcoya-Barrera J, Wollin B, Makarowski A, Nelson R, Field CJ, Jacobs RL, Richard C. (2023). A physiologically relevant dose of 50% egg-phosphatidylcholine is sufficient in improving gut permeability while attenuating immune cell dysfunction induced by a high-fat diet in male Wistar rats. Content in this chapter was presented at the American Society of Nutrition Virtual Conference (2021) and the Canadian Society for Nutrition Conference in Gatineau, QC (2022).

## 4.1 Introduction

Obesity is associated with diminished immune responses and an increased risk of infection (She, Mangat et al. 2022). Some of the mechanisms proposed to explain obesity-related immune dysfunction include high-fat diet (HFD) feeding leading to lipotoxicity and insulin resistance, and increased gut permeability. Metabolism and immune responses are highly interwoven, and an increase in lipid deposition in ectopic tissue has been shown to disrupt metabolic function and stimulate an immune response (Ertunc & Hotamisligil 2016). Patients with obesity have also been shown to have a higher degree of gut permeability (Genser, Aguanno et al. 2018). An increase in gut permeability leads to an increase in bacterial lipopolysaccharide (LPS) translocation from the gut to the bloodstream. In circulation LPSs can bind to toll-like receptor (TLR)-4, which upon activation increases the expression of several pro-inflammatory cytokines (tumor necrosis factor-alpha [TNF-α], interleukin [IL]-6, IL-Iβ, interferon gamma

[IFN-γ]) (Bischoff, Barbara et al. 2014, Moreira, Texeira et al. 2012). It has been suggested that this higher level of basal inflammation prevents immune cells from mounting an adequate response upon challenge (Richard, Wadowski et al. 2017).

Choline is an essential nutrient required for numerous processes in the body, including lipid transport and brain development (Zeisel 2000, Zeisel 2004, Zeisel & da Costa 2009). Choline is found in the diet as water-soluble forms (free choline [FC], glycerophosphocholine, and phosphocholine), and lipid-soluble forms (phosphatidylcholine [PC] and sphingomyelin [SM]) (Lewis, Field et al. 2015). Water- and lipid-soluble forms of choline are metabolized in the body differently and thus may impact metabolic processes differently (Lewis, Field et al. 2015). PC can be found in many foods but is richest in meat and eggs (Lewis, Field et al. 2015). PC plays an important role in intestinal permeability and maintaining cell membrane integrity. Indeed, it was shown that providing 100 mg/kg PC per day for 21 days improved intestinal barrier function and reduced serum TNF-α and plasma LPS levels in rats (Chen, Huang et al. 2019). PC along with phosphatidylethanolamine (PE) are the most abundant phospholipids in mammalian cell membranes, and the molar ratio of PC to PE has been proposed as a relevant marker of liver health (van der Veen, Kennelly et al. 2017, Ling, Chaba et al. 2012, Kharbanda, Maillard et al. 2007). There is accumulating evidence that both PC and PE metabolism play important roles throughout the body due to both an abnormally high or low PC:PE ratio disrupting cell membrane, mitochondria, and endoplasmic reticulum function (Li, Agellon et al. 2006, Tasseva, Bai et al., 2013, Fu, Yang et al. 2011). Therefore, dietary PC may play a role in immune function by modulating phospholipid composition of cell membranes to support cell function, and indirectly by preventing endotoxin translocation and subsequent inflammatory responses in obesity.

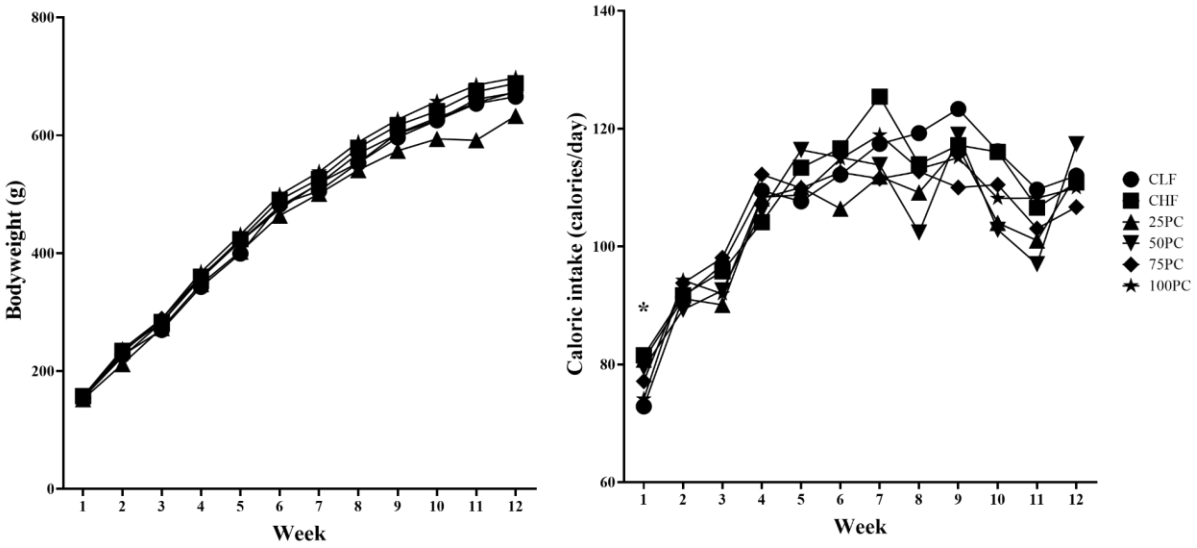
Studies from our group have consistently reported that rodent diets containing higher proportions of lipid soluble forms of choline relative to the standard form of choline, FC, in rodent diets beneficially modulates immune function during different life stages (Azarcoya-Barrera, Lewis et al. 2022, Azarcoya-Barrera, Field et al. 2021, Dellschaft, Richard et al. 2018, Azaroya-Barrera, Wollin et al. 2022). In Sprague-Dawley rats, we have shown that feeding a diet high in both PC and SM (around 66%) or a 50% PC diet during the suckling and weaning periods increases IL-2 and TNF-α production after T cell stimulation when compared to a diet containing only FC (Azarcoya-Barrera, Field et al. 2021). Recently, in Wistar rats, we have also shown that feeding a diet containing 100% PC from egg attenuates T cell dysfunction in male fed a HFD by increasing the production of IL-2 and TNF-α by stimulated splenocytes (Azarcoya-Barrera, Wollin et al. 2022). However, feeding a diet of 100% egg-derived PC is of limited physiological relevance as no human diet would contain choline only in the form of PC. Therefore, the objective of this study was to determine the effect of feeding varying doses of egg-PC on immune function in the context of a HFD when compared to low-fat and high-fat control diets. We hypothesized that feeding a diet reflecting a healthy omnivorous diet of about 50% of total choline (Lewis, Subhan et al. 2014) from egg-PC would be sufficient to improve the HFD-induced immune dysfunction.

## 4.2 Results

### 4.2.1 Anthropometric characteristics, caloric intake, and plasma cytokines

After 12 weeks of feeding, no significant difference in body weight between the diet groups existed ($p > 0.05$, **Figure 4-1A)**. Since the rats ate to satisfy caloric and protein requirements, there was no difference in caloric intake between the diet groups after week 1 ($p > 0.05$, **Figure 4-1B**). Intestinal length and organ weight (spleen and liver) were not affected by the dietary treatments (both $p > 0.05$, **Table 4-1**). The number of splenocytes were also not different among groups ($p > 0.05$). There was no significant difference between the measured plasma cytokine concentrations ($p > 0.05$, **Table 4-1**).

**Figure 4-1: (A) Body weight; (B) caloric intake of male Wistar rats fed the six experimental diets.**




Values are presented as mean ± SEM. *Means significantly different ($p < 0.05$) based on the one-way ANOVA test with the Duncan adjustment for multiple comparisons. CLF, control low-fat; CHF, control high-fat; 25PC, 25% egg-PC high-fat; 50PC, 50% egg-PC high-fat; 75PC, 75% egg-PC high-fat; 100PC, 100% egg-PC high-fat; PC, phosphatidylcholine

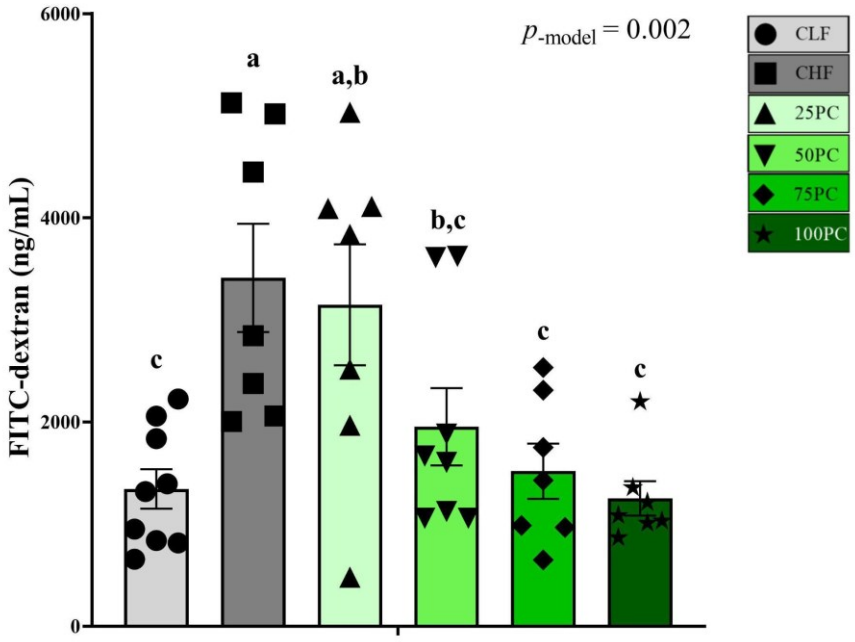
### 4.2.2 Effect of PC on liver total fatty acid composition

Several changes in liver total fatty acid (FA) composition were observed, even though the FA composition of the six diets were closely matched (**Table 4-2**). The proportion of total SFA in the liver was higher in rats fed the CLF diet compared to all other diets, and the lowest proportion was observed in the 50PC diet group (all $p < 0.05$, **Table 4-3**). The 100PC diet group had a significantly lower proportion of total monounsaturated FAs (MUFAs) and higher total polyunsaturated FAs (PUFAs) compared to all diet groups ($p < 0.05$). Total proportion of PUFAs were significantly lower in the CLF diet fed group compared to all other diet groups ($p < 0.05$). The n-6 to n-3 PUFA ratio was significantly lower in the CLF diet group compared to the rest of the diet groups ($p < 0.05$). Further, the SFA to PUFA ratio was significantly higher in the CLF diet compared to the rest of the diet groups ($p < 0.05$).

### 4.2.3 Intestinal permeability

The effect of the diets on intestinal permeability as assessed by FITC-dextran concentration is presented in **Figure 4-2**. The CHF diet fed rats had significantly higher intestinal permeability compared to the CLF diet group ($p < 0.05$). Starting at the 50PC diet, increasing the dosage of PC in the diet resulted in intestinal permeability being similar to the CLF diet fed rats (all $p > 0.05$). There was no difference in intestinal permeability between the CHF and 25PC diet ($p > 0.05$).

**Figure 4-2: Effect of diet on intestinal permeability in male Wistar rats**



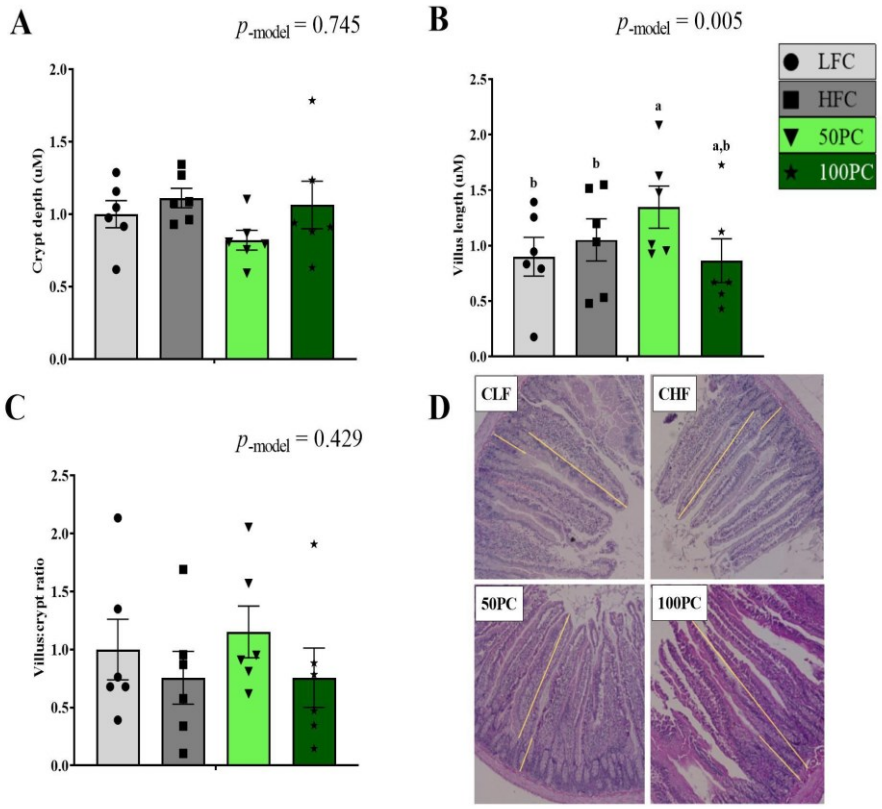

Values are means ± SEM. Groups that do not share a common letter are significantly different based on the one-way ANOVA with the Duncan adjustment for multiple comparison analysis. p-model = 0.002. CLF, control low-fat; CHF, control high-fat; 25PC, 25% egg-PC high-fat; 50PC, 50% egg-PC high-fat; 75PC, 75% egg-PC high-fat; 100PC, 100% egg-PC high-fat; PC, phosphatidylcholine

### 4.2.4 Intestinal morphology and mRNA of jejunum tissue

There was no difference in villi height in the jejunum between the CLF and CHF diet fed rats (**Figure 4-3**, $p > 0.05$). Between the 50PC and 100PC diets, only the 50PC diet significantly increased the villi height compared to the control diets ($p < 0.05$). There was no difference in both the crypt depth and villi:crypt ratio across all diets (both $p > 0.05$). The effect of the CLF, CHF, 50PC and 100PC diets on jejunum mRNA levels of genes associated with intestinal integrity and inflammation are presented in **Figure 4-4.** There was no significant effect of diet on the mRNA levels of *GFi1*, *Muc2*, *Spdef*, and *Tff3*, genes associated with goblet cell structure and production ($p > 0.05$). Further, there was no significant difference between diet groups for a gene associated with inflammation-related cell death, *Casp4* ($p > 0.05$). We also examined mRNA

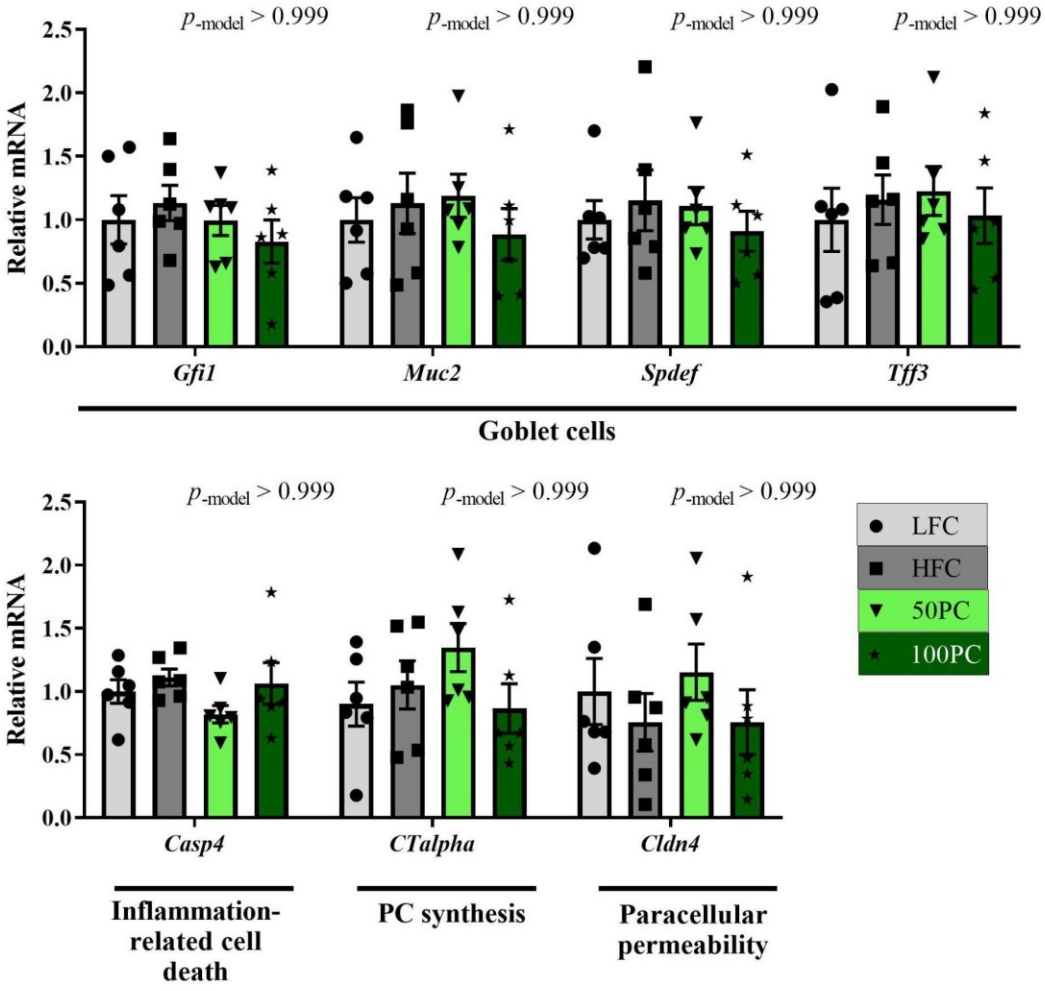
levels of *Cldn4*, a tight junction protein involved in intestinal permeability, which was not affected by diet ($p > 0.05$). Diet also had no significant effect on *Pcyt1a,* a gene coding for the rate limiting enzyme CTalpha of the Kennedy pathway of *de novo* phosphatidylcholine synthesis ($p > 0.05$).

**Figure 4-3: Effect of diet on (A) crypt depth, (B) villus height, and (C) villus height:crypt depth ratio of the jejunum.**




Values are means ± SEM. p-model < 0.05 based on the one-way ANOVA test with the Duncan adjustment for multiple comparisons test. CLF, control low-fat; CHF, control high-fat; 50PC, 50% egg-PC high-fat; 100PC, 100% egg-PC high-fat; PC, phosphatidylcholine

**Figure 4-4: Effect of diet on jejunum mRNA levels of genes related to goblet cells, cell death from inflammation, *de novo* PC synthesis, and paracellular permeability in male Wistar rats**



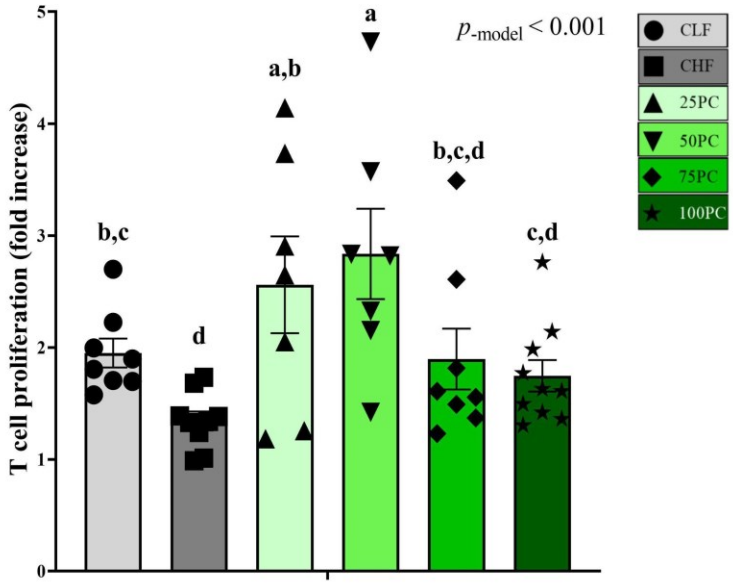

Values are means ± SEM. p-model < 0.05 based on the one-way ANOVA test with the Duncan adjustment for multiple comparisons test. CLF, control low-fat; CHF, control high-fat; 50PC, 50% egg-PC high-fat; 100PC, 100% egg-PC high-fat; PC, phosphatidylcholine

### 4.2.5 T cell proliferation assay and *ex vivo* cytokine production by splenocytes

Compared to the CLF diet group, T cell proliferation by splenocytes after stimulation with anti-CD3/anti-CD28 was significantly lower in the CHF group ($p < 0.001$) as shown in **Figure 4-5**. Feeding the 25PC and 50PC diet significantly increased T cell proliferation compared to both the CHF and CLF diet ($p < 0.05$) while the 75PC and 100PC had a normalizing

effect relative to the CLF diet ($p > 0.05$). *Ex-vivo* cytokine production in splenocytes after mitogen stimulation is presented in **Figure 4-6** and **Table 4-4**. After stimulation with the T cell mitogen PMA+I, the rats fed the CHF diet had significantly lower IL-10 production compared to the CLF diet ($p < 0.05$). The 50PC diet significantly increased IL-10 production compared to the CHF diet after PMA+I stimulation ($p < 0.05$), while the 25PC and 100PC diets normalized IL-10 production relative to the CLF diet ($p > 0.05$). After PMA+I stimulation, there was no significant difference in TNF-α production between the CLF and CHF group ($p > 0.05$). Of the PC diets, only the 50PC diet group produced higher TNF-α levels than the CHF group ($p < 0.05$). While there was no significant difference in IL-2 production between the CLF and CHF diet groups after PMA+I production, the CHF diet trended toward having lower IL-2 production (1289 ± 304 pg/mL and 489 ± 96 pg/mL for CLF and CHF respectively). Of the PC groups, IL-2 production was higher in only the 50PC diet compared to the control diets ($p < 0.05$; **Table 4-3**).

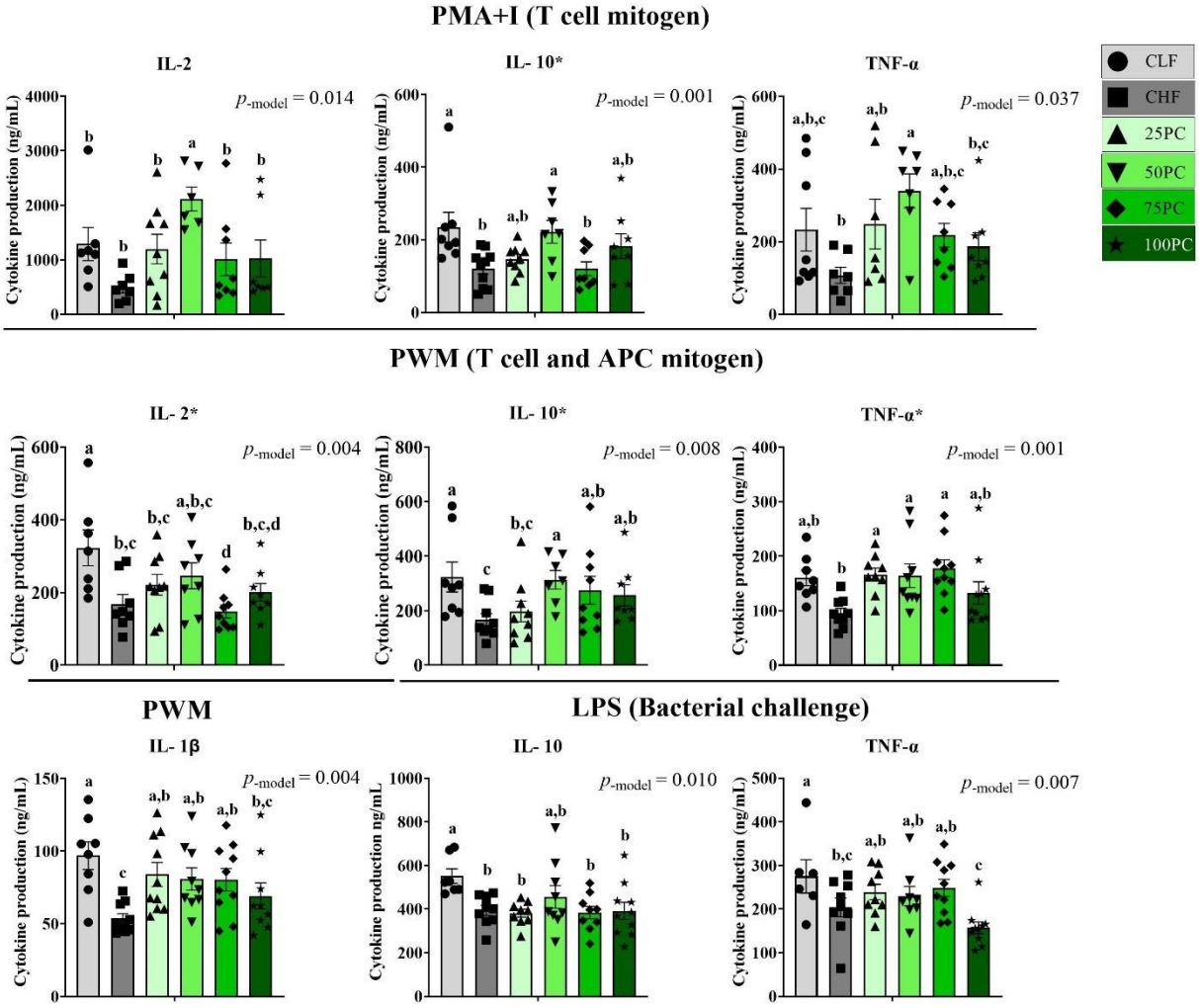
**Figure 4-5: Effect of diet on T cell proliferation in splenocytes following stimulation with anti-CD3/anti-CD28 in male Wistar rats.**




Values are means ± SEM. *p*-model < 0.0001. CLF, control low-fat; CHF, control high-fat; 25PC, 25% egg-PC high-fat; 50PC, 50% egg-PC high-fat; 75PC, 75% egg-PC high-fat; 100PC, 100% egg-PC high-fat; PC, phosphatidylcholine

Following PWM stimulation, rats fed the CHF diet produced significantly less IL-1β, IL-2, IL-10, and TNF-α when compared to the CLF diet (all $p < 0.05$, **Figure 4-6**). For the PC diets, the 25PC, 50PC, and 75PC diet groups produced similar levels of IL-1β to the CLF diet ($p > 0.05$). IL-2 production was normalized only in the 50PC diet relative to the CLF diet ($p > 0.05$). For IL-10 production, the 50PC diet produced levels similar to the CLF diet, while the 75PC and 100PC diet groups normalized production relative to the CLF diet group after PWM stimulation ($p > 0.05$). The 25PC, 50PC, and 75PC diet groups produced higher TNF-α levels than the CHF diet group, and the 100PC diet normalized TNF-α production relative to the CLF diet ($p < 0.05$). There was no significant difference in IL-6 production between the diet groups after PWM stimulation ($p > 0.05$, **Table 4-4**). After LPS stimulation, rats fed the CLF produced more IL-10 and TNF-α than rats fed the CHF diet ($p < 0.05$, **Figure 4-6**). Of the PC diets, only the 50PC diet increased IL-10 production similar to the CLF diet after LPS stimulation. A normalizing effect in TNF-α production after LPS stimulation was observed in rats fed 25PC, 50PC, and 75PC diets relative to the CLF diet fed rats ($p < 0.05$). There was no significant difference in IL-6 production between the diet groups after LPS stimulation ($p > 0.05$, **Table 4-4**).

**Figure 4-6: Significant effect of diet on *ex vivo* cytokine production in mitogen stimulated splenocytes of male Wistar rats**




Values are means ± SEM. p-model < 0.05. 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; 25PC, 25% egg-PC high-fat; 50PC, 50% egg-PC high-fat; 75PC, 75% egg-PC high-fat; 100PC, 100% egg-PC high-fat; IFN-γ, interferon gamma; IL, interleukin; PC, phosphatidylcholine; TNF-α, tumor necrosis factor-alpha.
*Analysis performed on log-transformed values.

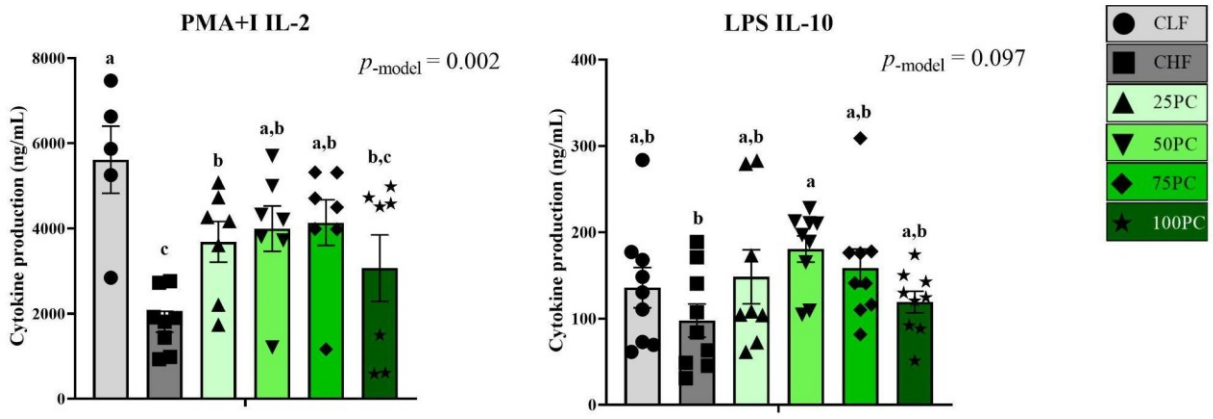
### 4.2.6 *Ex vivo* cytokine production by MLN cells

*Ex-vivo* cytokine production in MLNs after mitogen stimulation is presented in **Figure 4-7** and

**Table 4-4**. After stimulation with the T cell mitogen PMA+I, the rats fed the CHF diet had

significantly lower IL-2 production compared to the CLF diet fed rats ($p < 0.05$). The 25PC diet fed rats produced higher IL-2 levels than the CHF diet ($p < 0.05$), and the 50PC and 75PC diet fed rats produced similarly high levels of IL-2 relative to the CLF diet ($p > 0.05$). The 100PC diet fed rats produced IL-2 levels that were not significantly different from the CHF diet ($p > 0.05$). There was no significant difference in IL-10 or TNF-α production after PMA+I production between any of the diet groups ($p > 0.05$). After LPS stimulation, there was no significant difference in IL-10 or TNF-α between the diet groups ($p > 0.05$), although IL-10 levels in PC diets, namely the 50PC diet, tended to be higher than the CHF diet.

**Figure 4-7: Significant and trending effect of diet on *ex vivo* cytokine production in mitogen stimulated MLN cells of male Wistar rats.**




Values are means ± SEM. p-model < 0.05. 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; 25PC, 25% egg-PC high-fat; 50PC, 50% egg-PC high-fat; 75PC, 75% egg-PC high-fat; 100PC, 100% egg-PC high-fat; IFN-γ, interferon gamma; IL, interleukin; MLN, mesenteric lymph nodes; PC, phosphatidylcholine; TNF-α, tumor necrosis factor-alpha

### 4.2.7 Splenocyte phenotypes

Proportions of immune cell phenotypes isolated from the spleen from rats fed the 6 experimental diets are presented in **Figure 4-8A** and **Table 4-5**. Rats fed the 25PC and 100PC diets had a higher proportion of CD45RA+ B cells compared to the CHF diet fed rats ($p < 0.05$),

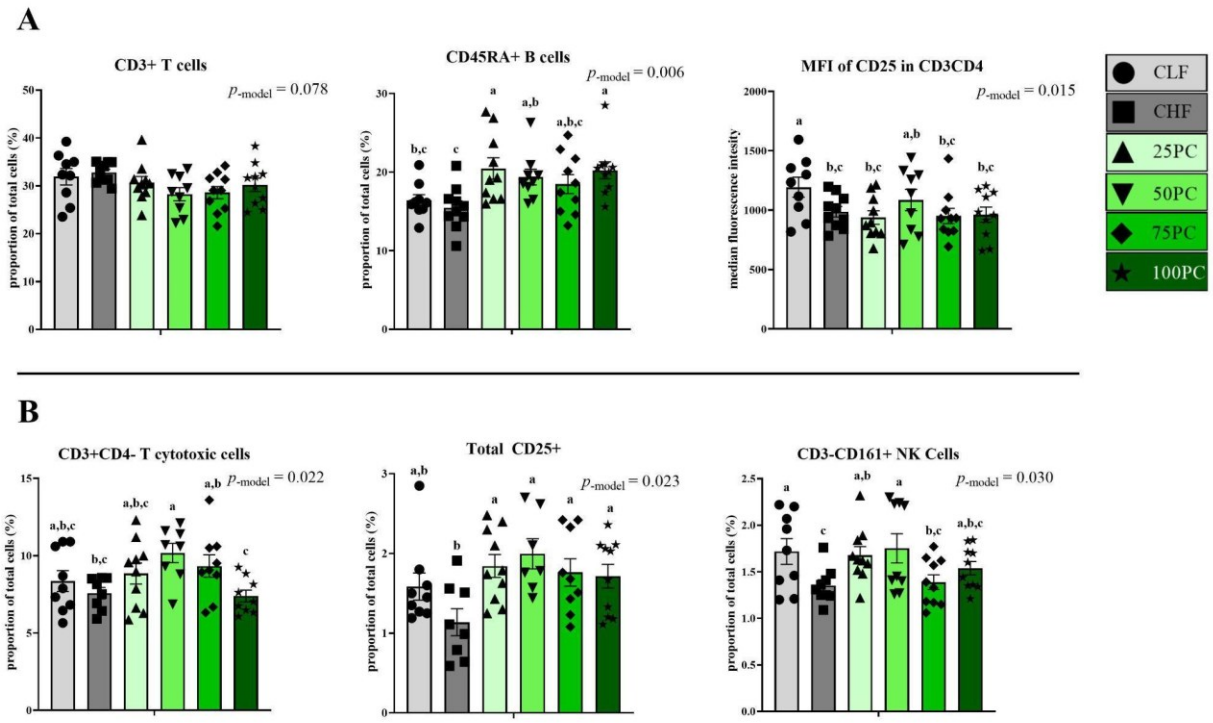
while the 50PC and 75PC were not different from the CLF. There was a trend toward a slightly lower proportion of CD3+ T cells with egg-PC compared to the CLF and CHF diet, but it did not reach statistical significance ($p > 0.05$). There were no significant differences between the diets for any of the following T cell populations ($p > 0.05$): CD3+CD4+ (T helper cells), CD3+CD4- (T cytotoxic cells), and CD3+CD4+FoxP3+ (T regulatory cells), CD3+CD4+CD25+ (T helper cells with an IL-2 receptor), CD3+CD4-CD25+ (T cytotoxic cells with an IL-2 receptor). There was no significant difference in OX6+ (MHC class II cells), CD68+ (macrophages), CD68+CD11b/c+ or CD68+TLR4+ (both activated macrophages), and CD161+CD3- (NK cells) between the diet groups ($p > 0.05$, **Table 4-5**). The median fluorescence intensity (MFI) of CD25+ in CD3+CD4+ was lower in the CHF compared to the CLF diet ($p < 0.05$, **Figure 4-8A**). Of the experimental diets, only the 50PC group increased the MFI of CD25+ in CD3+CD4+ to levels similar to that of the CLF group ($p < 0.05$). There was no difference in the MFI of CD25+ in CD3+ or CD3+CD4-, and no difference in the MFI of total TLR4+ and TLR4+ in CD68+ ($p > 0.053$).

### 4.2.8 MLN immune cell phenotypes

Proportions of immune cell phenotypes isolated from MLN of the rats fed the 6 experimental diets are presented in **Figure 4-8B** and **Table 4-6**. There was no significant difference between the CLF and CHF diets as it pertains to the expression of CD25+ (IL-2 receptor), whereas all the PC diets increased the proportion of cells expressing CD25+ when compared to the CHF diet fed rats ($p > 0.05$, **Figure 4-8B**). While there was no difference between the CLF and CHF diets, the 50PC diet was the only PC diet that increased the proportion of CD3+CD4- (T cytotoxic cells) compared to the CHF diet ($p < 0.05$, **Figure 4-8B**). Feeding a CHF diet significantly reduced the proportion of CD3-CD161+ (NK cells) compared

to the CLF diet. Both the 25PC and 50PC diets increased the NK cell proportion to levels similar to the CLF diet ($p < 0.05$). There was no significant difference in diet groups for the MFI of CD3+CD4+CD25+ and CD3+CD4-CD25+ in MLN. There was no diet effect on proportions of CD3+ (T cells), CD3+CD4+ (T helper cells) and CD3+CD4+FoxP3+ (T regulatory cells), CD45RA+ cell (B cells), CD68+ cells (macrophages), and OX6+ cells (MHC class II cells) (all $p > 0.05$, **Table 4-6**).

**Figure 4-8: Significant and trending effect of diet on (A) splenocyte phenotype and (B) MLN cell phenotype of male Wistar rats.**

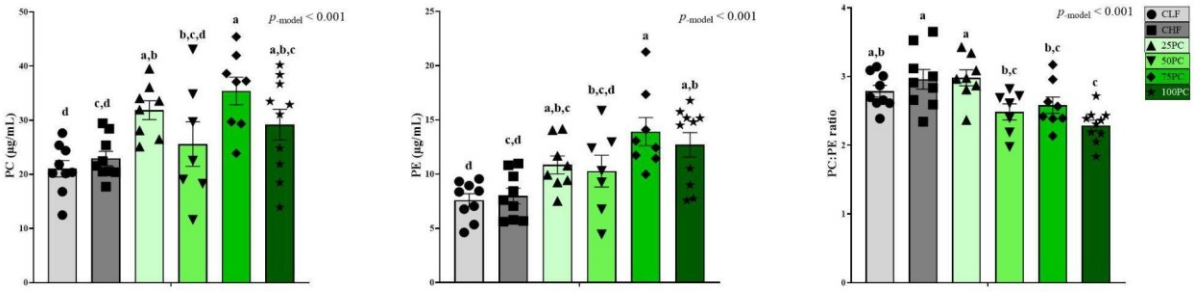



Values are means ± SEM. p-model < 0.05. Groups that do not share the same letter are significantly different based on the one-way ANOVA test with the Duncan adjustment for multiple comparisons test ($p < 0.05$). CLF, control low-fat; CHF, control high-fat; 25PC, 25% egg-PC high-fat; 50PC, 50% egg-PC high-fat; 75PC, 75% egg-PC high-fat; 100PC, 100% egg-PC high-fat; IFN-γ, interferon gamma; IL, interleukin; MLN, mesenteric lymph nodes; PC, phosphatidylcholine; TNF-α, tumor necrosis factor-alpha.

### 4.2.9 PC:PE ratio in splenocytes

The concentration of PE and PE, as well as the PC:PE ratio in the cell membrane of splenocytes are presented in **Figure 4-9**. There was no significant difference between the CLF and CHF diets for PC or PE concentration, or the PC:PE ratio. Rats fed the 25PC and 75PC diets had increased PC concentrations compared to the CHF diet ($p < 0.05$). Rats fed the 75PC and 100PC diets had increased PE concentrations compared to the CHF diet ($p < 0.05$). Rats fed the 50PC, 75PC, and 100PC diets had a decreased PC:PE ratio compared to the CHF diet ($p < 0.05$).

**Figure 4-9: Effect of diet on PC and PE in splenocytes of male Wistar rats**




Values are means ± SEM. Groups that do not share a common letter are significantly different based on the one-way ANOVA with the Duncan adjustment for multiple comparison analysis. p-model < 0.001. CLF, control low-fat; CHF, control high-fat; 25PC, 25% egg-PC high-fat; 50PC, 50% egg-PC high-fat; 75PC, 75% egg-PC high-fat; 100PC, 100% egg-PC high-fat; PC, phosphatidylcholine, PE, phosphatidylethanolamine

### 4.3 Discussion

In this study, we aim to determine the dose effect of egg-PC supplementation on immune function in male Wistar rats fed a HFD. Overall, our data supports our hypothesis that feeding a HFD impairs immune responses and that supplementing a HFD with a dose reflecting a healthy omnivorous diet of 50% total choline in the form of egg-PC improves intestinal permeability and attenuates HFD-related immune cell dysfunction (Lewis, Subhan et al. 2014). However, the mechanisms underlying the beneficial effect of PC in lowering gut permeability remain to be elucidated as none of the genes we assessed were affected by the diets.

The model and diets have been previously published and discussed in (Azarcoya-Barrera, Wollin et al. 2022), and as per all our choline studies, were designed to be metabolically matched for the proportion of FA in both the low-fat and high-fat diets. This is important because several FAs, such as SFAs and n-3 FAs, are known to be immunomodulatory (Calder 1998). Despite diets being matched for overall FA composition, analysis of lipids in the liver indicated that consuming a CLF increased the proportion of SFA and lowered the proportion of total PUFA including EPA, DPA and DHA. This is due primarily to the higher carbohydrate content of the CLF, which leads to the synthesis of palmitic acids in the liver and the subsequent elongation into longer chains such as stearic acid (da Silva-Santi, Antunes et al. 2016). We use two controls, a CLF and CHF, to understand both the effect of a HFD on immune function (the model *per se*), and the extent to which PC intake can normalize or not relative to a control low-fat diet (LFD). We have been documenting this increased proportion of SFAs in the liver consistently across our pre-clinical models of obesity (She, Mangat et al. 2022, Azarcoya-Barrera et al. 2022) suggesting that LFDs such as chow are likely not the best representation of a healthy control diet, even

though universally recognized as a proper control in pre-clinical studies of obesity. These findings strongly suggest that the forms of choline matter for optimal immune function, and therefore current rodent diets which only contain FC may not represent the best "healthy" control. Further study should examine the importance of giving a mixture of choline forms in chow and similar control LFDs rather than just FC to further understand the role of choline forms on immune function.

A growing body of literature has shown a link between obesity and intestinal permeability (Cani, Possemiers et al. 2009, Damms-Machado et al. 2017, Fasano 2017, Teixeira, Collado et al. 2012). An increase in intestinal permeability can lead to an increased risk of disorders related to systemic inflammation, such as T2D and cardiovascular disease (Ding & Lund 2011, Vetrani, Di Nisio et al. 2022). We showed that feeding a HFD containing only FC significantly increased intestinal permeability when compared to a LFD containing only FC. Interestingly, adding PC to the diet reduces intestinal permeability in a dose wise manner. PC interacts with mucus in the intestinal lumen to form a protective mucosal layer preventing bacteria from attaching and penetrating the epithelium (Korytowski, Abuillan et al. 2017). This can have an indirect effect on immune function by lowering the amount of circulating endotoxin entering the system. Indeed, several studies have shown that dietary PC can ameliorate LPS induced systemic inflammation and subsequently lower neuroinflammation, cognitive impairments, and multiple organ injuries (Tokés, Eros et al. 2011, Jung, Nam et al. 2013, Tan, Zhang et al. 2020). Moreover, dietary PC has been shown to improve intestinal barrier defense against *Clostridium difficile* toxin A *in vitro* (Olson, Diebel et al. 2015). We can now confirm that dietary PC can also protect against obesity induced gut permeability.

We examined how dietary PC may be modulating intestinal barrier integrity by histologically analyzing crypt depth and villus height in the CLF, CHF, 50PC, and 100PC groups. We found that villi height increased in the 50PC group compared to the control diets, but there was no significant difference in villus:crypt ratio between diet groups. A greater villus length suggests a greater capacity for nutrient absorption, which may aid in overall metabolic function (Furuya & Furuya 2013). Further, we examined gene expression of several genes associated with intestinal barrier function by RT-qPCR. We found no difference in the expression levels of genes associated with goblet cell function, inflammation related pyroptosis, PC synthesis, or tight junction proteins. This suggests that the interaction between dietary PC and intestinal barrier function does not result in changes at the gene level. Further study is required to elucidate the mechanism by which PC exhibits a protective effect on the intestinal barrier.

We demonstrated that feeding a HFD containing only FC impairs T cell function, and all doses of PC were able to improve immune function to some extent, as measured by *ex vivo* cytokine production. T cell response was impaired in the CHF fed rats after stimulation with T cell mitogens by producing less IL-2, IL-10 and TNF-α. TNF-α is a key Th1 cytokine known to induce IL-2 production, and IL-10 is a key Th2 cytokine which can down regulate the Th1 response (Raphael, Nalawade et al. 2015). IL-2 is a pleiotropic cytokine that is involved in T cell proliferation of both Th1 and Th2 cell differentiation (Liao, Lin et al. 2011). Interestingly, there was no significant difference in the proportion of CD3+CD4+ T helper cells between CHF and CLF groups despite a reduction in cytokine production. Of all the PC diets, only the 50PC diet increased IL-2, IL-10, and TNF-α production to levels significantly greater than the CHF diet

after PMA+I stimulation. Similarly, replacing a portion of FC with PC in a HFD increased IL-2 production in MLN cells in the 25-75% PC dose range, which is partly explained by the observed increase in cells expressing CD25+ (IL-2 receptor) in the PC diets. We also observed an enhanced IL-10 production in APC from the MLN with the 50% dose of PC. IL-10 is secreted by Th2 and Treg cells involved in suppressing immune response (Lopez-Castejon, Brough et al. 2011), which suggests that consuming choline in the form of PC may improve to some extent the resolution phase of inflammation in the gut.

We also demonstrated that feeding a HFD containing only FC reduced cytokine production from APC. After stimulation with PWM, a T cell and APC mitogen, IL-1β, IL-10 and TNF-α were significantly lower in the CHF diet than the CLF diet group. IL-1β is a potent pro-inflammatory cytokine produced mostly by monocytes/macrophages and dendritic cells in response to recognition of pathogens through pattern recognition receptors (PRR) such as TLR4 (Lopez-Castejon, Brough et al. 2011, Garlanda & Jaillon 2016), suggesting that HFD feeding can also impair APC function. For all the examined cytokines, adding PC at least partly ameliorated the observed immune dysfunction of feeding a HFD. Similar patterns of cytokines production were also observed after LPS stimulation, a ligand for the TLR4 (Park & Lee 2013). Interestingly, neither the proportion nor the MFI of cells expressing the TLR4 was affected by diet, suggesting that the observed effect of the diets on IL-1β occurs independently of PRR activation. Along with the observed increase in proportions of B cells in the PC diets, this also suggests that PC may support better APC capacity to activate T cells which could also explain the improved T cell function discussed previously. We previously showed that feeding a HFD containing choline 100% in the form of PC did not affect APC cell function after stimulation

with both PWM and LPS when compared to a HFD containing only FC (Azarcoya-Barrera et al. 2022). Our results are consistent with this study in that the measured cytokine levels after both mitogen stimulation were not different between the CHF and 100PC groups, but the lower dose diets did increase cytokine production in several cytokines that were measured, thus further confirming that a diet containing mixed forms of choline is most beneficial for immune cell function.

Since the PC:PE ratio has been shown to be a relevant marker of metabolic health in hepatocytes, for the first time we examined both the PC and PE composition of splenocyte membranes as well as the PC:PE ratio. We had previously shown that feeding a diet containing 100% PC to lactating Sprague Dawley rats increased the PC content splenocytes membrane isolated from their offspring (Lewis, Richard et al. 2016). We further demonstrated *in vitro* that increasing the supply of PC in the form of lyso-PC to splenocytes increased T cell proliferation and IL-2 production (Lewis, Richard et al. 2016). In the current study, we confirmed that increasing PC intake increases both the PC and PE content in splenocytes while reducing the PC:PE ratio. It has been shown that perturbations in the PC:PE ratio causes cellular dysfunction including endoplasmic reticulum (ER) stress which has been implicated in inflammatory responses (Fu, Yang et al. 2011). Mitochondrial dysfunction, ER stress, and dyslipidemia have all been implicated in the development of insulin resistance and diabetes in obesity (Fu, Yang et al. 2011, Supale, Li et al. 2012, Goldberg 2001). Literature suggests that lipid disequilibrium brought on by HFDs increases the PC:PE ratio in hepatocytes and contributes to ER stress (van der Veen, Kennelly et al. 2017). Although not significant, we showed a trend toward an

increased PC:PE ratio in the CHF vs. the CLF diet. Further studies are required to elucidate the relationship between the PC:PE ratio in splenocytes and immune function.

Since both lipid- and water- soluble forms of choline are required for several functions in the body, it is possible that providing a diet that contains both forms allows for proper metabolic function more than solely providing PC or FC (Wallace, Blusztajn et al. 2018). As a lipid soluble form of choline, dietary PC is delivered directly to peripheral organs (adipose, muscle, lymph nodes) before reaching the liver (Lewis, Field et al. 2015) whereas FC goes directly to the liver. In the liver, both dietary and endogenously synthesized PC can then be utilized in the synthesis of lipoproteins (Lewis, Field et al. 2015). Since PC can be acquired from both dietary and *de novo* sources, it is possible that the 50PC diet provides the optimal proportion of dietary PC while also allowing for appropriate synthesis to occur. For example, PE can be methylated through the PEMT pathway to synthesize PC in the liver, and this process can help maintain PC in hepatocyte membranes when dietary choline is reduced (St. Germain, Iraji et al. 2023). This may explain why the 50PC dose provides a more beneficial effect than the 100PC diet.

Our study has many strengths including the design of the diets to ensure choline content and FA composition are closely matched, and the assessment of many factors that have been proposed to be involved in modulating immune function. However, some limitations should also be stressed. This study was carried out in male Wistar rats only; thus sex differences were not assessed although immunological differences do exist in this model (Tibaes, Azarcoya-Barrera et al. 2022). Further, while the CHF diet clearly induced immune dysfunction and increased intestinal permeability, we did not measure body composition (i.e. fat pad weight) or plasma

metabolites such as glucose or insulin to confirm that obesity-related metabolic dysfunction was present. PC and FC are only two of the five forms of choline that can be found in the diet and our data should be interpreted as such in that they do not reflect a human diet containing a mixture of choline forms. As noted above, our results, along with previously published results (Azarcoya-Barrera, Wollin et al. 2022), suggest that a CLF diet containing only FC may not reflect a true "healthy" diet. Considering that commercial rodent research diets contain only FC, future study should investigate the ideal proportion of FC and PC in a CLF. Moreover, the 25% and 100% PC doses are less relevant for human consumption as most cohort studies report consumption of PC at about 40-60% of total choline (Lewis, Subhan et al. 2014, Wiedeman, Barr et al. 2018). Secretory IgA plays a pivotal role in gut-associated immune function by providing protection against pathogens and contributing to oral tolerance, which was not measured in this study (Childs, Calder et al. 2019). Finally, we did not measure LPS in circulation which could have helped confirm that PC improved intestinal barrier function and subsequently reduced circulating bacterial content (Gordon, Parker et al. 2005). This would also provide confirmation of our data in an inflammatory model *in vivo*.

## 4.4 Conclusion

In summary, our results suggest that a HFD impairs both peripheral and gut-associated T cell and APC function by lowering cytokine response upon stimulation. We showed that adding PC in any doses improved cytokine production. Potential mechanisms were identified that may explain the beneficial effect of PC, such as an increase in IL-2 receptor, a higher proportion of B cells, and a decrease in intestinal permeability. Despite seeing some beneficial effect in all doses of PC, the 50% dose is the most relevant for human consumption while also providing the most consistent beneficial effect on immune function.

## 4.5 References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## 4.6 Tables

**Table 4-1:** Anthropometric and plasma cytokine data of male Wistar rats fed the six experimental diets[1].

| Variables | CLF diet | CHF diet | 25PC diet | 50PC diet | 75PC diet | 100PC diet | *p* model |
|---|---|---|---|---|---|---|---|
| Body weight (g) | 666 ± 20.5 | 688 ± 16.6 | 633 ± 20.8 | 674 ± 23.6 | 673 ± 36.2 | 697 ± 16.5 | 0.553 |
| Spleen weight (g) | 1.59 ± 0.09 | 1.53 ± 0.06 | 1.58 ± 0.06 | 1.58 ± 0.12 | 1.61 ± 0.08 | 1.53 ± 0.03 | 0.986 |
| Splenocytes, n x $10^6$/100 g tissue[2] | 19.8 ± 1.37 | 19.9 ± 1.70 | 16.5 ± 1.86 | 16.3 ± 1.54 | 16.4 ± 1.14 | 18.8 ± 1.19 | 0.098 |
| Liver weight (g) | 27.5 ± 1.28 | 27.4 ± 1.65 | 25.9 ± 1.14 | 26.5 ± 1.68 | 27.1 ± 1.7 | 26.9 ± 1.05 | 0.898 |
| Intestine length (cm) | 1245 ± 2.15 | 127 ± 2.52 | 127 ± 2.20 | 124 ± 2.60 | 125 ± 2.80 | 126 ± 2.00 | 0.560 |
| Plasma cytokines[3] | | | | | | | |
| IL-13 (pg/mL)[4] | 0.51 ± 0.03 | 0.96 ± 0.18 | 1.79 ± 1.17 | 0.95 ± 0.53 | 0.53 ± 0.02 | 0.572 ± 0.06 | 0.403 |
| IL-6 (pg/mL)[4] | 4.95 ± 2.71 | 11.4 ± 8.2 | 5.71 ± 1.03 | 9.70 ± 0.85 | 9.61 ± 2.85 | 5.99 ± 0.93 | 0.792 |
| TNF-α (pg/mL) | 1.78 ± 0.14 | 2.21 ± 0.11 | 2.06 ± 0.18 | 2.26 ± 0.20 | 2.32 ± 0.14 | 2.17 ± 0.14 | 0.174 |

[1]Values are means ± SEM, n=10. 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; 25PC, 25% egg-PC high-fat; 50PC, 50% egg-PC high-fat; 75PC, 75% egg-PC high-fat; 100PC, 100% egg-PC high-fat; IFN-γ, interferon gamma; IL, interleukin; PCHF, PC high-fat; PC, phosphatidylcholine; TNF-α, tumor necrosis factor-alpha.
[2]Analysis performed on log-transformed values.
[3]The following plasma cytokines were tested, but resulted in non-detectable levels in the samples: IFN-γ, IL-1β, IL-4, IL-5, IL-10
[4]Analysis performed using non-parametric testing

**Table 4-2: Fatty acid composition of experimental diets[1]**

| Fatty Acid | CLF diet | CHF diet | 25PC diet | 50PC diet | 75PC diet | 100PCdiet |
|---|---|---|---|---|---|---|
| (% of total fatty acids) | | | | | | |
| **14:0** | 0.72 ± 0.06 | 0.72 ± 0.03 | 0.66 ± 0.01 | 0.65 ± 0.0 | 0.61 ± 0.01 | 0.63 ± 0.02 |
| **16:0** | 18.0 ± 0.74 | 18.1 ± 0.58 | 17.9 ± 0.15 | 17.8 ± 0.12 | 17.6 ± 0.41 | 18.0 ± 0.12 |
| **16:1n9** | 0.15 ± 0.02 | 0.18 ± 0.03 | 0.15 ± 0.01 | 0.14 ± 0.01 | 0.14 ± 0.02 | 0.18 ± 0.02 |
| **16:1n7** | 0.97 ± 0.09 | 0.96 ± 0.07 | 0.93 ± 0.01 | 0.92 ± 0.01 | 0.88 ± 0.04 | 0.89 ± 0.01 |
| **18:0** | 10.1 ± 1.00 | 10.0 ± 0.59 | 9.87 ± 0.10 | 9.82 ± 0.09 | 9.52 ± 0.34 | 9.77 ± 0.16 |
| **18:1n9** | 32.0 ± 0.23 | 31.8 ± 0.52 | 31.6 ± 0.36 | 31.4 ± 0.01 | 31.4 ± 0.29 | 31.3 ± 0.39 |
| **18:1t11** | 1.00 ± 0.29 | 1.22 ± 0.18 | 1.07 ± 0.35 | 1.26 ± 0.04 | 1.12 ± 0.38 | 1.17 ± 0.26 |
| **C18:2n-6** | 30.9 ± 0.33 | 29.7 ± 1.25 | 30.5 ± 0.27 | 30.6 ± 0.25 | 31.4 ± 0.29 | 30.5 ± 0.07 |
| **20:0** | 0.31 ± 0.02 | 0.31 ± 0.0 | 0.31 ± 0.01 | 0.31 ± 0.0 | 0.31 ± 0.01 | 0.30 ± 0.0 |
| **18:3n-3 (ALA)** | 7.00 ± 0.56 | 6.35 ± 0.35 | 6.38 ± 0.02 | 6.50 ± 0.01 | 6.40 ± 0.14 | 6.54 ± 0.03 |
| **20:2n-6** | 0.31 ± 0.02 | 0.30 ± 0.02 | 0.28 ± 0.0 | 0.28 ± 0.0 | 0.26 ± 0.01 | 0.27 ± 0.0 |
| **20:3n-6** | 0.07 ± 0.01 | 0.07 ± 0.01 | 0.07 ± 0.0 | 0.07 ± 0.0 | 0.08 ± 0.0 | 0.08 ± 0.0 |
| **20:4n-6** | 0.80 ± 0.01 | 0.07 ± 0.0 | 0.14 ± 0.06 | 0.14 ± 0.04 | 0.18 ± 0.04 | 0.26 ± 0.0 |
| **20:5n-6** | 0.07 ± 0.01 | 0.07 ± 0.01 | 0.07 ± 0.0 | 0.07 ± 0.0 | 0.07 ± 0.0 | 0.07 ± 0.0 |
| **22:6n-3 (DHA)** | 0.01 ± 0.01 | 0.09 ± 0.10 | 0.03 ± 0.02 | 0.03 ± 0.02 | 0.03 ± 0.0 | 0.08 ± 0.02 |
| **Total SFA** | 29.2 ± 1.76 | 29.2 ± 1.18 | 28.8 ± 0.24 | 28.6 ± 0.21 | 28.1 ± 0.75 | 28.7 ± 0.26 |
| **Total MUFA** | 34.1 ± 0.61 | 34.2 ± 0.48 | 33.7 ± 0.02 | 33.7 ± 0.04 | 33.5 ± 0.11 | 33.5 ± 0.14 |
| **Total PUFA** | 38.4 ± 0.44 | 36.6 ± 1.66 | 37.5 ± 0.21 | 37.7 ± 0.17 | 38.4 ± 0.87 | 37.8 ± 0.12 |
| **n-6:n-3** | 4.44 ± 0.02 | 4.62 ± 0.12 | 4.79 ± 0.03 | 4.71 ± 0.06 | 4.90 ± 0.03 | 4.65 ± 0.02 |
| **SFA:PUFA** | 0.86 ± 0.04 | 0.85 ± 0.02 | 0.85 ± 0.01 | 0.85 ± 0.01 | 0.84 ± 0.02 | 0.86 ± 0.01 |

[1]Analysis of the fatty acid composition of the six experimental diets collected weekly (n=2 batches for experimental diets; n=3 for control diets).

**Table 4-3: Fatty acid composition of liver tissue.[1]**

| Fatty acid (FA) | CLF diet | CHF diet | 25PC diet | 50PC diet | 75PC diet | 100PCdiet | pValue |
|---|---|---|---|---|---|---|---|
| | | | % of total FAs | | | | |
| 14:0 | 0.64 ± 0.03$^{a}$ | 0.41 ± 0.02$^{b,c}$ | 0.36 ± 0.02$^{d}$ | 0.45 ± 0.02$^{b}$ | 0.37 ± 0.01$^{c,d}$ | 0.33 ± 0.01$^{d}$ | <0.001 |
| 14:1 | 0.17 ± 0.02$^{a,b}$ | 0.14 ± 0.01$^{b}$ | 0.15 ± 0.01$^{b}$ | 0.16 ± 0.01$^{b}$ | 0.15 ± 0.02$^{b}$ | 0.15 ± 0.01$^{b}$ | 0.020 |
| 16:0 | 23.8± 0.44$^{a}$ | 21.2 ± 0.38$^{b}$ | 20.3 ± 0.43$^{b,c}$ | 20.3 ± 0.26$^{b,c}$ | 20.6 ± 0.35$^{b,c}$ | 19.8 ± 0.29$^{c}$ | <0.001 |
| 16:1n9 | 0.37 ± 0.03$^{a}$ | 0.36 ± 0.05$^{a}$ | 0.30 ± 0.04$^{a,b}$ | 0.26 ± 0.04$^{a,b}$ | 0.27 ± 0.04$^{a,b}$ | 0.32 ± 0.03$^{a}$ | 0.017 |
| 16:1n7 | 2.57 ± 0.19$^{a}$ | 0.98 ± 0.07$^{b}$ | 0.29 ± 0.10$^{b}$ | 0.98 ± 0.07$^{b}$ | 0.86 ± 0.08$^{b}$ | 0.57 ± 0.06$^{c}$ | <0.001 |
| 17:0 | 0.20 ± 0.01$^{a,b}$ | 0.18 ± 0.01$^{b,c}$ | 0.16 ± 0.02$^{b,c}$ | 0.14± 0.02$^{c}$ | 0.14 ± 0.01$^{c}$ | 0.18 ± 0.01$^{b,c}$ | 0.001 |
| 18:0 | 15.2 ± 0.71$^{a}$ | 12.5 ± 1.18$^{b}$ | 13.7 ± 0.77$^{a,b}$ | 11.6 ± 0.63$^{b}$ | 12.5 ± 0.84$^{b}$ | 15.2 ± 0.74$^{a}$ | 0.003 |
| 18:1n9 | 17.8 ± 0.95$^{b,c}$ | 21.6 ± 1.10$^{a}$ | 19.8 ± 1.13$^{a,b}$ | 21.7 ± 0.94$^{a}$ | 20.5 ± 1.10$^{a,b}$ | 15.9 ± 0.85$^{c,d}$ | <0.001 |
| 18:1n7 | 2.39 ± 0.26$^{a}$ | 1.06 ± 0.07$^{b}$ | 0.97 ± 0.04$^{b}$ | 0.98 ± 0.10$^{b}$ | 1.04 ± 0.08$^{b}$ | 1.02 ± 0.06$^{b}$ | <0.001 |
| 18:2n6 (LA) | 13.8 ± 1.90$^{c}$ | 21.8 ± 0.85$^{b}$ | 21.7 ± 0.59$^{b}$ | 23.6 ± 0.86$^{a,b}$ | 23.2 ± 0.86$^{a,b}$ | 23.0 ± 0.57$^{a,b}$ | <0.001 |
| 18:3n3 (ALA) | 0.84 ± 0.08$^{c}$ | 1.66 ± 0.13$^{a,b}$ | 1.53 ± 0.10$^{b}$ | 2.01 ± 0.19$^{a}$ | 1.88 ± 0.07$^{a,b}$ | 1.88 ± 0.11$^{a,b}$ | <0.001 |
| 20:0 | 0.18 ± 0.03$^{c}$ | 0.26 ± 0.04$^{b,c}$ | 0.34 ± 0.02$^{a,b}$ | 0.37 ± 0.04$^{a}$ | 0.32 ± 0.02$^{a,b}$ | 0.31 ± 0.03$^{a,b}$ | 0.001 |
| 20:2n6 | 0.27 ± 0.05$^{b}$ | 0.40 ± 0.02$^{a}$ | 0.38 ± 0.03$^{a}$ | 0.42 ± 0.02$^{a}$ | 0.42 ± 0.01$^{a}$ | 0.38 ± 0.03$^{a}$ | 0.006 |
| 20:3n6 | 0.67 ± 0.08 | 0.58 ± 0.02 | 0.58 ± 0.04 | 0.62 ± 0.05 | 0.59 ± 0.03 | 0.57 ± 0.04 | 0.275 |
| 20:4n6 (ARA) | 13.0 ± 1.48 | 11.5 ± 0.83 | 11.8 ± 1.44 | 10.97 ± 0.75 | 11.7 ± 0.81 | 13.0 ± 1.49 | 0.988 |
| 20:5n3 (EPA) | 0.32 ± 0.03$^{a}$ | 0.43 ± 0.03$^{b}$ | 0.43 ± 0.02$^{b}$ | 0.47 ± 0.03$^{b}$ | 0.46 ± 0.03$^{b}$ | 0.47 ± 0.03$^{b}$ | 0.050 |
| 24:1n9 | 0.07 ± 0.02$^{a}$ | 0.04 ± 0.01$^{b}$ | 0.01 ± 0.0$^{b}$ | 0.01 ± 0.0$^{b}$ | 0.02 ± 0.0$^{b}$ | 0.03 ± 0.01$^{b}$ | 0.001 |
| 22:4n6 | 0.30 ± 0.02$^{b}$ | 0.47 ± 0.06$^{a}$ | 0.45 ± 0.04$^{a}$ | 0.45 ± 0.04$^{a}$ | 0.47 ± 0.04$^{a}$ | 0.48 ± 0.04$^{a}$ | 0.018 |
| 22:5n6 | 0.10 ± 0.01$^{a,b}$ | 0.09 ± 0.02$^{a,b}$ | 0.09 ± 0.01$^{a,b}$ | 0.11 ± 0.02$^{a,b}$ | 0.11 ± 0.01$^{a,b}$ | 0.13 ± 0.02$^{a}$ | 0.158 |
| 22:5n3 (DPA) | 0.63 ± 0.07$^{c}$ | 0.88 ± 0.08$^{b}$ | 0.87 ± 0.07$^{b}$ | 0.94 ± 0.07$^{b}$ | 0.92 ± 0.06$^{b}$ | 0.98 ± 0.05$^{b}$ | <0.001 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 22:6n3 (DHA) | 4.61 ± 0.25[a] | 3.52 ± 0.24[b] | 3.58 ± 0.17[b] | 3.48 ± 0.21[b] | 3.56 ± 0.29[b] | 3.89 ± 0.18[b] | 0.001 |
| Total SFA | 40.3 ± 0.88[a] | 34.5 ± 1.02[c,d] | 34.9 ± 0.48[b,c] | 32.9 ± 0.61[d] | 33.9 ± 0.61[c,d] | 35.9 ± 0.58[b,c] | <0.001 |
| Total MUFA | 23.6 ± 1.20[a] | 24.2 ± 1.13[a] | 22.7 ± 1.01[a] | 24.1 ± 0.96[a] | 22.8 ± 1.10[a] | 18.4 ± 0.86[b] | <0.001 |
| Total PUFA | 36.1 ± 1.10[c] | 41.3 ± 0.72[b] | 42.4 ± 0.80[b] | 43.1 ± 0.71[b] | 43.2 ± 0.68[b] | 45.7 ± 0.47[a] | <0.001 |
| Total n6 | 30.4 ± 0.78[d] | 34.8 ± 0.49[c] | 35.1 ± 1.09[c] | 36.2 ± 0.55[b,c] | 36.4 ± 0.44[b,c] | 37.5 ± 1.10[a,b] | <0.001 |
| Total n3 | 6.43 ± 0.38[b] | 6.50 ± 0.25[a,b] | 6.44 ± 0.26[b] | 6.90 ± 0.22[a,b] | 6.81 ± 0.29[a,b] | 7.30 ± 0.19[a] | 0.235 |
| n-6:n-3 | 4.69 ± 0.18[c] | 5.40 ± 0.15[a,b] | 5.63 ± 0.13[a,b] | 5.40 ± 0.18[b] | 5.41 ± 0.18[a,b] | 5.31 ± 0.12[b] | 0.001 |
| SFA:PUFA | 1.13 ± 0.05[a] | 0.84 ± 0.03[b] | 0.83 ± 0.02[b] | 0.76 ± 0.02[b] | 0.79 ± 0.01[b] | 0.79 ± 0.01[b] | <0.001 |

[1]Analysis of the FA composition of the total lipids extracted from liver tissue (n=10 per group); ALA, α-linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; FA, fatty acid; EPA, eicosapentaenoic acid; LA, linoleic acid; MUFA, monounsaturated FAs; n, omega; PUFA, polyunsaturated FAs; SFA, saturated FAs; CLF, control low-fat; CHF, control high-fat; 25PC, 25% egg-PC high-fat; 50PC, 50% egg-PC high-fat; 75PC, 75% egg-PC high-fat; 100PC, 100% egg-PC high-fat; PC, phosphatidylcholine.

**Table 4-4: *Ex vivo* cytokine production by mitogen-stimulated splenocytes and MLN cells of male Wistar rats fed the six experimental diets.[1]**

| Cytokine (pg/ml)[1] | CLF diet | CHF diet | 25PC diet | 50PC diet | 75PC diet | 100PC diet | *p* Value |
|---|---|---|---|---|---|---|---|
| **Splenocytes** | | | | | | | |
| PMA+I (T cell mitogen) | | | | | | | |
| IL-2 | 1289 ± 304$^{b}$ | 490 ± 96.1$^{b}$ | 1198 ± 270$^{b}$ | 2115 ± 220$^{a}$ | 1010 ± 300$^{b}$ | 1024 ± 340$^{b}$ | 0.014 |
| IL-10[2] | 235 ± 40.9$^{a}$ | 122 ± 15.8$^{b}$ | 150 ± 14.7$^{a,b}$ | 220 ± 31.4$^{a}$ | 120 ± 19.3$^{b}$ | 183 ± 33.9$^{a,b}$ | 0.001 |
| TNF-α | 233 ± 58.8$^{a,b,c}$ | 107 ± 22.0$^{b}$ | 273 ± 75.4$^{a,b}$ | 340 ± 46.5$^{a}$ | 218 ± 32.8$^{a,b,c}$ | 186 ± 38.0$^{b,c}$ | 0.037 |
| PWM (T and APC mitogen) | | | | | | | |
| IL-1β[2] | 96.9 ± 9.52$^{a}$ | 51.7 ± 2.60$^{c}$ | 83.9 ± 8.20$^{a,b}$ | 80.9 ± 7.65$^{a,b}$ | 80.3 ± 7.70$^{a,b}$ | 69.1 ±8.96$^{b,c}$ | 0.004 |
| IL-2[2] | 323 ± 49.0$^{a}$ | 152 ± 23.1$^{b,c}$ | 204 ± 26.1$^{b,c}$ | 246 ± 35.5$^{a,b,c}$ | 148 ± 17.8$^{d}$ | 200 ± 24.1$^{b,c,d}$ | 0.004 |
| IL-10[2] | 323 ± 54.9$^{a}$ | 134 ± 13.0$^{c}$ | 165 ± 23.5$^{b,c}$ | 313 ± 33.9$^{a}$ | 275 ± 51.2$^{a,b}$ | 257 ± 38.6$^{a,b}$ | 0.008 |
| TNF-α[2] | 160 ± 14.2$^{a,b}$ | 89.4 ± 7.30$^{b}$ | 166 ± 12.3$^{a}$ | 164 ± 21.7$^{a}$ | 177 ± 16.2$^{a}$ | 133 ± 20.3$^{a,b}$ | 0.001 |
| IL-6 | 594 ± 49.1 | 463 ± 60.7 | 528 ± 35.9 | 657 ± 68.8 | 530 ± 56.5 | 588 ± 43.8 | 0.258 |
| LPS (Bacterial challenge) | | | | | | | |
| IL-10 | 551 ± 33.6$^{a}$ | 394 ± 23.3$^{b}$ | 355 ± 18.0$^{b}$ | 456 ± 52.6$^{a,b}$ | 384 ± 28.5$^{b}$ | 392 ± 39.2$^{b}$ | 0.010 |
| TNF-α | 275 ± 38.2$^{a}$ | 204 ± 21.6$^{b,c}$ | 241 ± 19.3$^{a,b}$ | 230 ± 22.4$^{a,b}$ | 248 ± 19.7$^{a,b}$ | 157 ± 13.6$^{c}$ | 0.007 |
| IL-6 | 486 ± 61.9 | 381 ± 42.7 | 347 ± 45.0 | 443 ± 60.5 | 506 ± 61.2 | 402 ± 45.0 | 0.266 |
| **MLN cells** | | | | | | | |
| PMA+I (T cell mitogen) | | | | | | | |
| IL-2 | 5613 ± 786$^{a}$ | 1808 ± 245$^{c}$ | 3686 ± 478$^{b}$ | 3994 ± 534$^{a,b}$ | 4136 ± 537$^{a,b}$ | 3067 ± 779$^{b,c}$ | 0.002 |
| IL-10 | 125.8 ± 48.8 | 103.2 ± 30.2 | 172 ± 51.3 | 241.2 ± 57.5 | 120.2 ± 21.8 | 93.7 ± 14.6 | 0.118 |
| TNF-α | 118.9 ± 30.7 | 118.9 ± 35.8 | 168.6 ± 39.9 | 227.3 ± 37.4 | 174.8 ± 21.7 | 125.2 ± 30.0 | 0.611 |
| LPS (Bacterial challenge) | | | | | | | |
| IL-10 | 135.8 ± 23.4$^{a,b}$ | 97.7 ± 19.2$^{b}$ | 148.3 ± 31.3$^{a,b}$ | 180.6 ± 15.1$^{a}$ | 158.7 ± 21.8$^{a,b}$ | 119.2 ± 12.4$^{a,b}$ | 0.097 |
| TNF-α | 39.4 ± 7.02 | 38.7 ± 2.76 | 31.3 ± 7.61 | 37.7 ± 13.1 | 35.6 ± 5.47 | 67.5 ± 15.0 | 0.187 |

[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; 25PC, 25% egg-PC high-fat; 50PC, 50% egg-PC high-fat; 75PC, 75% egg-PC high-fat; 100PC, 100% egg-PC high-fat; PC, phosphatidylcholine; IFN-γ, interferon gamma; IL, interleukin; TNF-α, tumor necrosis factor-alpha.

[2]Analysis performed on log-transformed values.

**Table 4-5:** Splenocyte phenotype of male Wistar rats fed the six experimental diets.[1]

| Phenotype | CLF diet | CHF diet | 25PC diet | 50PC diets | 75PC diet | 100PC diet | *p* Value |
|---|---|---|---|---|---|---|---|
| | | | % of total gated cells | | | | |
| Total CD3+ | 32.7 ± 1.72 | 32.8 ± 0.66 | 30.7 ± 1.30 | 28.3 ± 1.37 | 28.6 ± 1.28 | 30.2 ± 1.41 | 0.078 |
| | | | % of CD3+ gated cells | | | | |
| CD4+ (T helper cells) | 66.6 ± 1.25 | 66.8 ± 1.46 | 66.2 ± 1.39 | 63.0 ± 1.88 | 69.1 ± 1.66 | 68.1 ± 2.03 | 0.202 |
| CD4- (T cytotoxic cells) | 33.3 ± 1.26 | 33.1 ± 1.46 | 33.7 ± 1.39 | 36.8 ± 1.87 | 30.9 ± 1.65 | 31.8 ± 2.03 | 0.209 |
| | | | % of CD3+CD4+ gated cells | | | | |
| FoxP3+ (T regulatory cells) | 5.46 ± 1.33 | 5.53 ± 1.14 | 6.98 ± 0.98 | 5.47 ± 1.21 | 6.04 ± 1.17 | 3.89 ± 0.87 | 0.541 |
| CD25+ | 8.33 ± 0.54 | 7.47 ± 0.25 | 7.50 ± 0.72 | 9.41 ± 0.57 | 8.05 ± 0.59 | 7.95 ± 0.68 | 0.224 |
| | | | %of CD3+CD4- gated cells | | | | |
| CD25+ | 3.05 ± 0.23 | 3.08 ± 0.25 | 3.36 ± 0.49 | 3.67 ± 0.41 | 4.10 ± 0.42 | 3.73 ± 0.30 | 0.216 |
| | | | % of total gated cells | | | | |
| Total CD45RA+ (B cells) | 16.7 ± 0.72$^{b,c}$ | 15.5 ± 0.87$^{c}$ | 20.5 ± 1.36$^{a}$ | 19.4 ± 1.01$^{a,b}$ | 18.5 ± 1.21$^{a,b,c}$ | 20.2 ± 1.07$^{a}$ | 0.006 |
| Total CD68+ (macrophages) | 7.21 ± 0.32 | 7.13 ± 0.29 | 6.66 ± 0.38 | 7.65 ± 0.38 | 6.94 ± 0.55 | 7.15 ± 0.51 | 0.688 |
| CD68+CD11bc+ | 1.52 ± 0.20 | 1.34 ± 0.21 | 1.72 ± 0.14 | 1.87 ± 0.16 | 1.49 ± 0.21 | 1.58 ± 0.20 | 0.457 |
| CD68+TLR4+ | 3.67 ± 0.17 | 3.56 ± 0.27 | 2.96 ± 0.31 | 3.63 ± 0.29 | 2.97 ± 0.33 | 3.23 ± 0.28 | 0.239 |
| Total OX6+ (MHC class II+) | 58.1 ± 1.69 | 58.7 ± 1.37 | 57.9 ± 1.71 | 58.2 ± 1.01 | 58.2 ± 1.97 | 59.5 ± 1.71 | 0.986 |
| CD3+CD161+ (NK cells) | 3.04 ± 0.28 | 2.92 ± 0.43 | 3.88 ± 0.47 | 3.99 ± 0.33 | 3.49 ± 0.29 | 2.80 ± 0.31 | 0.099 |
| Total TLR4+ | 4.87 ± 0.31 | 4.85 ± 0.49 | 4.20 ± 0.50 | 5.44 ± 0.45 | 4.14 ± 0.52 | 4.31 ± 0.45 | 0.343 |
| MFI of CD25+ in: | | | | | | | |
| CD3+ | 846 ± 44.0 | 766 ± 22.2 | 700 ± 41.7 | 795 ± 61.7 | 719 ± 29.5 | 747 ± 49.0 | 0.199 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| CD3+CD4+ | 1193 ± 75.5[a] | 987 ± 44.2[b,c] | 892 ± 52.5[b,c] | 1132 ± 86.4[a,b] | 980 ± 64.6[b,c] | 962 ± 63.9[b,c] | 0.015 |
| CD3+CD4- | 477 ± 14.9 | 464 ± 12.5 | 445 ± 11.9 | 445 ± 12.3 | 464 ± 19.9 | 484 ± 21.8 | 0.426 |
| MFI TLR4+ | 951 ± 15.0 | 974 ± 17.3 | 917 ± 17.8 | 938 ± 18.3 | 908 ± 11.1 | 936 ± 22.5 | 0.115 |
| MFI CD68+TLR4+ | 1055 ± 30.1 | 1055 ± 40.1 | 1009 ± 32.7 | 1041 ± 26.4 | 1014 ± 30.8 | 1060 ± 39.9 | 0.825 |

[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 ($p < 0.05$). No significant differences observed in the following: CD45RA+CD80+ (1.23 ± 0.13), OX6+CD80+ (1.55 ± 0.15), Total CD25+ (6.70 ± 0.39), Total CD80+ (2.62 ± 0.31), % of CD27+ in CD3+CD4+ (56.3 ± 1.84) and CD3+CD4- (74.1 ± 2.17); CLF, control low CLF, control low-fat; CHF, control high-fat; 25PC, 25% egg-PC high-fat; 50PC, 50% egg-PC high-fat; 75PC, 75% egg-PC high-fat; 100PC, 100% egg-PC high-fat; MFI, median fluorescence intensity; PC, phosphatidylcholine.

**Table 4-6:** MLN cell phenotype of male Wistar rats fed the six experimental diets.[1]

| Phenotype | CLF diet | CHF diet | 25PC diet | 50PC diet | 75PC diet | 100PC diet | *p* Value |
|---|---|---|---|---|---|---|---|
| | | | % of total gated cells | | | | |
| Total CD25+ | 1.58 ± 0.17$^{a,b}$ | 1.14 ± 0.17$^{b}$ | 1.84 ± 0.14$^{a}$ | 2.00 ± 0.19$^{a}$ | 1.76 ± 0.17$^{a}$ | 1.72 ± 0.15$^{a}$ | 0.023 |
| Total CD3+ | 44.6 ± 2.43 | 48.2 ± 2.12 | 46.7 ± 2.03 | 42.3 ± 1.56 | 45.6 ± 1.82 | 44.5 ± 2.25 | 0.453 |
| | | | % of CD3+ gated cells | | | | |
| CD4+ (helper T cells) | 35.4 ± 2.00 | 37.9 ± 2.04 | 36.5 ± 2.07 | 33.0 ± 1.31 | 36.2 ± 1.98 | 37.1 ± 1.85 | 0.542 |
| CD4- (cytotoxic T cells) | 8.38 ± 0.67$^{a,b,c}$ | 7.57 ± 0.37$^{b,c}$ | 8.85 ± 0.68$^{a,b,c}$ | 10.2 ± 0.62$^{a}$ | 9.33 ± 0.73$^{a,b}$ | 7.39 ± 0.38$^{c}$ | 0.022 |
| CD25+ | 1.71 ± 0.15 | 1.63 ± 0.25 | 1.93 ± 0.22 | 2.09 ± 0.21 | 2.52 ± 0.21 | 2.02 ± 0.43 | 0.236 |
| | | | % of CD3+CD4+ gated cells | | | | |
| FoxP3+ (regulatory T cells) | 5.68 ± 0.86 | 5.65 ± 0.73 | 7.37 ± 0.95 | 7.04 ± 0.67 | 6.78 ± 0.70 | 5.00 ± 0.49 | 0.188 |
| MFI CD25+ | | | | | | | |
| CD3+CD4+ | 548.3 ± 28.3 | 517.1 ± 20.7 | 498.6 ± 13.2 | 499.1 ± 15.1 | 504.3 ± 14.0 | 527.3 ± 17.8 | 0.403 |
| CD3+CD4- | 532.4 ± 43.4 | 559.5 ± 26.3 | 496.1 ± 21.3 | 564.8 ± 47.7 | 510.2 ± 20.6 | 549.7 ± 28.3 | 0.564 |
| | | | % of total gated cells | | | | |
| Total CD45RA+ (B cells) | 26.4 ± 0.76 | 28.3 ± 1.48 | 26.4 ± 1.88 | 30.0 ± 2.09 | 24.3 ± 1.14 | 28.2 ± 1.38 | 0.144 |
| Total CD68+ (macrophages) | 1.09 ± 0.10 | 0.98 ± 0.10 | 1.07 ± 0.10 | 1.15 ± 0.04 | 1.04 ± 0.05 | 1.02 ± 0.05 | 0.748 |
| Total OX6+ (MHC class II+) | 53.3 ± 2.20 | 52.6 ± 2.07 | 52.3 ± 1.84 | 55.8 ± 2.18 | 52.2 ± 2.06 | 52.3 ± 2.12 | 0.814 |
| CD161+CD3-(NK cells) | 1.72 ± 0.14$^{a}$ | 1.35 ± 0.06$^{c}$ | 1.68 ± 0.09$^{a,b}$ | 1.75 ± 0.16$^{a}$ | 1.39 ± 0.08$^{b,c}$ | 1.54 ± 0.07$^{a,b,c}$ | 0.030 |

[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 ($p < 0.05$). CLF, control low CLF, control low-fat; CHF,

control high-fat; 25PC, 25% egg-PC high-fat; 50PC, 50% egg-PC high-fat; 75PC, 75% egg-PC high-fat; 100PC, 100% egg-PC high-fat; MFI, median fluorescence intensity; PC, phosphatidylcholine.

# CHAPTER 5: Comparing the effect of plant- vs. animal-derived dietary sources of phosphatidylcholine on immune function in the context of a high-fat diet in male Wistar rats

Content from this chapter was presented at the Canadian Nutrition Society Annual Meeting in Gatineau, QC (2022) and the American Society of Nutrition Annual Meeting in Boston, MA (2023).

## 5.1 Introduction

High-fat diet (HFD) consumption leading to obesity has been associated with lower immune response, despite higher tissue inflammation (She, Mangat et al. 2022). It has been shown that HFD and obesity lead to an increase in number and size of adipocytes resulting in a spillover of fatty acids (FA) into the bloodstream leading to lipid deposition into other tissues such as liver and muscle (Ertunc & Hotamisligil 2016). This process impedes metabolic function, which can promote local inflammation and attract immune cells, such as macrophages, in trying to restore tissue homeostasis (McNelis & Olefsky 2014, Ip, Hogan et al. 2015). Further, HFDs have been associated with changes in gut microbiota composition, which can degrade the mucosal barrier and lead to increased intestinal permeability (Bischoff, Barbara et al. 2014). Bacterial content, primarily endotoxin known as LPS, can then translocate to the bloodstream from the gut (Ghosh, Wang et al. 2020). In circulation, endotoxin binds to toll-like receptor 4 (TLR-4) present on immune cells to increase the production of several pro-inflammatory cytokines (tumor necrosis factor-alpha [TNF-α], interleukin [IL]-6, IL-1β, interferon gamma [IFN-γ]) (Horioka, Tanaka et al. 2020). Altogether, this leads to higher levels of systemic inflammation, which can increase risk of poorer outcomes in metabolic and infectious disease.

Phosphatidylcholine (PC), a lipid-soluble form of the essential nutrient choline, has shown promise as an immune protective nutrient under the context of obesity. PC is an integral phospholipid in mammalian cell membranes, and thus can directly support immune cell function by contributing to immune cell structure and proliferation (van der Veen, Kennelly et al. 2017). Further, PC is an important constituent of the mucus barrier (Korytowski, Abuillan et al. 2017). PC has been shown to inhibit TNF-α induced activation of the inflammatory pathway NF-κB in plasma membranes of enterocytes (Treede, Braun et al. 2007). We have also shown that providing a diet containing choline 100% in the form of PC attenuates obesity induced T cell dysfunction, specifically by improving IL-2 and TNF-α production after T cell stimulation (Azarcoya-Barrera, Wollin et al. 2022).

PC is found in the diet in several foods. Egg yolk is by far the richest source of dietary PC, but milk and beef are also widely consumed sources of PC (Smolders, Wit et al. 2019, Lewis, Subhan et al. 2014). In plant sources, soybeans also provide a source of PC (Wiedman, Barr et al. 2018). The general structure of phosphatidylcholine is a glycerol moiety with two FAs linked by ester bonds in the sn-1 and sn-2 position and the third carbon of glycerol esterified to phosphorylcholine (Kanno, Wu et al. 2007). The FAs in the sn-1 and sn-2 position can differ between PC molecules from different sources (Sugasini, Yalagala et al. 2019). We have shown that feeding choline in a maternal diet as 100% egg-PC as opposed to free choline (FC, a water-soluble form of choline that is the standard form of choline in rodent diets) in Sprague-Dawley rats enhanced T cell function in lactating dams (Dellschaft, Richard et al. 2018, Lewis, Richard et al. 2017) and their offspring (Lewis, Richard et al. 2016) as assessed by increased IL-2

production upon stimulation. We have also shown that feeding a mixture of choline forms in the maternal diet composed of 50% soy-PC compared to 100% FC had no effect on IL-2 production but did lead to an overall more efficient immune response (Richard, Lewis et al. 2017). Altogether, this suggests that there may be a differential effect on immune function between egg- and soy-derived PC.

The comparison between both control diets and the 100PC diet, which will be labeled as the EPC diet for this chapter, was explored in Chapter 4. Therefore, the objective of this study was to focus on the effect of feeding PC derived from different dietary forms (egg- and soy-PC) on immune function in the context of a HFD. We hypothesized that egg-PC will improve immune cell function to a greater extent than soy-PC.

## 5.2 Results

### 5.2.1 Anthropometric characteristics and plasma cytokines

No significant difference in body weight between the diet groups were observed ($p > 0.05$, **Table 5-1)**. Intestinal length and organ weight (spleen and liver) were not affected by the dietary treatments ($p > 0.05$). The number of splenocytes were also not different among groups ($p > 0.05$). We did not observe changes in plasma IL-10, IL-13, or TNF-α ($p > 0.05$).

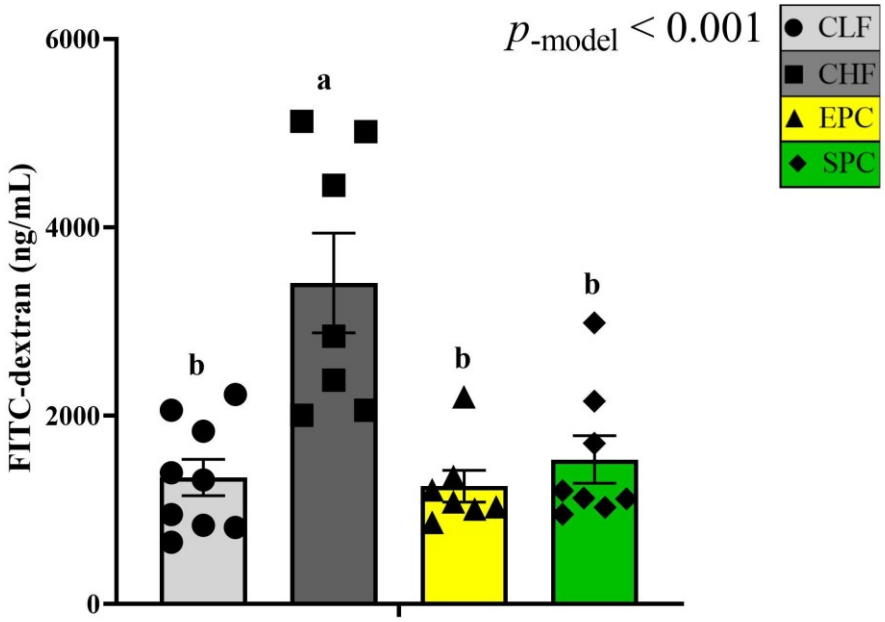
### 5.2.2 Effect of PC form on liver total FA composition

Several changes in liver total FA composition were observed, even though the FA composition of the four diets were closely matched **(Table 5-1)**. The proportion of total SFA in the liver was higher in rats fed the CLF diet compared to all other diets ($p < 0.05$), and no significant difference between the CHF, EPC, and SPC diets was observed ($p > 0.05$, **Table 5-2**). The SPC diet group had a higher n-6:n-3 ratio than the EPC and CLF diet ($p < 0.05$). This is in part due to the higher proportion of linoleic acid (LA) compared to the other diet groups ($p < 0.05$). Further, the SPC diet group had a lower proportion of docosahexaenoic acid (DHA) compared to the CLF and EPC diet group ($p < 0.05$).

### 5.2.3 Intestinal Permeability

The effect of the diets on intestinal permeability as assessed by FITC-dextran concentration is presented in **Figure 5-1**. The CHF diet fed rats had significantly higher intestinal permeability compared to the CLF diet group ($p < 0.05$). Both the EPC and SPC diets had similar intestinal permeability to the CLF diet group ($p > 0.05$).

**Figure 5-1: Effect of diet on intestinal permeability in male Wistar rats**

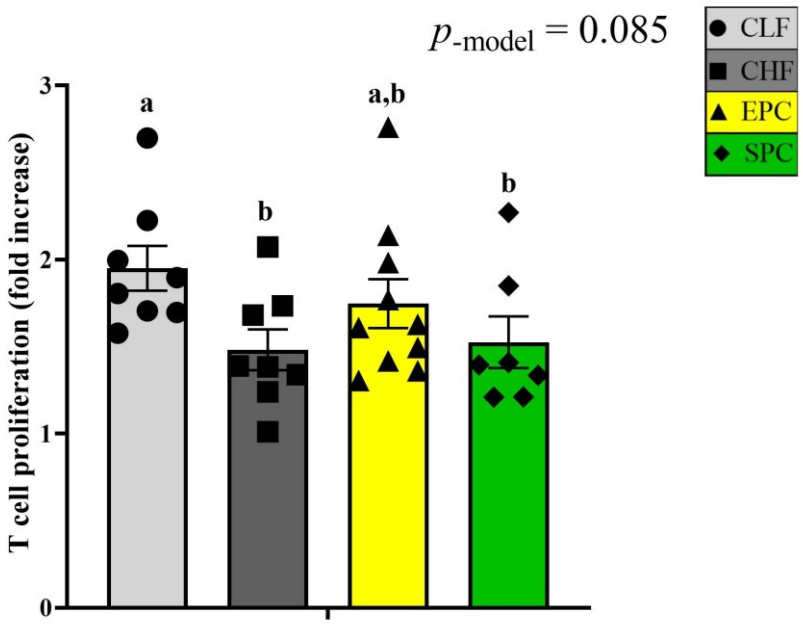



Values are presented as mean ± SEM. Bars that do not share the same letter are significantly different ($p < 0.05$). CLF, control low-fat; CHF, control high-fat; EPC, 100% egg-PC high-fat; SPC, 100% soy-PC high-fat; PC, phosphatidylcholine.

### 5.2.4 T cell proliferation assay and *ex vivo* cytokine production by splenocytes

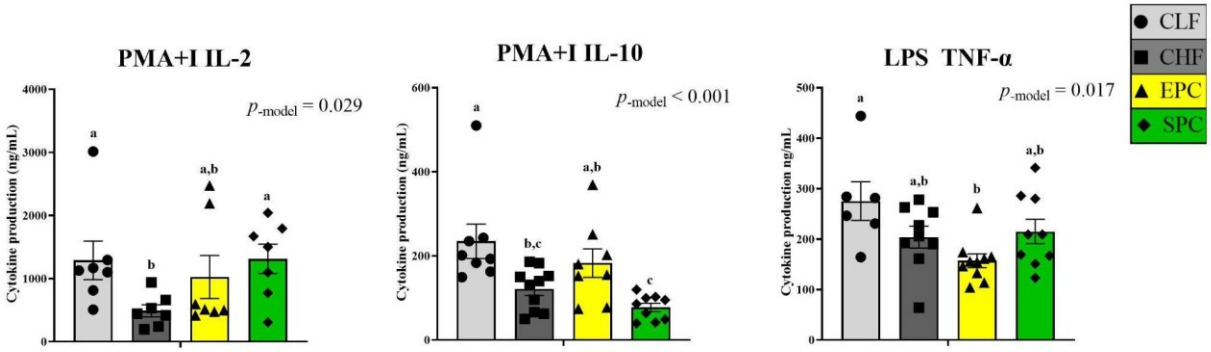
While the CHF diet lowered T cell proliferation compared to the CLF diet, feeding the EPC diet increased T cell proliferation to levels similar to the CLF diet, where the same effect was not observed in the SPC diet ($p < 0.05$, **Figure 5-2**). *Ex-vivo* cytokine production in splenocytes after mitogen stimulation is presented in **Figure 5-3** and **Table 5-3**. After stimulation with the T cell mitogen PMA+I, both the EPC and SPC diet groups produced IL-2 levels similar to that of the CLF diet, but only the SPC diet group increased IL-2 levels significantly higher than the CHF diet ($p < 0.05$). After PMA+I stimulation, the EPC diet fed group produced IL-10 to levels similar to that of the CLF diet group, but the SPC diet did not have the same effect ($p < 0.05$). After PMA+I stimulation, there was no significant difference in TNF-α production between the diet groups.

**Figure 5-2: Effect of diet on T cell proliferation of splenocytes stimulated by anti-CD3/anti-CD28 in male Wistar rats**




Values are presented as mean ± SEM. Bars that do not share the same letter are significantly different ($p < 0.05$). CLF, control low-fat; CHF, control high-fat; EPC, 100% egg-PC high-fat; SPC, 100% soy-PC high-fat; PC, phosphatidylcholine.

**Figure 5-3: Significant effect of diet on *ex vivo* cytokine production from splenocytes of male Wistar rats**




Values are presented as mean ± SEM. Bars that do not share the same letter are significantly different ($p < 0.05$). CLF, control low-fat; CHF, control high-fat; EPC, 100% egg-PC high-fat; SPC, 100% soy-PC high-fat; PC, phosphatidylcholine.

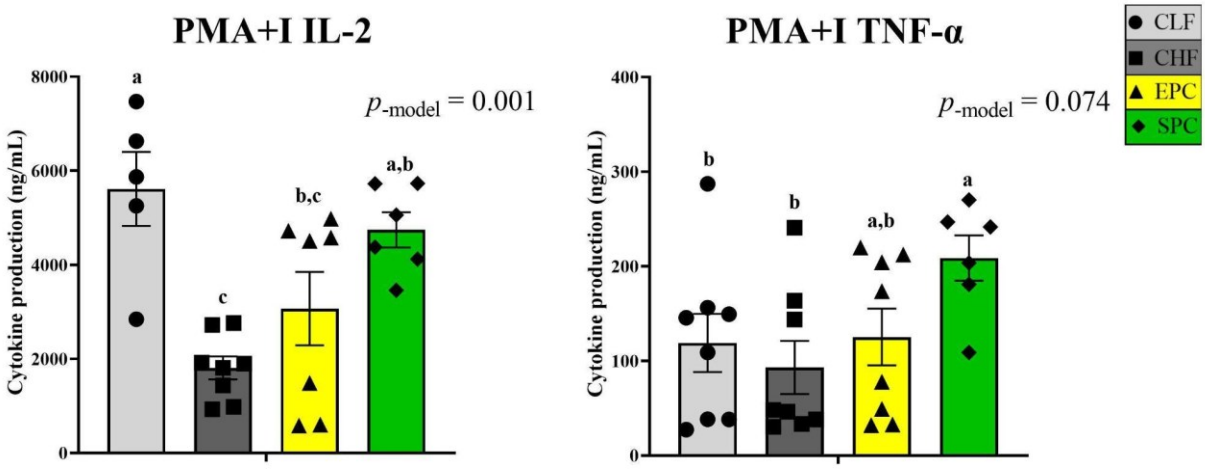
Following PWM stimulation, both the EPC and SPC diet produced similar IL-1β levels to the CHF diet ($p > 0.05$) which were all lower than the CLF diet. The EPC diet group tended to produce levels of IL-2 similar to the CLF diet, while the SPC diet group did not ($p = 0.074$). For IL-10 production, only the EPC diet group produced levels similar to the CLF diet group after PWM stimulation ($p > 0.05$). There was no significant difference in IL-6 production between the diet groups after PWM stimulation.

After LPS stimulation, both PC diets produced IL-10 levels similar to the CHF diet ($p < 0.05$). There was no difference in TNF-α levels between the CLF, CHF, and SPC diet while the EPC diet produced lower TNF-α than the CLF diet ($p < 0.05$). There was no significant difference in IL-6 production between the diet groups after LPS stimulation.

### 5.2.5 *Ex vivo* cytokine production by MLN cells

*Ex-vivo* cytokine production in MLN after mitogen stimulation is presented in **Figure 5-4** and **Table 5-3**. After stimulation with the T cell mitogen PMA+I, only the SPC diet produced IL-2 levels similar to the CLF diet ($p < 0.05$). After PMA+I stimulation, there was no difference in TNF-α production between the CLF, CHF, and EPC diet group, but the SPC diet group produced significantly higher TNF-α levels than the rest of the groups ($p < 0.05$). After PMA+I stimulation, there was no significant difference in IL-10 production between the diet groups.

**Figure 5-4: Significant or trending effect of diet on *ex vivo* cytokine production from MLN cells of male Wistar rats**



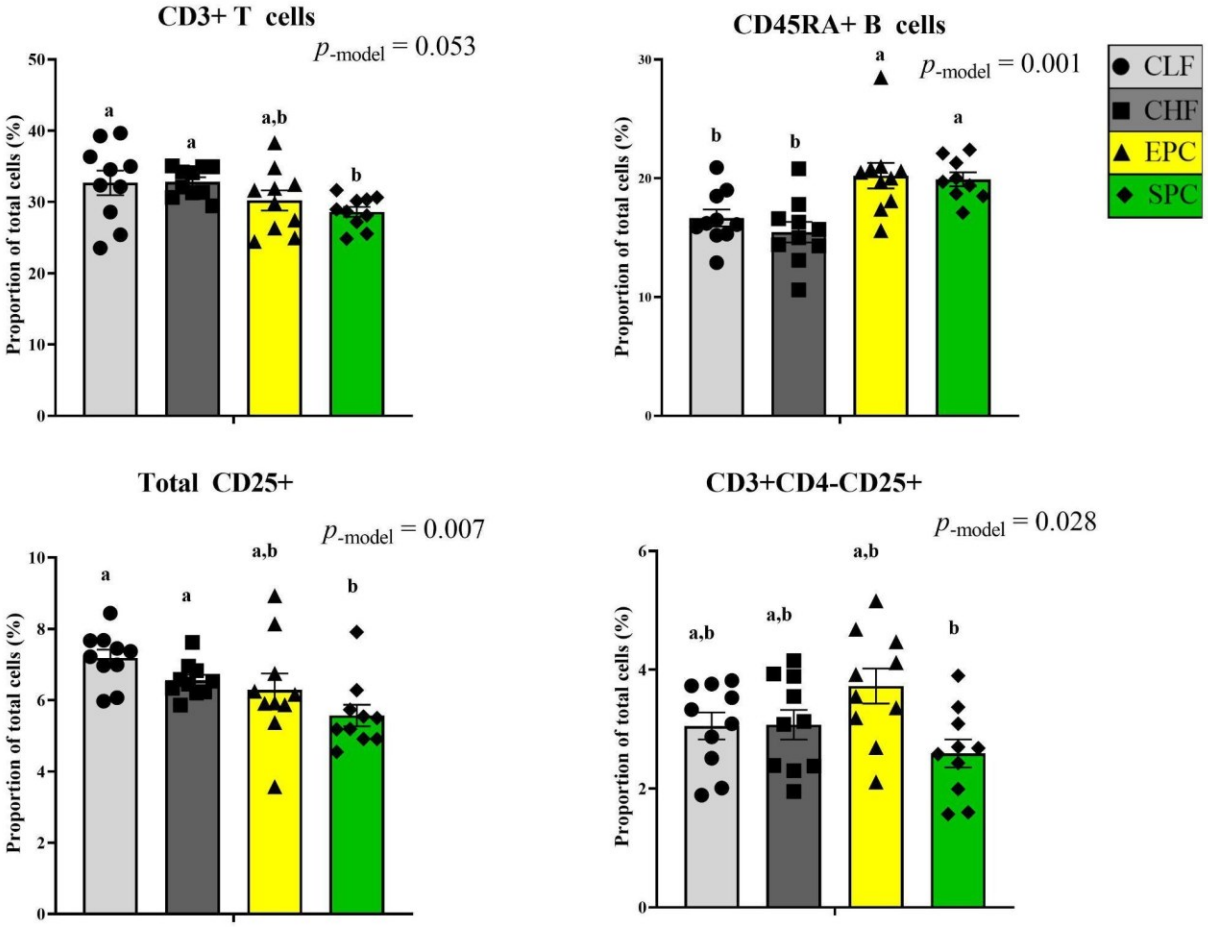

Values are presented as mean ± SEM. Bars that do not share the same letter are significantly different ($p < 0.05$). CLF, control low-fat; CHF, control high-fat; EPC, 100% egg-PC high-fat; SPC, 100% soy-PC high-fat; PC, phosphatidylcholine.

### 5.2.6 Splenocyte phenotypes

Proportions of immune cell phenotypes isolated from the spleen of rats fed the 4 experimental diets are presented in **Table 5-4**. Rats fed the SPC diet had significantly lower total CD3+ T cell and CD25+ (cells expressing the IL-2 receptor) proportions than the CLF and CHF diets ($p < 0.05$). Further, the EPC diet had a higher proportion of CD3+CD4-CD25+ (T cytotoxic cells expressing the IL-2 receptor) than the SPC diet ($p < 0.05$). While not significant, the SPC diet group tended to have a lower proportion of CD3+CD4+CD25+ (T helper cells expressing the IL-2 receptor) than the rest of the diet groups ($p = 0.180$). There were no significant differences between the diets for any of the remaining T cell phenotypes ($p > 0.05$). Rats fed the EPC and SPC diets had a higher proportion of CD45RA+ B cells compared to the CLF and CHF

diet fed rats ($p < 0.05$). Rats fed the EPC diet had lower CD68+TLR4+ (activated macrophages) proportions than both the CLF and CHF groups ($p < 0.05$). There was no significant difference in OX6+ (MHC class II cells), CD68+ (macrophages), CD68+CD11b/c+ (activated macrophages), and CD161+CD3- (NK cells) between the diet groups ($p > 0.05$).

**Figure 5-5: Significant effect of diet on splenocyte phenotypes of male Wistar rats**




Values are presented as mean ± SEM. Bars that do not share the same letter are significantly different ($p < 0.05$). CLF, control low-fat; CHF, control high-fat; EPC, 100% egg-PC high-fat; SPC, 100% soy-PC high-fat; PC, phosphatidylcholine.

## 5.3 Discussion

In this study, we aim to determine the effect of different forms of dietary PC on immune function in male Wistar rats fed a HFD. The model, and CLF, CHF, and EPC diets have been previously published and discussed (Azarcoya Barrera, Wollin et al. 2022). Overall, our data partly supports our hypothesis that egg-PC is more beneficial to T cell function than soy-PC. While egg-PC did enhance the production of several cytokines, some of the examined cytokines were more enhanced by feeding soy-PC, suggesting that different sources of PC modulate immune responses differently.

Despite diets being well matched for overall FA composition, analysis of lipids in the liver indicated several differences between the diets. Since one of the FA chains of PC is cleaved to be absorbed (Law, Chan et al. 2019), it is possible that PC from different sources influence lipids absorption and incorporation differently. Indeed, the n-6:n-3 ratio was higher in the SPC diet compared to the EPC. This may primarily be explained by the higher proportion of LA and lower proportion of DHA in the SPC diet group compared to the EPC diet group. LA is a precursor to several pro-inflammatory mediators, which could explain the observed increase in TNF-α levels in both splenocytes and MLN in the SPC diet (Innes & Calder 2018). Further, DHA has been shown to increase IL-10 production in monocytes (Jaudszus, Gruen et al. 2013). Since DHA was lower in the SPC diet group, this may explain the observed reduction in IL-10 in mitogen-stimulated splenocytes relative to the EPC group. Altogether, this may have contributed to explain the different effect on immune function between the two dietary forms.

We examined different mechanisms by which differing dietary forms of PC may affect immune function. First, we looked at how dietary PC affects intestinal permeability. We demonstrated that feeding PC regardless of the source was able to normalize intestinal permeability in the context of a HFD suggesting that PC in general can maintain gut barrier to a greater extent when compared to FC. This is consistent with other PC studies that have shown improvements in intestinal barrier function using several PC sources (Dial, Mayssa et al. 2008, Zhou & Ward, 2019, Tan, Zhang et al. 2020). However, when assessing gut-associated immune function, the SPC diet group produced more IL-2 and TNF-α in mitogen-stimulated MLN. This suggests that although both dietary forms of PC affect the gut barrier similarly, the FA composition of the soy-PC might have promoted a better Th1 response locally in the MLN.

On the other hand, the phospholipid composition of the EPC and SPC diet may also partially explain the differences observed in immune cell response. For the EPC diet, Lipoid 80 was used as the source of PC, which contains 85% PC+ Lyso-PC, 9.5% PE and 3% SM. For the soy-PC diet, soy lecithin was used as the source of PC, which contains about 42% PC, 36% PE, and 22% PI. Thus, while PC amount was matched between the EPC and SPC diet, this inherently led to the PE and PI content of the SPC being higher as the soy lecithin was lower in PC. Both PE and PI are involved in the synthesis of glycosylphosphatidylinositol-anchored proteins, which have a wide range of biological functions, including modulating immune response (Patel & Witt 2017, Mei, Ning et al. 2022). It has also been shown that PE co-ordinates the expression and localization of CXCR5 within T follicular helper (Tfh) cells, which modulates humoral immunity (Fu, Guy et al. 2021, Crotty 2014). Further, the toll/interleukin 1 receptor domain-containing adaptor protein (TIRAP) anchors to phosphatidylinositol species on plasma and

endosomal membranes and plays a role in several TLR signaling pathways (Patra & Choi 2018). Binding of LPS to TLR-4 stimulates a signaling cascade that triggers an immune response, including the release of IL-2 and TNF-α (Swanson, Katkar et al. 2020, Costalonga & Zell, 2007). Thus, the higher proportion of PI in the SPC diet may partly explain the increased production of IL-2 and TNF-α in both splenocytes and MLN regardless of the FA composition. Further examination is required to determine the effect of PE and PI on T cell function.

Regarding peripheral immune function, we observed similar changes in cytokine production between the EPC and SPC diet groups. When stimulated with PMA+I, a T cell mitogen, the EPC diet group produced significantly higher levels of IL-10 compared to the SPC group. IL-10 is an integral Th2 cytokine involved in downregulating host immune response (and the resolution phase of inflammation) as well as maintaining tolerance (Iyer & Cheng 2012). Although both PC diets normalized IL-2 production relative to the CLF, only the SPC diet increased IL-2 production to levels similar to the CLF diet suggesting a more enhanced capacity for T cell proliferation. Yet, when stimulated with anti-CD3 and anti-CD28, a specific T cell mitogen, T cell proliferation rate was higher in the EPC than the SPC group. This discrepancy could be explained at least partly by the fact that the SPC group had a lower proportion of total CD3+ T cells and T cells expressing CD25 (the IL-2 receptor). It is possible that while the SPC diet group produced more IL-2, the reduction in IL-2 receptor impeded T cell proliferation. After PWM stimulation, the EPC diet was also the only PC diet to produce levels of IL-10 similar to the CLF diet, suggesting an overall better inflammatory resolution capacity. Interestingly, after LPS stimulation, a mitogen that stimulated primarily APC, TNF-α production in the SPC diet was similar to the CLF diet, while the EPC diet did not have the same effect. Altogether, this

suggests that the EPC diet leads to an overall better T cell function while also promoting better resolution capacity as assessed by both T cell proliferation and cytokine production after T cell stimulation. Conversely, the SPC diet, as discussed previously, seems to enhance APC function and local immune cell function in the gut to a greater extent than EPC which may be attributable to the differences in phospholipids composition between diets.

While our study had several strengths there were also limitations present. Firstly, as discussed, the diets were significantly different in phospholipid content, which may in part have affected our results. Future study should examine a more purified form of soy-PC. However, the soy-PC used for this study is what is currently available on the market as soy lecithin, so the results we presented are relevant for human consumption. Further, while the objective of this study was to assess the effect of different dietary sources of PC, no human diet would contain choline 100% in the form of PC. Population based studies have shown typical consumption patterns of choline to be approximately 50% lipid-soluble forms and 50% water-soluble forms (Wiedeman, Barr et al. 2018). In Chapter 4 we showed that providing a diet of 50% egg-PC and 50% FC had a more pronounced beneficial effect on immune function than a diet containing only egg-PC. Therefore, it is possible that the effect of soy-PC may also be more pronounced when providing a diet of 50% soy-PC.

## 5.4 Conclusion

Our results indicate that egg- and soy-derived PC attenuate obesity induced immune cell dysfunction to some degree but have differential effects on T cell proliferation and Th1/Th2 response. Egg-PC showed a greater beneficial effect on IL-10 production, a key cytokine in the Th2 response, as well as on T cell proliferation stimulated by anti-CD3/anti-CD28 than soy-PC. While, both egg- and soy-PC showed a similar beneficial effect on gut permeability, soy-PC improved cytokines production from the MLN to a greater extent than EPC which may be attributable to the presence of other phospholipids in the soy lecithin used in this study.

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## Tables

**Table 5-1**: Fatty acid composition of experimental diets[1].

| Fatty Acid | CLF diet | CHF diet | EPC diet | SPC diet |
|---|---|---|---|---|
| | (% of total fatty acids) | | | |
| 14:0 | 0.72 ± 0.06 | 0.72 ± 0.03 | 0.63 ± 0.02 | 0.72 ± 0.03 |
| 16:0 | 18.0 ± 0.74 | 18.1 ± 0.58 | 18.0 ± 0.12 | 18.1 ± 0.19 |
| 16:1n9 | 0.15 ± 0.02 | 0.18 ± 0.03 | 0.18 ± 0.02 | 0.16 ± 0.02 |
| 16:1n7 | 0.97 ± 0.09 | 0.96 ± 0.07 | 0.89 ± 0.01 | 0.98 ± 0.02 |
| C18:0 | 10.1 ± 1.00 | 10.0 ± 0.59 | 9.77 ± 0.16 | 10.1 ± 0.30 |
| C18:1n9 | 32.0 ± 0.23 | 31.8 ± 0.52 | 31.3 ± 0.39 | 33.7 ± 0.46 |
| 18:1t11 | 1.00 ± 0.29 | 1.22 ± 0.18 | 1.17 ± 0.26 | 1.15 ± 0.19 |
| C18:2n-6 (LA) | 30.9 ± 0.33 | 29.7 ± 1.25 | 30.5 ± 0.07 | 27.7 ± 0.49 |
| 20:0 | 0.31 ± 0.02 | 0.31 ± 0.0 | 0.30 ± 0.0 | 0.30 ± 0.01 |
| C18:3n-3 (ALA) | 7.00 ± 0.56 | 6.35 ± 0.35 | 6.54 ± 0.03 | 6.53 ± 0.25 |
| 20:2n-6 | 0.31 ± 0.02 | 0.30 ± 0.02 | 0.27 ± 0.0 | 0.30 ± 0.0 |
| 20:3n-6 | 0.07 ± 0.01 | 0.07 ± 0.01 | 0.08 ± 0.0 | 0.08 ± 0.0 |
| 20:4n-6 (ARA) | 0.80 ± 0.01 | 0.07 ± 0.0 | 0.26 ± 0.0 | 0.08 ± 0.0 |
| 20:5n-3 | 0.07 ± 0.01 | 0.07 ± 0.01 | 0.07 ± 0.0 | 0.07 ± 0.0 |
| 22:6n-3 (DHA) | 0.01 ± 0.01 | 0.09 ± 0.10 | 0.08 ± 0.02 | 0.00 ± 0.0 |
| Total SFA | 29.2 ± 1.76 | 29.2 ± 1.18 | 28.7 ± 0.26 | 29.2 ± 0.45 |
| Total MUFA | 34.1 ± 0.61 | 34.2 ± 0.48 | 33.5 ± 0.14 | 36.0 ± 0.30 |
| Total PUFA | 38.4 ± 0.44 | 36.6 ± 1.66 | 37.8 ± 0.12 | 34.8 ± 0.70 |
| Ratio n-6:n-3 | 4.44 ± 0.02 | 4.62 ± 0.12 | 4.65 ± 0.02 | 4.27 ± 0.12 |
| Ratio SFA:PUFA | 0.86 ± 0.04 | 0.85 ± 0.02 | 0.86 ± 0.01 | 0.81 ± 0.01 |

[1]Analysis of the fatty acid composition of the four experimental diets collected weekly (n=2 batches for experimental diets; n=3 for control diets); 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; 25PC, 25% egg-PC high-fat; 50PC, 50% egg-PC high-fat; 75PC, 75% egg-PC high-fat; 100PC, 100% egg-PC high-fat; SPC, soy-PC high-fat; PC, phosphatidylcholine.

**Table 5-2: Fatty acid composition of liver tissue.[1]**

| Fatty Acid | CLF diet | CHF diet | EPC diet | SPCdiet | pValue |
|---|---|---|---|---|---|
| 14:0 | 0.64 ± 0.03[a] | 0.41 ± 0.02[b] | 0.33 ± 0.01[c] | 0.37 ± 0.02[b,c] | <0.001 |
| 14:1 | 0.17 ± 0.02[a,b] | 0.14 ± 0.01[b] | 0.15 ± 0.01[b] | 0.20 ± 0.01[a] | 0.008 |
| 16:0 | 23.8± 0.44[a] | 21.2 ± 0.38[b] | 19.8 ± 0.29[c] | 20.3 ± 0.30[b,c] | <0.001 |
| 16:1n9 | 0.37 ± 0.03[a] | 0.36 ± 0.05[a] | 0.32 ± 0.03[a] | 0.19 ± 0.02[b] | 0.004 |
| 16:1n7 | 2.57 ± 0.19[a] | 0.98 ± 0.07[b] | 0.57 ± 0.06[c] | 0.41± 0.04[c] | <0.001 |
| 17:0 | 0.20 ± 0.01 | 0.18 ± 0.01 | 0.18 ± 0.01 | 0.25 ± 0.03 | 0.112 |
| C18:0 | 15.2 ± 0.71[a] | 12.5 ± 1.18[b] | 15.2 ± 0.74[a] | 15.6 ± 0.76[a] | 0.050 |
| C18:1n9 | 17.8 ± 0.95[b] | 21.6 ± 1.10[a] | 15.9 ± 0.85[b,c] | 14.9 ± 0.56[c] | <0.001 |
| 18:1n7[2] | 2.39 ± 0.26[a] | 1.06 ± 0.07[b] | 1.02 ± 0.06[b] | 1.01 ± 0.30[b] | <0.001 |
| C18:2n-6 (LA) | 13.8 ± 1.90[c] | 21.8 ± 0.85[b] | 23.0 ± 0.57[b] | 25.2 ± 0.62[a] | <0.001 |
| C18:3n-3 (ALA) | 0.84 ± 0.08[b] | 1.66 ± 0.13[a] | 1.88 ± 0.11[a] | 1.96 ± 0.13[a] | <0.001 |
| 20:0 | 0.18 ± 0.03[b] | 0.26 ± 0.04[a,b] | 0.31 ± 0.03[a] | 0.28 ± 0.02[b] | 0.033 |
| 20:2n-6 | 0.27 ± 0.05[b] | 0.40 ± 0.02[a] | 0.38 ± 0.03[a] | 0.42 ± 0.01[a] | 0.011 |
| 20:3n-6 | 0.67 ± 0.08 | 0.58 ± 0.02 | 0.57 ± 0.04 | 0.69 ± 0.03 | 0.175 |
| 20:4n-6 (ARA) | 14.4 ± 0.66[a] | 11.5 ± 0.83[b] | 14.3 ± 0.73[a] | 12.7 ± 0.66[a,b] | 0.020 |
| 20:5n-3 (EPA) | 0.32 ± 0.03[b] | 0.43 ± 0.03[a] | 0.47 ± 0.03[a] | 0.44 ± 0.03[a] | 0.028 |
| 24:1n9 | 0.07 ± 0.02[a] | 0.04 ± 0.01[b] | 0.03 ± 0.01[b] | 0.02 ± 0.01[b] | 0.035 |
| 22:4n-6 | 0.30 ± 0.02[b] | 0.47 ± 0.06[a] | 0.48 ± 0.04[a] | 0.40 ± 0.02[a] | 0.002 |
| 22:5n-6 | 0.10 ± 0.01[a,b] | 0.09 ± 0.02[a,b] | 0.13 ± 0.02[a] | 0.08 ± 0.01[b] | 0.097 |
| 22:5n-3 (DPA) | 0.63 ± 0.07[c] | 0.88 ± 0.08[b] | 0.98 ± 0.05[b] | 1.19 ± 0.05[a] | <0.001 |
| 22:6n-3 (DHA) | 4.61 ± 0.25[a] | 3.52 ± 0.24[b,c] | 3.89 ± 0.18[b] | 3.25 ± 0.16[c] | <0.001 |
| Total SFA | 40.3 ± 0.88[a] | 34.5 ± 1.02[b] | 35.9 ± 0.58[b] | 36.9 ± 0.65[b] | <0.001 |
| Total MUFA | 23.6 ± 1.20[a] | 24.2 ± 1.13[a] | 18.4 ± 0.86[b] | 16.8 ± 0.58[b] | <0.001 |
| Total PUFA | 36.1 ± 1.10[c] | 41.3 ± 0.72[b] | 45.7 ± 0.47[a] | 46.3 ± 0.50[a] | <0.001 |
| Total n-6 | 30.4 ± 0.78[c] | 34.8 ± 0.49[b] | 37.5 ± 1.10[a] | 39.5 ± 0.32[a] | <0.001 |
| Total n-3 | 6.08 ± 0.32 | 6.06 ± 0.23 | 6.74 ± 0.18 | 6.40 ± 0.21 | 0.166 |
| n-6:n-3 | 4.94 ± 0.17[c] | 5.40 ± 0.15[a,b] | 5.31 ± 0.12[b] | 5.83 ± 0.18[a] | <0.001 |
| SFA:PUFA[2] | 1.13 ± 0.05[a] | 0.84 ± 0.03[b] | 0.79 ± 0.01[b] | 0.80 ± 0.02[b] | <0.001 |

[1]Analysis of the fatty acid composition of the total lipids extracted from liver tissue (n=2 per rat); ALA, α-linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; MUFA, monounsaturated fatty acids; n, omega; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; CLF, control low-fat; CHF, control high-fat; 25PC, 25% egg-PC high-fat; 50PC, 50% egg-PC high-fat; 75PC, 75% egg-PC high-fat; 100PC, 100% egg-PC high-fat; SPC, soy-PC high-fat; PC, phosphatidylcholine.
2 Analysis performed on log-transformed values.

**Table 5-3: *Ex vivo* cytokine production by mitogen-stimulated splenocytes and MLN cells of male Wistar rats fed the four experimental diets.[1]**

| Cytokine (pg/ml)[1] | CLF diet | CHF diet | EPC diet | SPC diet | *p* Value |
|---|---|---|---|---|---|
| **Splenocytes** | | | | | |
| PMA+I (T cell mitogen) | | | | | |
| IL-2[2] | 1289 ± 304[a] | 489.9 ± 96.1[b] | 1024 ± 340[a,b] | 1311 ± 233[a] | 0.029 |
| IL-10[2] | 234.8 ± 40.9[a] | 121.6 ± 15.8[b,c] | 182.9 ± 33.9[a,b] | 77.4 ± 9.88[c] | < 0.001 |
| TNF-α | 232.9 ± 58.8 | 107.4 ± 22.0 | 186.4 ± 38.0 | 237.5 ± 39.7 | 0.156 |
| PWM (T and APC mitogen) | | | | | |
| IL-1β[2] | 103.4 ± 8.00[a] | 53.8 ± 3.15[b] | 69.1 ± 8.96[b] | 64.1 ± 3.73[b] | < 0.001 |
| IL-2 | 283.9 ± 35.0[a] | 168.5 ± 26.0[b] | 200.6 ± 24.1[a,b] | 174.9 ± 34.8[b] | 0.074 |
| IL-10[2] | 323.4 ± 54.9[a] | 165.9 ± 23.3[c] | 256.6 ± 38.6[a,b] | 194.6 ± 11.2[b,c] | 0.008 |
| IL-6 | 593.9 ± 49.1 | 463.4 ± 60.7 | 588.1 ± 43.8 | 510.9 ± 47.1 | 0.214 |
| LPS (Bacterial challenge) | | | | | |
| IL-10 | 550.9 ± 33.6[a] | 394.0 ± 23.3[b] | 391.9 ± 39.2[b] | 391.5 ± 30.3[b] | 0.006 |
| TNF-α | 275.1 ± 38.2[a] | 203.9 ± 21.6[a,b] | 157.0 ± 13.6[b] | 215.0 ± 24.2[a,b] | 0.002 |
| IL-6 | 486.1 ± 61.9 | 381.4 ± 42.7 | 402.2 ± 45.0 | 327.4 ± 28.7 | 0.114 |
| **MLN Cells** | | | | | |
| PMA+I | | | | | |
| IL-2 | 5613 ± 786[a] | 1808 ± 245[c] | 3067 ± 779[b,c] | 4745 ± 375[a,b] | 0.001 |
| IL-10[2] | 125.8 ± 48.8 | 103.2 ± 30.4 | 93.7 ± 14.6 | 204.1 ± 63.9 | 0.226 |
| TNF-α | 119 ± 31[b] | 93.0 ± 28.1[b] | 125.2 ± 30.0[a,b] | 208.7 ± 23.8[a] | 0.074 |

[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; EPC, 100% egg-PC high-fat; SPC, soy-PC high-fat; PC, phosphatidylcholine; IFN-γ, interferon gamma; IL, interleukin; TNF-α, tumor necrosis factor-alpha.

[2] Analysis performed on log-transformed values.

**Table 5-4: Splenocyte phenotype of male Wistar rats fed the four experimental diets.[1]**

| Cell Phenotype[1] | CLF diet | CHF diet | EPC diet | SPC diet | *p* Value |
|---|---|---|---|---|---|
| **Splenocytes** | | | | | |
| % of total gated cells | | | | | |
| Total CD25+ | 7.18 ± 0.23[a] | 6.56 ± 0.15[a] | 6.29 ± 0.46[a,b] | 5.57 ± 0.30[b] | 0.008 |
| TotalCD3+ | 32.7 ± 1.72[a] | 32.8 ± 0.66[a] | 30.2 ± 1.41[a,b] | 28.6 ± 0.70[b] | 0.053 |
| % of CD3+ gated cells | | | | | |
| CD4+ (T helper cells) | 66.6 ± 1.25 | 66.8 ± 1.46 | 68.1 ± 2.03 | 68.9 ± 1.48 | 0.703 |
| CD8p+ (T cytotoxic cells) | 33.3 ± 1.26 | 33.1 ± 1.46 | 31.8 ± 2.03 | 31.0 ± 1.48 | 0.701 |
| % of CD3+CD4+ gated cells | | | | | |
| CD25+ | 8.33 ± 0.54 | 7.47 ± 0.25 | 7.95 ± 0.68 | 6.75 ± 0.52 | 0.180 |
| FoxP3+ ( T regulatory cells) | 5.46± 1.33 | 5.53 ± 1.14 | 3.89 ± 0.86 | 7.14 ± 1.52 | 0.348 |
| % of CD3+CD4- gated cells | | | | | |
| CD25+ | 3.05 ± 0.22[a,b] | 3.08 ± 0.25[a,b] | 3.73 ± 0.30[a] | 2.59 ± 0.24[b] | 0.028 |
| % of total gated cells | | | | | |
| CD45RA+ (B cells) | 16.7 ± 0.72[b] | 15.5 ± 0.87[b] | 20.2 ± 1.07[a] | 19.9 ± 0.58[a] | 0.001 |
| TotalOX6+ (MHC class II+) | 58.1 ± 1.69 | 58.7 ± 1.37 | 59.5 ± 1.71 | 61.1 ± 1.46 | 0.572 |
| Total CD68+ (macrophages) | 7.22 ± 0.32 | 7.13 ± 0.29 | 7.15 ± 0.51 | 6.07 ± 0.33 | 0.093 |
| CD68+TLR4+ | 51.1 ± 1.52[a] | 49.5 ± 2.30[a] | 42.4 ± 2.17[b] | 46.4 ± 2.14[a,b] | 0.031 |
| CD3-CD161+ (NK cells) | 3.04 ± 0.28 | 2.92 ± 0.43 | 2.80 ± 0.31 | 3.42 ± 0.45 | 0.681 |
| Total TLR4+ | 4.87 ± 0.31 | 4.85 ± 0.47 | 4.31 ± 0.45 | 3.90 ± 0.38 | 0.267 |

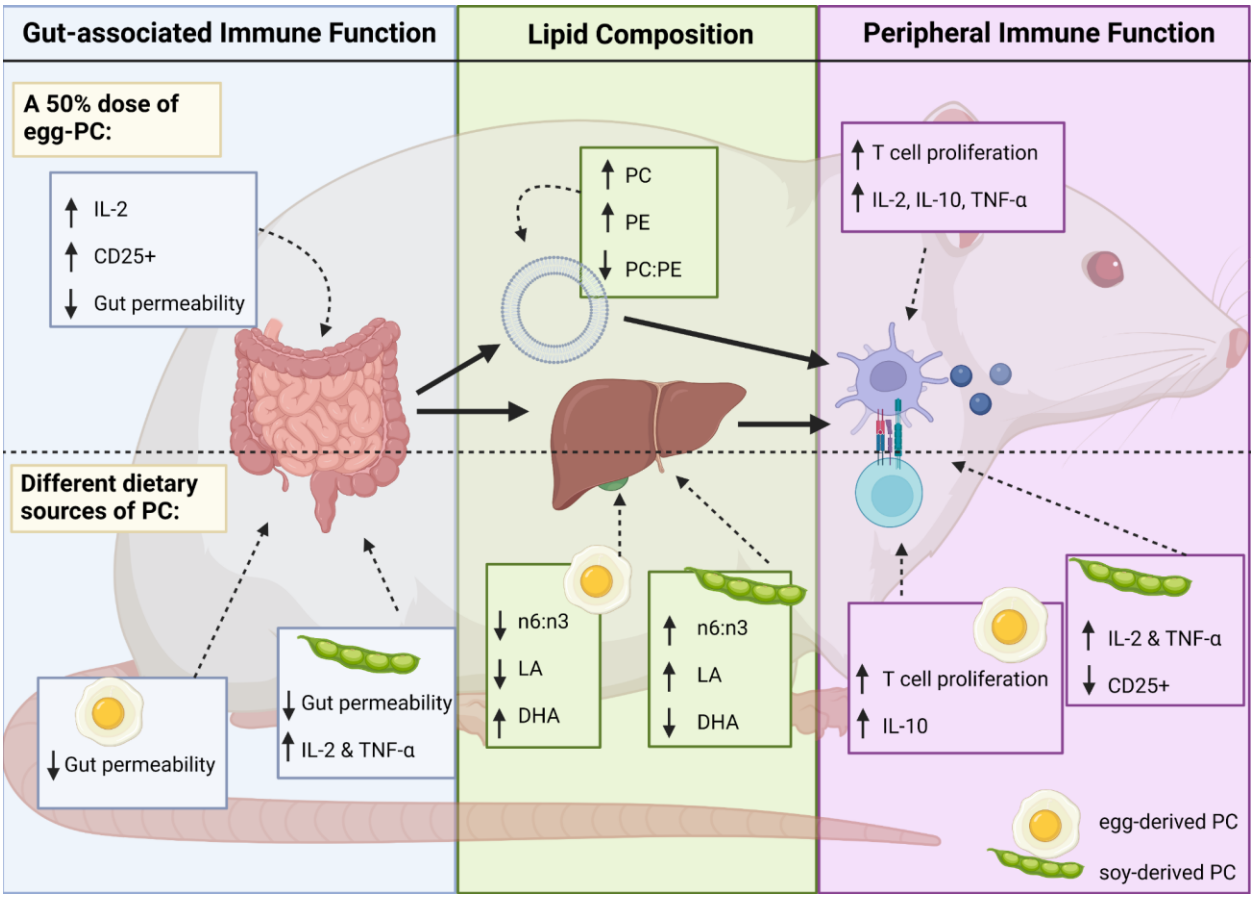
[1]Values are presented as mean ± SEM, n=10 per group. Groups that do not share the same letter are significantly different based on the one-way ANOVA test with the Duncan adjustment for multiple comparisons test ($p < 0.05$). No significant differences observed in the following: CD45RA+CD80+ (1.23 ± 0.13), OX6+CD80+ (1.55 ± 0.15), Total CD25+ (6.70 ± 0.39), Total CD80+ (2.62 ± 0.31), % of CD27+ in CD3+CD4+ (56.3 ± 1.84) and CD3+CD4- (74.1 ± 2.17); CLF, control low CLF, control low-fat; CHF, control high-fat; 25PC, 25% egg-PC high-fat; 50PC, 50% egg-PC high-fat; 75PC, 75% egg-PC high-fat; EPC, 100% egg-PC high-fat; SPC, soy-PC high-fat; MFI, median fluorescence intensity; PC, phosphatidylcholine.

## Chapter 6. General discussion and future directions

### 6.1 Executive summary of findings

In our studies we examined both the dose response of egg-derived PC as well as the dietary source effect of egg- and soy-derived PC under the context of HFD-induced immune dysfunction. **Figure 6-1** depicts a summary of our results.

**Figure 6-1. A summary of the dose and source effect of dietary PC within the context of a HFD[1]**




[1] Abbreviations: DHA, docosahexaenoic acid; IL, interleukin; LA, linoleic acid; n, omega; PC, phosphatidylcholine; PE, phosphatidylethanolamine. Created with Biorender.com

### 6.1.1 A physiologically relevant dose of 50% egg-phosphatidylcholine is sufficient in improving gut permeability while attenuating immune cell dysfunction induced by a high-fat diet in male Wistar rats

In Chapter 4, we investigated the impact of feeding varying doses of choline in a HFD as egg-derived PC on peripheral (spleen) and gut-associated immune responses (MLN) in male Wistar rats. Earlier work from our group suggested that PC could improve T cell function in obesity when provided as the sole source of choline in the diet (Azarcoya-Barrera, Wollin et al. 2022). However, since population studies report an average consumption of 40-60% PC, this is of no relevance to human consumption (Wiedeman, Barr et al. 2018, Lewis, Subhan et al. 2014). Therefore, we hypothesized that 1) a HFD leading to obesity reduces T cell function in splenocytes and MLN, but can be mitigated by egg-PC, 2) a dose of 50-75% egg-PC reflecting normal human consumption of choline will provide the most beneficial effect on T cell function compared to only FC, 3) feeding a HFD with egg-PC compared to FC will increase PC incorporation into cell membranes, thus directly supporting immune cell proliferation and function, and 4) feeding a HFD with egg-PC compared to FC will improve intestinal barrier protection, thus indirectly supporting immune function by reducing risk of endotoxemia. Our hypotheses were confirmed by our results that feeding a HFD containing only FC decreased IL-1β, IL-2, IL-10, and TNF-α following mitogen stimulation, and while adding PC in any dose increased cytokine production to some extent, the 50PC diet significantly improved the greatest number cytokine levels in comparison to the CHF diet. In MLN, we observed that there was an increase in total cells expressing CD25+ (IL-2 receptor) in the PC diets, which could partially explain the increase in IL-2 in these diet groups. We also observed that a HFD containing only FC tended to increase the PC:PE ratio in splenocyte membranes, while adding PC to a HFD in a physiologically relevant dose of 50-75% normalized the ratio relative to the CLF diet, suggesting

that dietary PC can modulate phospholipid composition of immune cell membranes. Our fourth hypothesis was confirmed by the significant increase in intestinal permeability in the CHF diet compared to the CLF diet, whereas doses of PC 50% or greater returned permeability to levels similar to that of the CLF diet. However, we measured mRNA expression in several genes associated with intestinal health and permeability and found no significant changes. Thus, the exact mechanism by which PC is improving intestinal permeability remains unclear.

### 6.1.2 Egg- and soy- derived phosphatidylcholine have differential effects on immune function in the context of a high-fat diet in male Wistar rats

In Chapter 5, we examined the differential effect of providing either egg- or soy-derived PC in the context of a HFD on immune responses in Wistar rats. Since earlier work suggested the egg-PC could improve T cell function (i.e., IL-2 production) to a greater extent than soy-PC (Dellschaft, Richard et al. 2018), we hypothesized that egg-PC will improve T cell function to a greater extent than soy-PC. Our results were partly confirmed in that egg-PC improved T cell proliferation and IL-10 production in splenocytes. This suggests that egg-PC not only enhances T cell proliferation in general but also enhances cytokine production involved in the resolution phase of inflammation. However, IL-2 production was higher after T cell mitogen stimulation in both splenocytes and MLN cells in the soy-PC diet group. This was partly explained by the lower proportion of cells expressing CD25+ (the IL-2 receptor) in that group. Further, TNF-α production in both splenocytes stimulated with an APC mitogen and MLN cells stimulated with a T cell mitogen increased to levels similar to the CLF diet only in the soy-PC diet group. Altogether the soy-PC diet was shown to enhance APC function and local immune cell function in the gut. Both the EPC and SPC effectively lowered intestinal permeability relative to the CHF diet, suggesting that PC in general can maintain the gut barrier. Thus, we suggest that the

observed differences in FA composition in the liver partly explain the differences in effect on immune cell function. Despite diets being well matched for overall FA composition, there was a higher proportion of LA, a precursor to several proinflammatory mediators, and lower proportion of anti-inflammatory DHA in the liver tissue of the SPC diet compared to the EPC diet. Further, we noted that while the soy lecithin used to provide soy-PC is reflective of what is on the market, it contained other phospholipids, which have been shown to have immunomodulatory effects (Patel & Witt 2017, Fu, Guy et al. 2021). Thus, further study will require the isolation of soy-derived PC to fully tease out the specific differential effects of egg- and soy-PC.

### 6.2 General discussion and future directions

Overall, our study showed both a PC and FA specific effect on HFD induced T cell dysfunction. We demonstrated that a HFD containing only FC impaired T and APC cell responses by lowering IL-2, TNF-α, and IL-10 production in splenocytes and IL-2 in MLN, and that adding egg-PC in the diet at any dose counteracted the effect of feeding a HFD on immune responses in male Wistar rats to some degree. We observed that PC modulated the expression of the IL-2 receptor, which may partially explain the enhanced T cell response. While each dose of PC influenced HFD induced immune cell dysfunction, the 50% dose most consistently improved T cell response. A 50% dose of choline in lipid soluble forms is reflective of average human consumption. While the population in general does not consume the AI of choline, our study confirms that the proportions of lipid soluble and water-soluble forms in an average human diet appear optimal for immune function since humans consume on average 50% of their choline as PC (Wiedeman, Barr et al. 2018). Future clinical study should examine the immunological effects of providing a choline sufficient diet containing approximately 50% lipid-soluble forms such as PC in individuals with obesity.

We observed a FA effect on immune function in both of our studies. When comparing two dietary sources of PC that have been matched for FA composition, we saw that liver tissue of rats fed the SPC diet resulted in a higher n-6:n-3 ratio and a differential effect on immune function. It was shown that FA may modulate local immune function to a greater degree than peripheral immune function, as evidenced by the observed increase in IL-2 production in MLN cells of SPC relative to the EPC diet. Interestingly, FAs also seem to modulate IL-2 receptor expression, which should be further investigated. Altogether this confirms that both PC and the respective FA that they contain have immunomodulatory effects and further study should clarify the mechanism by which these effects occur. We also observed an increase in SFA content of the liver tissue of the CLF diet relative to the rest of the diets. We have been documenting this increased proportion of SFAs in the liver consistently across our studies and pre-clinical models of obesity (Azarcoya-Barrera, Wollin et al. 2022, She, Wang et al. 2022) suggesting that current rodent research diets containing only FC may not accurately reflect a true "healthy" control. While FC-only LFDs are currently universally recognized as a proper control, future study should investigate the ideal proportion of choline forms in CLF diet. It should also be noted that we used liver tissue as a surrogate to understand FA incorporation into tissue since we didn't have enough splenocytes. Further study should confirm this in both MLN cells and splenocytes due to the observed differential effect. While we observed a differential effect of dietary forms of PC on FA incorporation in liver tissue, we also noted the limitation that the soy lecithin we used contained higher proportions of other phospholipids, such as PE and PI. Both PE and PI have been shown to exert important immunomodulatory properties, thus further study should examine their exact effect on T cell function (Patel & Witt 2017, Mei, Ning et al. 2022). In the future,

understanding the interplay between different phospholipids and FA composition could lead to the development of an ideal PL mixture with ideal FA composition to beneficially modulate immune function in obesity.

Mechanistically, we showed that providing PC in the diet may have a beneficial effect on HFD induced immune dysfunction due to the observed effect on intestinal permeability. Obesity is associated with an increase in intestinal permeability, and our study showed that providing a dose of 50% or greater of PC lowered HFD induced intestinal permeability (Khan, Luck et al. 2018). We did not observe differences in mRNA levels of several genes associated with intestinal barrier function. Our collaborator has previously shown that impaired *de novo* PC synthesis in the gut is linked to higher abundance of transcripts related to ER stress and cell death together with loss of goblet cells from the small intestinal epithelium (Carlin, Kennelly et al. 2022). Thus, there may be a differential effect on gene expression between exogenous and endogenous sources of PC. Further study should examine the effect of dietary PC on other genes and goblet cell morphology to further understand how diet affects the intestinal barrier. As for gut morphology, we showed the 50% dose of PC significantly increased jejunal villus height relative to the control diets. Our collaborator has previously shown that a choline deficient (CD) diet reduced intestinal villus height in lactating dams when compared to a choline sufficient and PC-supplemented diet, which indicated a lower absorptive surface area of the CD diet fed group (da Silva, Kelly et al. 2015). Clinical trials have shown that PC is low in patients with ulcerative colitis (UC), and providing PC endogenously improves clinical and endoscopic outcomes (Stremmel, Vural et al. 2021). Since a reduction in villus height is observed in patients with UC the beneficial effect of dietary PC may partially be explained by ameliorating villus atrophy and

altogether supporting better nutrient absorption (Mourad, Barada et al. 2017). While our lab has assessed PC content in the stomach of rat offspring from dams fed PC and FC diets (Dellschaft, Richard et al. 2018), we did not measure intestinal PC content in our study. Future studies should examine the effect of PC on the PC content in the mucosal layer of rats fed a HFD. This may provide insight into the specific mechanism by which PC is integrated into the mucosal layer, as well whether this occurs independently of intestinal morphology changes.

Another mechanism we explored was the effect dietary PC had on phospholipid composition of splenocyte membranes. PC and PE are both integral phospholipids in mammalian cell membranes, and a growing body of literature has shown that the PC:PE ratio in hepatocytes is a relevant marker for metabolic health (van der Veen, Kennelley et al. 2017). Previously, we have shown that providing a diet containing 100% PC increased PC incorporation into splenocytes of lactating dams (Lewis, Richard et al. 2016). In the current study we showed that providing a diet containing only FC resulted in a higher PC:PE ratio, reflective of what is observed in hepatocytes in obese models (Patel & Witt 2017). Further, we showed that providing PC, especially in higher doses, significantly decreased the PC:PE ratio. As this is one of the first studies to examine the effect of diet on the PC:PE ratio in splenocytes, the relationship between PC:PE ratio and immune function should be further investigated.

Although our study had many strengths, some limitations should be addressed in future studies. While PC and FC are the two forms of choline that make up the largest percentage of total choline consumption, other forms of choline exist that have also been shown to be immunomodulatory, such as SM (Norris, Porter et al. 2017). Future studies should examine the

effect of a HFD containing a mixture of choline high in lipid soluble forms, such as buttermilk derived choline that contains SM. Our group has previously shown that providing a diet containing buttermilk provided greater benefit than a PC only diet on T cell responses of lactating dams (Azarcoya-Barrera, Field et al. 2021). Therefore, providing mixed lipid-soluble choline forms including PC and SM may lead to greater immune benefit in the context of HFD feeding. Another limitation of our study is the absence of *in vivo* analysis of inflammation. Our studies showed that PC contributed to overall immune function by production of both Th1 and Th2 cytokines upon mitogen stimulation. Since classically pro- and anti- inflammatory cytokines are important in both the onset and resolution phase of inflammation respectively, an increase in pro-inflammatory cytokine levels upon mitogen stimulation is indicative of an attenuation of immune dysfunction. However, in circulation, higher levels of IL-6, TNF-α, and CRP are indicative of a higher degree of systemic inflammation (She, Mangat et al. 2022). We used plasma cytokine levels to assess systemic inflammation at the end of our diet treatment. Most of the cytokines we assessed were not detectable, suggesting our model did not lead to high levels of chronic systemic inflammation. Thus, understanding the immunomodulatory properties of PC in the context of an inflammatory state still needs to be investigated. Another limitation of our study is the absence of female animals. We have previously shown that female Wistar rats are resistant to developing an obesity phenotype and are prone to a higher Th1 response (Tibaes, Azarcoya-Barrera et al. 2022). Since we observed differences in Th1 and Th2 responses between different dietary sources of PC, it is prudent to examine potential sex differences of dose and dietary source of PC. However, it has been demonstrated that PEMT gene expression is upregulated by estrogen, suggesting that females may be less sensitive to choline modulation in the diet. Indeed, Fischer *et al*., (2010) showed after feeding a low-choline diet for 42 days,

significantly less premenopausal women developed reversible hepatic and muscle tissue dysfunction compared to men and postmenopausal women. Finally, Wistar rats were selected as an animal model for their observed similar immune dysfunction, such as lower IL-2 production and T cell proliferation, as reported with individuals with obesity and T2D (Richard, Wadowski et al. 2017, Lamas, Martinez et al. 2002). Further, Wistar rats provide the additional benefit of developing the obesity phenotype more rapidly than Sprague-Dawley (Marques, Meireles et al. 2015). However, we had a significantly lower amount of isolated MLN cells than expected, which resulted in low cell counts and subsequent reduction in the number of cytokines assessed. Thus, future study should optimize the required cell numbers, stimulation time and ideal concentration for each mitogen in each tissue (i.e., spleen and MLN tissues). This is especially important as we observed a differential effect of PC and FA on both Th1 and Th2 responses as well as T and APC function, suggesting that mitogens stimulating different cells and cytokines may also have differential effects. We also chose a 48-hour stimulation time to balance between APC and T cell response, but it is possible that shorter or longer incubation times may give more insight into specific cell responses. For example, 12- and 24-hour stimulation could be used to understand early APC cell response from a bacterial challenge, while the 72-hour stimulation for a T cell mitogen could be used to better understand the resolution phase for IL-10 production. Further, intracellular staining could also be used post stimulation to give more insight into which immune cell populations are producing which cytokines. Since most of our previous studies have used Sprague-Dawley rats, we recommend further troubleshooting in determining laboratory analysis differences between the two models.

Altogether our study showed a beneficial effect of PC on obesity-induced immune dysfunction. Further, we provided evidence that the FA that remains upon absorption varies between dietary sources, thus providing further evidence of a FA effect on immune function. Since we showed that a physiologically relevant dose of 50% PC provided the most optimal immunological response, and we know that the population in general consumes less than the AI of total choline, further clinical study should assess choline sufficient dietary interventions on obesity-induced immune dysfunction (Wiedeman, Barr et al. 2018). Additionally, this dietary pattern should be explored in patients with inflammatory gastrointestinal conditions, such as UC. Further, since both PC and FA have beneficial effects on immune function, personalized nutrition research should examine specific phospholipid and FA proportions that would be ideal in supporting immune health. The current market primarily offers choline supplements in the form of water-soluble forms, such as choline bitartrate. Since we have shown immunological benefit for providing both mixed choline forms, further development should focus on providing mixed choline form supplements.

### 6.3 Conclusion

Overall, the research presented in this thesis demonstrated that a diet providing PC attenuated HFD induced immune dysfunction. We showed that a physiologically relevant dose of 50% PC decreased intestinal permeability brought on by a HFD and subsequently improved T cell and APC response. Further, we showed that while both egg- and soy-PC have beneficial effects on immune function, egg-PC exerts a greater beneficial effect on T cell proliferation and Th2 responses, while soy-PC exerts preferential effects on gut-associated and Th1 immune responses. Altogether this evidence suggests that designing healthy diets, both in research environments and to the general population, should consider mixed forms of choline.

Specifically, animal research diets typically only contain water-soluble FC. Our results show that a mixture of lipid- and water-soluble forms are essential for adequate immune function. PC is a promising form of choline to counteract the effects of feeding a HFD, and thus further studies should be conducted to determine the mechanism behind PC and other lipid soluble forms of choline's effect on T cell and APC response and intestinal permeability.

## 6.4 References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