Evaluating the effects of dietary docosahexaenoic acid along with arachidonic acid during the development of the immune system in early life.

by

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Abstract

The immune system is immature at birth and the dietary fats provided during early life play an important role in the development of the infant's immune system. The precursor omega-3 (n-3) and n-6 polyunsaturated fatty acids (PUFAs) α-linolenic acid and linoleic acids, respectively, are essential for our body. The bioconversion of precursors into long-chain PUFA (LCPUFA), docosahexaenoic (DHA, n-3) and arachidonic acids (ARA, n-6) is ineffective. Therefore, dietary supply of LCPUFAs is vital. It is hypothesized that the immunomodulatory properties of LCPUFAs can influence the development of immune system during early life. Evidence suggests that infants with high n-3 LCPUFA status are less likely to develop atopic conditions such as asthma and food allergies. In infants with genetic predispositions to developing food allergies, a T-helper-type-2 (Th2) skewed condition, supplementation of fish oil (containing DHA) has been reported to reduce allergen-specific immunoglobulin-(Ig) response associated with food allergies. The specific suppression of immune response is known as oral tolerance (OT). However, the effect of DHA supplementation, along with ARA, on the development of the infant immune system has not been fully elucidated. Thus, this thesis aims to understand the essentiality of dietary LCPUFAs in the development of the immune system and oral tolerance in healthy and allergy-prone conditions using pre-clinical rodent models during early life.

We conducted a series of animal experiments to study the effects of LCPUFAs supplementation during early life (suckling and/or weaning period) on the development of the immune system and OT in rodent offspring. OT was induced using repetitive oral exposure to ovalbumin (ova) followed by systemic challenge with ova and adjuvant. First, using a healthy Sprague Dawley rat model, we determine the effect of feeding stearidonic acid (SDA)-enriched diet (3% of total fat) on the LCPUFA status and immune functions in dams and offspring after

suckling and weaning. During lactation, supplementing the maternal diet with SDA increased the breastmilk levels of n-3 LCPUFAs (eicosapentaenoic (EPA) and docosapentaenoic acid (DPA) and DHA). Providing an SDA-diet during suckling and weaning only increased the splenocyte phospholipid levels of EPA and DPA while DHA and ARA showed no difference. In 6-week-old offspring supplemented with SDA-diet, lower levels of inflammatory cytokines; interleukin(IL)-6 and tumor necrosis factor-alpha (TNF-α), and higher anti-inflammatory cytokine (IL-10) were seen in splenocytes stimulated with lipopolysaccharide (LPS) *ex-vivo* than the control group (all *P*'s < 0.05). Therefore, SDA may have increased *in-vivo* biosynthesis of EPA and DHA but not DHA, which could have promoted an anti-inflammatory effect in 6-week offspring.

Next, we used allergy-prone rodent models to evaluate the effects of LCPUFAs, DHA and ARA on the development of the immune system and OT. Using Brown Norway rat offspring, we showed that dietary DHA (0.8% in suckling and 0.5% in weaning period) alongside ARA (0.5% in suckling and weaning), promoted OT development (35% lower plasma ovalbumin (ova)-specific-IgG, $P = 0.04$) in the offspring fed DHA+ARA-diet compared to control. More importantly, DHA+ARA during weaning promoted higher Th1 specific cytokines (TNF-α and interferon(IFN)-γ) by splenocytes stimulated with phorbol-myristate-acetate and ionomycin (PMAi, non-specific lymphocyte stimulant), compared to control group. Further, DHA+ARA during weaning was associated with a higher proportion of adaptive immune cells such as OX12+ and IgG+ B-cells in the spleen.

We then used suckling and weaning diets supplemented with DHA (derived from a plant-based source) and ARA (1% of total fat each) to study the immune system and OT development using another allergy-prone model, BALB/c mouse. Feeding lactating dams DHA+ARA showed an increase in breast milk DHA level (60%, P<0.001). In pups (3-wk and 6-wk), DHA+ARA

increased the splenocyte level of DHA without affecting ARA. Suckling period supplementation showed higher inflammatory cytokines (IL-1β, IFN-γ and TNF-α) production by LPS-stimulated splenocytes from 3-week offspring in comparison to controls. Furthermore, DHA+ARA resulted in higher T-cell cytokines (IL-2, IFN-γ and IL-1β) upon stimulation with anti-CD3/anti-CD28 and lower inflammatory-response by LPS-stimulated splenocytes. Last, DHA+ARA during weaning resulted in lower Th2 cytokines (IL-4 and IL-6) by ova-stimulated splenocytes and plasma levels of ova-IgE compared to the control, which may be beneficial for OT.

Overall, we showed that dietary supplementation of n-3 and n-6 LCPUFAs during early life can positively modulate the development of the immune system and OT in neonates prone to developing allergies.

Preface

This thesis is original work by Dhruvesh Patel. The research projects involving animals received research ethics approval from the Committee of Animal Policy and Welfare of the Faculty of Agriculture, Life and Environmental Sciences at the University of Alberta. This is listed as "Modulation of T cell function and development through the manipulation of diet-derived long-chain polyunsaturated fatty acids" with protocol number AUP00000125, June 2015. The contributions made by the candidate, Dhruvesh Patel, and the co-authors in completing this work are described here.

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Dedication

In memory of my grandmother, Madhuben Manubhai Patel. Om Shanti.

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ALA, alpha-linolenic acid

APC, antigen presenting cells

ARA, arachidonic acid

CD, cluster of differentiation

COX, cyclooxygenase

CTL, cytotoxic T cell

CTLA4, cytotoxic T lymphocyte-associated protein-4

DC, dendritic cell

DHA, docosahexaenoic acid

DPA, docosapentaenoic acid

DRI, dietary reference intake

EFSA, European Food and Safety Authority

EPA, eicosapentaenoic acid

GALT, gut associated lymphoid tissue

IEL, intraepithelial lymphocytes

IFN-γ, Interferon-gamma

Ig, immunoglobulin

IL, interleukin

LA, linoleic acid

LCPUFA, long chain polyunsaturated fatty acids

LOX, lipoxygenase

LPS, lipopolysaccharide

LT, thromboxane

MHC, major histocompatibility complex

MLN, mesenteric lymph node

MUFA, monounsaturated fatty acid

N-3, omega-3

N-6, omega-6

NK, natural killer

OT, oral tolerance

Ova, ovalbumin

PBMC, peripheral blood mononuclear cell

PG, prostaglandin

PL, phospholipid

PMAi, phorbol-myristate-acetate and ionomycin

PP, Peyer's patches

PUFA, polyunsaturated fatty acid

RBC, red blood cell

SD, suckling diet

SDA, stearidonic acid

SFA, saturated fatty acid

SPD, suckling period diet

TCR, T-cell receptor

TGF-β, transforming growth factor-beta

Th, T helper cell

Th1, T helper type-1

Th2, T helper type-2

TLR, toll-like receptor

TNF-α, tumor necrosis factor-alpha

Treg, T regulatory cell

TX, thromboxane

WBC, white blood cell

WD, weaning diet

LIST OF APPENDICES

Chapter 1. Introduction and background literature

1.1. Background on immune system development

The immune system is responsible for protecting against infectious agents, identifying and eliminating cancerous cells, and restoring homeostasis. It has two closely functioning arms, namely innate and adaptive (or acquired) immune systems. These two arms of immunity also work closely with other body systems; lymphatic, endocrine, neural, and circulatory (Murphy et al., 2017).

The immune cells are derived from hematopoietic stem cells of bone marrow that further differentiate into common lymphoid and common myeloid progenitors (Simon et al., 2015). The natural killer cells (NKs), B cells, and T cells are derived from common lymphoid progenitors, and the granulocytes (neutrophil, eosinophil, and basophil), monocytes (macrophages and mast cells) and dendritic cells (DCs) are derived from common myeloid progenitor (Murphy et al., 2017). The innate immune cells consist of neutrophils, eosinophils, basophils, mast cells, dendritic cells, macrophages, monocytes, and NKs. In contrast, adaptive immune cells consist of T and B cells (Marshall et al., 2018).

The organs of the immune system are classified into central (primary) immune organs and peripheral (secondary) immune (or lymphoid) organs based on their role in the development and maturation of immune cells (Ruddle & Akirav, 2009). The central immune organs consist of bone marrow and thymus, and the peripheral immune organs consist of the spleen, lymph nodes and mucosal-associated lymphoid tissue (located in the gut, respiratory tract, nasal tract, etc.) (Murphy et al., 2017). The naïve lymphocytes (of the adaptive immune arm) develop in the bone marrow and thymus. These cells then travel to peripheral immune organs, where they undergo

maturation. These immune cells provide long-lasting, antigen-specific and robust immunity. The innate immune system functions to protect through various mechanism, which includes an anatomical barrier, chemical barrier, humoral components (complement, coagulation, and cytokine system) and cellular components (i.e., phagocytosis) (Akira et al., 2006; Gasteiger et al., 2017). The protection provided by the innate immune system is quick, short-lived, and broadly specific to the pathogens.

An overtly strong response to harmless agents results in autoimmune and allergic (hypersensitivity) disorders (Marshall et al., 2018). For instance, the development of food allergies occurs due to the overactivation of the immune response toward food antigens. When these antigens are exposed to the mucosal membranes, they bind to IgE and trigger the activation of mast cells and the Th2 type response (Satitsuksanoa et al., 2018; Vickery et al., 2011). This only occurs if the immune system is sensitized toward that specific food antigen due to previous exposure. Atopic individuals are predisposed to developing allergies and are likely to develop sensitization towards various antigens, including the ones present in food. Upon initial exposure to food antigens, the sensitization process involves the differentiation of naïve T cells into Th2 cells (Burks et al., 2008; Chin & Vickery, 2012). Subsequently, the activation of B cells to antigen-specific IgE-producing B cells under the exposure of Th2 type cytokines (IL-4, IL-5, IL-9 and IL-13) (Johnston et al., 2014). Some types of food allergies can also be classified as non-IgE mediated and do not involve a characteristic IgE response associated with food allergies (Cianferoni, 2020).

1.1.1. Innate immune cells

During the first few days of an infection, the innate immune system plays a crucial role in controlling infection and activating the adaptive immune system through producing cytokine, chemokines, and presentation of an infectious agent to the lymphocytes. The innate immune system responds within minutes to hours of infection, and it generates a non-specific response toward foreign threats. Emerging evidence suggests that there might be some overlap between innate and adaptive immune cell functions. For example, innate lymphoid cells (ILCs) are derived from common lymphoid progenitor but generate non-specific immune responses like innate immune cells and help in the activation of T cells (Gasteiger et al., 2017).

Granulocytes: The neutrophil, eosinophil and basophil comprise the granulocyte portion of white blood cells (WBCs). These cells derive their name from their small intracellular particles (granules) and function as effector cells of the innate immune system (Marshall et al., 2018). The neutrophils are the most abundant immune cells in the bloodstream. The function of neutrophils is phagocytosis of pathogens that may be coated with antibodies and complements, the release of anti-microbial proteins and neutrophil extracellular traps. These cells are the first ones to reach the site of infection and initiate an immune response, which involves the recruitment of monocytes and other cells (Gasteiger et al., 2017). The eosinophils also contain granules that have basic protein crucial in fighting parasitic, viral, bacterial and fungal infections. In allergic disease, eosinophils become activated and produce inflammation by producing IL-4, IL-5 and IL-13 (Th2 cytokines) (Bae et al., 1999). Akin to neutrophils, antigen presentation can also be performed by these cells. The basophils become activated in the blood by the cross-linking of the FceRI receptor upon binding to IgE and begin degranulation. The granules in basophils contain histamines, major basic proteins, as well as cytokines like IL-4 and IL-13. The functioning of

mast cells are similar to basophils; both are involved in the allergic response (Johnston et al., 2014).

Dendritic cells (DC): These immune cells are known as professional antigen-presenting cells (APCs) due to their crucial role in the activation of the adaptive immune system through presenting novel antigens to T cells (Merad et al., 2013). DCs detect foreign antigen through pattern recognition receptors (PRR), such as Toll-like receptor (TLR), NOD-like receptor (NLR), etc. Their primary function is to capture, process and present antigens on major histocompatibility complex (MHC) molecules for T cells. The phenotype of DC involved in the activation of T cells can predict the polarization of T cells into either T regulatory cells (Tregs), Th1, Th2, or Th17. The role of DC in the development of tolerogenic responses and Treg cells has been widely reported (Merad et al., 2013; Nagatani et al., 2006; Papaioannou et al., 2021). More importantly, DC in the gut mucosa is specialized to sample and present many antigens derived from dietary proteins (Stagg, 2018). These DC are understood to be bone-marrow-derived conventional DC (cDC), and they are implicated in the development of oral tolerance (OT) through the induction of peripheral Treg cells (Esterházy et al., 2016). The plasmacytoid DCs (pDCs) are 0.1 to 0.5% PBMC and are also present in lymphoid organs. The pDCs are involved in a high level of type-1 interferon production and are implicated in autoimmune disease (Merad et al., 2013).

Macrophages: Most macrophages are long-lived, tissue-resident and terminally differentiated immune cells (Ginhoux & Jung, 2014). However, during the course of life, they can also be derived from other bone-marrow-derived myeloid progenitors such as monocyte-derived DC or monocyte-derived macrophages. The developmental origins of various macrophage phenotypes have been described elsewhere (Ginhoux & Jung, 2014). Macrophages can detect products of

bacterial and other microorganisms through signals from various receptors such as TLR, NLR, scavenger receptor, mannose receptor, and more (Balestrieri et al., 2021). Upon detection, the harmful microorganism is cleared through phagocytosis, followed by processing and presenting antigen-specific to the microorganism through cell surface molecule MHC (Mills et al., 2000; Muraille et al., 2014). This process is crucial for activating antigen-specific T cells (Guerriero, 2019). Macrophages also play an essential role in tissue homeostasis through the phagocytic removal of apoptotic cells and providing survival signals to other cells in the tissue. The monocytes leave the blood to enter the tissue during infection, where they differentiate into monocyte-derived macrophages. These cells produce inflammatory markers (IL(interleukin)-1β, IL-6, TNF (tumor necrosis factor)-α, IL-8 and IL-12) that contribute to local and systemic inflammation (Arango Duque & Descoteaux, 2014). Tissue resident macrophages evolve to acquire specialized functions within the environment of the tissue. For instance, the macrophages present in the gut may contribute to the development of OT by transferring food antigens to CD103+ DC of the gut (Mazzini et al., 2014).

Natural killer (NK) cells: Although derived from the common lymphoid progenitors (akin to B and T cells), NK cells function like innate immune cells (Guilmot et al., 2011). Their primary function involves directly killing virally infected and cancerous cells (Vivier et al., 2008). NK cells possess activating and inhibiting surface receptors, and the cumulative signals from these receptors dictate the NK cells killing outcome. The NK cells release cytolytic granules, containing perforin and granzymes, to kill the target cell through lysis.

1.1.2. Adaptive immune cells

The adaptive immune system generates antigen-specific and long-term responses with immunological memory. Lymphocytes function through various mechanisms to neutralize and eliminate harmful threats. The B cells of adaptive immunity can undergo cell receptor maturation (B-cell receptors, immunoglobulins(Ig)) upon exposure to unknown antigens so that they can effectively bind to the foreign antigen during subsequent exposure. Similarly, the T cell receptor (TCR) comprises two TCR chains (α and β chains or γ and δ chains) and six CD3 chains (1 CD3δ, 2 CD3ε, 1CD3 γ and 2ζ chains). The antigen presented by APC is recognized by the antigen-binding site on α:β chains of TCR. The antigen-binding site undergoes gene rearrangement during T cell development in the thymus and generates a diverse repertoire with each cell expressing unique TCR with a single antigen-binding site.

T cells: The T lymphocytes (or T cells) undergo development in the thymus before entering circulation as naïve T cells (Luckheeram et al., 2012). These naïve T cells undergo an additional transformation when they recognize a foreign antigen presented in the context of MHC by APCs, where they become activated and differentiate into effector T cells. This process is called clonal selection (Murphy et al., 2017). The differentiation of T cells into specific subtypes is a complex process and relies on signals from APC, co-stimulatory signal, cytokine environment, nature of the antigen, etc. (Smith-Garvin et al., 2009). The naïve CD8+ T cells upon activation differentiate into cytotoxic T (CTL) cells. CTLs identify and kill virally infected cells by recognizing foreign antigens presented on MHC-I molecules (Smith-Garvin et al., 2009). The T helper (Th) cells do not directly kill target cells but generate cytokines that activate other immune cells such as B cells, macrophages, etc. (Zhu, 2018). The role of Tregs involves the suppression of the activity of other lymphocytes through the production of transforming growth

factor (TGF)-β, IL-10 and other tolerogenic signalling molecules. The activation of T cells is an intricate process and is still a subject of ongoing research (Hwang et al., 2020; Smith-Garvin et al., 2009). However, the simultaneous presence of three signals is necessary for the activation of naïve T cells. The first signal is received upon correct recognition of the antigen and MHC combination on the APC by the T-cell receptor complex (CD3 along with co-receptors; CD4 or CD8). The second signal is received from the co-stimulatory molecule upon binding to their ligand on APC. For instance, the binding of the co-stimulatory molecule CD28 of T cell to CD80/CD86 on APC provides the second co-stimulatory signal. The third signal is received through the binding of cytokine released by the APC to the cytokine receptor on the T cells. For instance, the activation and differentiation of naïve T cells into Th1 cells occur under IL-12, Th2 under IL-4 and Th17 under IL-6 and IL-23 (Smith-Garvin et al., 2009; Zhu, 2018).

B cells: The B cells (B lymphocytes) develop in the bone marrow and enter the circulation with surface-bound primary immunoglobulin (Murphy et al., 2017). B cells at this stage are still considered naïve and undergo additional editing of B-cell receptors (also known as immunoglobulins) (Harwood & Batista, 2010). Upon encountering the antigen, the B cell receptors undergo further affinity maturation towards select antigen and isotype switching to produce effective antigen-specific responses. The mature B cells differentiate into effector plasma cells (or memory cells) that can release a large number of immunoglobulins (Harwood & Batista, 2010). Primary B cells start expressing primary immunoglobulins (IgM and IgD), which become secondary immunoglobulins (IgG, IgA or IgE) upon isotype switching. Each isotype of B cell immunoglobulins has unique effector functions. For instance, IgM predominantly activates the complement system, IgE binds to mast cells and eosinophils involved in allergic responses, and IgA neutralizes pathogens in the mucosal membrane (Vazquez et al., 2015). The

B cell activation process called T-cell dependent B cell activation involves receiving signals from B-cell receptors and from T helper cells that recognize the antigen on the B cell (presented in the context of MHC-II). The B cells can also be activated by cross-linking of B-cell receptors bound to multivalent antigens or pathogens, through a process called T-independent B cell activation.

1.1.3. Central and peripheral immune organs

Central immune organs, thymus and bone marrow, are involved in producing, developing, and educating immune cells. The hematopoietic stem cells originate from bone marrow and develop into common lymphoid progenitors and myeloid progenitors. The lymphoid progenitors develop into B, T, NK, and innate lymphoid cells. The T cell undergoes an additional development phase in the thymus where it develops T cell receptors specific for a wide range of antigens before entering the circulation. In the bone marrow, the common myeloid progenitors develop into dendritic cells, macrophages, neutrophils, eosinophils, basophils and mast cells. Peripheral immune organs include lymphoid organs such as the spleen, lymph nodes, tonsils, and mucosal membranes. These organs function to capture and present foreign antigens from circulation. Here the mature APC present the foreign antigen to lymphocytes in the context of MHC molecules which provides early signals for their activation and polarization into various specialized cells.

Lymph nodes: These are small bean-shaped structured distributed throughout the body and connected by a network of lymphatic vessels responsible for draining lymphatic fluid from nearby peripheral regions. The lymph nodes are vital for initiating adaptive immune response as they provide a crossroad between antigens and lymphocytes (Ruddle & Akirav, 2009). This occurs when the foreign antigen is drained from the site of infection by APC or lymphatic fluid

to the lymph node where the lymphocytes are present. The anatomy of a lymph node is broadly divided into the outer cortex, paracortical and inner medulla areas (Macpherson & Smith, 2006). During infection, lymphatic fluid drains into the lymph node, passing through lymphoid follicles, B cell-rich area followed by T cell-rich area in the paracortical area and into the medulla where the macrophages and plasma cells are present (Murphy et al., 2017). Here the antigen naïve T cells encounter DCs and macrophages presenting antigen resulting in their activation and subsequently activating B cells.

Spleen: The function of the spleen is to drain the blood and identify pathogens present in the bloodstream (Lewis et al., 2019). It is also known as the graveyard of red blood cells (RBC) as it functions to remove RBC from circulation. The spleen has two distinct regions; the red pulp area, where RBCs are removed and the white pulp area, where the lymphocyte regions surround the arterioles (Lewis et al., 2019; Murphy et al., 2017). The white pulp region of the spleen consists of areas rich in T cells, B cells (plasma cells) and APCs (DC, macrophages, and B cells). Akin to lymph nodes, these spleen regions are responsible for the activation of T cells by the APC specific to blood-borne antigens, resulting in their differentiation into effector cells (Ruddle & Akirav, 2009).

Gut Associate Lymphoid Tissue (GALT): The mucosal membranes are present in regions that are open to the air, food, and foreign microorganisms. Therefore, our body has specialized features of the immune system to protect us from exposure to potentially harmful agents. The mucosal membrane of the gut is protected by a specialized set of immune organs collectively known as gut-associated lymphoid tissues (GALT) (Murphy et al., 2017). The gut comprises of several lymphoid structures such as Peyer's patches, Microfold (M) cells, mesenteric lymph nodes (MLN), and cells of intraepithelial membrane and lamina propria. The primary function of

the GALT is to protect the body from the foreign antigen and microbiome that enters the gut through the oral route (Spahn & Kucharzik, 2004). All the antigens from the epithelial membrane of the gastrointestinal tract are captured and processed by the APC and subsequently presented to the lymphocytes (Castro-Sánchez & Martín-Villa, 2013; Shi et al., 2017; Stagg, 2018; Uematsu et al., 2008). The M cells are present in the epithelial membrane lining and are specialized in collecting free-floating antigens from the lumen (Castro-Sánchez & Martín-Villa, 2013). The Peyer's patch is present right under the epithelial membrane. It contains B cell follicles, germinal centers, T-cell-rich areas and tissue-resident APCs (DCs and macrophages) (Castro-Sánchez & Martín-Villa, 2013). The antigens are immediately sampled by the DCs of the Peyer's patches and can activate lymphocytes, allowing them to protect the mucosal tissues. In addition to Peyer's patches, the mesentery of the gut houses a series of lymph nodes identified as MLNs (Castro-Sánchez & Martín-Villa, 2013). The MLN drains foreign antigens in the lymphatic fluids of the gut that may have escaped the Peyer's patches (Spahn et al., 2002). Here these foreign antigens, either free-floating or presented in the context of APCs, can interact with lymphocytes to promote their activation and differentiation into effector cells.

Emerging evidence has suggested the role of a specialized category of heterogeneous lymphocytes in the intraepithelial region, known as intraepithelial lymphocytes (IEL) (Haitao Ma et al., 2021). These cells reside in the gut and provide the first line of defence against foreign antigens and regulate gut immunological responses (Haitao Ma et al., 2021). Similarly, the cells of lamina propria also support the immune responses to antigens entering from the gut. The lamina propria form the loose connective tissue at the base of the mucosal membrane and provides nutrition and support to the epithelial cells. The immune cells of lamina propria includes adaptive and innate immune cells and functions to protect by maintaining the mucosal

membrane and regulating the plethora of commensal microorganisms of the gut (Atarashi et al., 2010; Shi et al., 2017; Uematsu et al., 2008). It is essential to highlight that the development of OT is also initiated in the gut and will be described later (Castro-Sánchez & Martín-Villa, 2013).

1.1.4. Central tolerance begins in primary lymphoid organs – bone marrow and thymus.

T-cell development in the thymus ensures that the T-cell receptor (TCR) is functional but tolerant to self-antigens (Smith-Garvin et al., 2009). The T cell development undergoes rearrangement of TCRβ and TCRα chains, positive selection of T cells with functional TCR, and negative selection of TCR with very high affinity to self-antigen. After the development of functional TCRαβ, the T cell is called double positive as they express both co-receptors, CD4 and CD8. The positive selection process promotes the survival and selection of T cells that can successfully bind a self-antigen presented in the context of an MHC with moderate to high affinity. If the T cell recognizes the antigen presented in the context of MHC-I, it becomes a CD8+ T cell, but when the T cell recognizes the antigen in MHC-II, it becomes a CD4+ T cell. Further, the TCR with high affinity to self-antigen undergoes negative selection resulting in self-induced apoptosis (Palmer, 2003). Removing auto-reactive T cells is called clonal deletion and ensures the lymphocytes are self-tolerant. The last step before they can be fully functional involves T cell activation by APCs in circulation when a specific antigen is presented in the context of a select MHC molecule. This results in the polarization of CD4+ T cells into Th1, Th2, Th17, Treg, and T helper follicular cells (T_{FH}) and the CD8+ T cells into effector cells and CTL with cell-mediated immunity, effector functions or memory functions (Zhu, 2018).

The process of central tolerance for B cells begins in the bone marrow where the autoreactive B-cell receptor undergoes editing of B-cell receptor to generate surface-bound IgM that is tolerant

to self-antigens (Harwood & Batista, 2010). The specific mechanisms behind self-tolerance induced in the bone marrow have been described elsewhere (Nemazee, 2017). A breach in the maintenance of self-tolerance can result in the development of autoimmune diseases. Since central tolerance is not perfect, the lymphocytes undergo additional development through exposure to self-antigen in peripheral lymphoid organs, inducing peripheral tolerance.

1.1.5. Peripheral tolerance

Upon entering peripheral lymphoid organs, the potentially self-reactive lymphocytes undergo deletion to ensure self-antigen tolerance (Randall & Mebius, 2014). The induction of peripheral tolerance can occur through various mechanisms. The clonal deletion of T cells can also be induced in the peripheral lymphoid organs. When the T cell receives a signal from TCR without necessary co-stimulatory signals, a state of anergy is induced, by which no activation signals are initiated (Schwartz, 2003). Additionally, the role of Tregs in developing peripheral tolerance is well described and the subject of ongoing research (Vignali et al., 2008). The T regulatory cells aim to induce an immunosuppressive response in lymphocytes and other immune cells to promote peripheral tolerance. The Treg cells that develop in the thymus are called natural Tregs (nTregs), whereas Tregs derived from naïve T cells in the peripheral lymphoid organs are called inducible Tregs (iTregs).

The implication of tolerance is not only restricted to protection from autoimmunity but also involves preventing unnecessary immune responses to harmless dietary antigens. The phenomenon of OT, also referred to as mucosal tolerance, is an outcome of peripheral tolerance induced through the exposure of food antigens via the oral route. The development of OT helps prevent food allergies.

1.1.5.1. Oral (mucosal) tolerance (OT)

OT is defined as an active suppression of systemic immune responses towards a harmless food antigen that is previously introduced to the immune system through the oral route. The development of OT involves immune cells of peripheral lymphoid organs, especially GALT (Wu & Weiner, 2003). Neonates are exposed to foreign antigens soon after birth, and their immune system needs to differentiate between harmful and harmless antigens to provide optimal immunity. The development of OT towards some of these harmless antigens begins early in life through exposure to breastmilk and later through weaning food (Field, 2005; Kizu et al., 2015). The immune system during early life possesses some unique characteristics that allow the successful development of OT that will last for life (Adkins et al., 2004; Basha et al., 2014; Verhasselt, 2010). Briefly, the immune component transferred through the mother's breastmilk provides passive immunity and an environment to support a tolerogenic response (Field, 2005; Verhasselt, 2010). Breastfed infants are exposed to residual dietary proteins in the maternal diet since they can enter the breastmilk through APC with peptide-bound MHC molecules, antigens bound to secretory IgG or free-floating peptides derived from dietary protein. The breastmilk also contains cytokines like IL-10, TGF-β and growth factors that may be important for developing mucosal membranes in the neonates. The GALT (Peyer's patches and MLN) of neonates contains a higher proportion of CD103+ DC, IL-10-producing B cells and TGF-β-producing epithelial cells, all of which play a crucial role in the development of Tregs suppressive function and OT. The development of OT in neonates is essential in preventing food allergies.

The development of OT requires exposure of antigens through the oral route, i.e., akin to the consumption of food protein in our diet. This dietary protein gets digested and absorbed through

the gut mucosal membrane. One such protein commonly used in research is ovalbumin (ova), found in egg white. Ova gets digested and absorbed in the small intestine where it is sampled by APCs such as dendritic cells (DCs), macrophages and B cells. Subsequently, these cells further break down the digested ova peptides into a small sequence of amino acids that can be presented on the major histocompatibility complex-II (MHC-II) of APC. The APCs in the gut-associated lymphoid tissues, mesenteric lymph nodes (MLNs) and Peyer's patches (PP), are primarily responsible for inducing tolerogenic responses towards specific food proteins, such as ova. Here the MHC-II of APC interacts with T cell receptors of naïve T cells and starts developing antigen-specific maturation and differentiating into specialized mature T cells, that can generate a robust immunosuppressing response. In the development of OT, the T cell-based suppression of immune response can occur through different mechanisms based on the quantity of antigen during primary exposure (Rezende & Weiner, 2017; Weiner et al., 2011). Briefly, a high dose of ova exposure (>0.5mg ova /g of bodyweight) induces OT by T cell anergy/deletion, described elsewhere (Strobel, 2001; Weiner et al., 2011). Whereas a low dose continuous exposure of ova (<0.1mg ova /g of bodyweight) induces OT by antigen-specific suppression of regulatory T cells (Tregs) (note; although inducible Tregs and natural Tregs have different activation sites for this review they are considered to have similar immunosuppressive functions). For the purpose of this review, where OT in neonates is considered, Tregs-based immunosuppression in the development of OT will be prioritized. In neonates, Tregs specific to ova is passively transferred through breastmilk from dams tolerized to ova, resulting in OT development during early life (Ray et al., 2011). However, several other factors also influence the outcome of OT induction, which are described later.

Factors like intestinal mucosal immunity, food immunomodulatory effects, and gut microbiota composition can affect OT development (Berin & Mayer, 2013; Yang et al., 2021). Although the usefulness of OT in preventing food allergy has reached clinical trials from pre-clinical animal models, still there is no standardized protocol developed to study OT development in neonates (Rezende & Weiner, 2017; Romantsik et al., 2018). This may be due to the challenges involved in using a pre-clinical model of food allergy to study OT development toward food antigens (Bøgh et al., 2016). Multiple factors can influence the successful induction of OT, and though many are relatively well understood, the effect of nutrition remains to be explored (Garside & Mowat, 2001; Weiner et al., 2011).

1.1.5.2. Hypersensitivity and food allergies

On the other end of the spectrum, a heightened immune response towards food antigens results in the development of food allergies or food hypersensitivities. Food allergy prevalence during early infants has been a growing cause of concern (Elghoudi & Narchi, 2022). Unlike OT, infants that develop allergies get sensitized towards orally introduced food antigens and elicit an antigen-specific immune response. The characteristic sign of food allergy is the activation of the Th2 immune response resulting in a high level of antigen-specific IgE production (Chin & Vickery, 2012). Due to genetic factors, some infants are more likely to generate a prolonged Th2-biased response (IL-4, IL-5 and IL-13), predisposing them to develop allergies (Van Der Velden et al., 2001). Additionally, a breakdown of Th17 responses in allergic children has also been reported (Dhuban et al., 2013). The role of mucosal permeability during early infancy has also been suggested, as an underdeveloped gut could allow a large concentration of antigens to enter the gut and promote immune responses (Johnston et al., 2014). Some dietary changes are

also implicated in promoting food allergies, such as Vitamin D deficiency, a diet with a high n-6 to n-3 fat ratio, a low antioxidant diet and obesity (Lack, 2012).

The use of rodent models in immunology literature has enabled us to understand the human immune system. Rats and mice share several basic aspects of the immune system with humans. This allows us to translate findings from rodents to humans, especially when research in humans is ethically challenging. The strain of rodent models used in research can be broadly classified into outbred and inbred based on their genetic variability. Outbred strains such as Sprague-Dawley rats, Wistar rats and CD-1 mice, are used to study complex diseases such as cancer, and drug testing (Wildner, 2019), while inbred strains, such as Brown Norway rat, Lewis rat, BALB/c mouse, and C57BL/6 mouse are used when limited genetic variability is required. Further, the genetic background of the rodent model is also selected based on the pattern of their immune responses. In general, outbred models can generate a balanced Th1/Th2 immune response while the inbred models generate either a Th2 or Th1 biased immune response, as seen in BALB/c mouse and C57BL/6 (Watanabe et al., 2004). Using a model with a Th2-biased immune response can allow us to study immunological disorders associated with a skewed Th2 response, i.e., asthma, allergies and hypersensitivity reactions (Burns-Naas et al., 2008). Further, the human immune system also becomes Th2 dominant during early life, pregnancy and lactation and using Th2-biased models can be useful (Burns-Naas et al., 2008). Mice models compared to rats are more suitable to study mechanisms behind the development of OT since the immune system of mice is more characterized and offer a wide range of species-specific antibodies and reagents for immune assays. However, there are potential limitations to using young mice pups due to their relatively smaller size when compared to rat pups. This can sometimes restrict the quantity of tissue and cells available for laboratory analysis. The Th2 skewed immune system of

BALB/c mice and Brown Norway rats provides an environment akin to the immune system observed in early life. Further, these inbred rodent strains (i.e., Brown Norway and BALB/c) can be useful as they limit differences in immune parameters due to genetic differences compared to outbred strains (i.e., Sprague Dawley rat).

1.2. Immune system during early life

The mother's immune system during pregnancy undergoes major changes to protect the growing fetus from harmful foreign microorganisms and adverse effects of the immune response. The prenatal and postnatal transfer of passive immunity through the placenta and later through breastmilk provides immune protection during early life. In the prenatal period, the fetal immune system comprises maternal IgG complexes transferred through the placenta along with other immune responses generated by the maternal immune system during pregnancy. Additionally, the exposure of the maternal immune system to various challenges or vaccination also prime the developing immunity of the fetus (Wilcox & Jones, 2018). An observational study by Song et al. (2021) reported maternal SARS-CoV-2 specific IgG in infants, passively transferred through the placenta, born to mothers infected with COVID-19 during pregnancy.

The passive transfer of immune components through breast milk provides protection during early life in neonates. In the first six months, neonates can generate immunoglobulin responses to various antigens, in the form of plasma IgM, IgG, and secretory IgA (Basha et al., 2014). During this period, the antigen naïve neonatal immune system is exposed to large amounts of foreign antigens and will need to develop robust long-term immunity against them. However, the immune responses generated during early infancy rely heavily on innate immune cells and passive immunity transferred from the mother. Although the adaptive immune cells are present

and functional at birth, their proportions are significantly lower compared to adults (Adkins et al., 2004). During the first year of life, the inexperienced immune system encounters a large amount of foreign- and self-antigen in circulation leading to the development of lymphocytes with a highly diverse repertoire of receptors (Foth et al., 2021; Wedderburn et al., 2001). This has also been shown with rodents, where the proportion of lymphocytes in spleen (T and B cells) gradually increase, and the proportion of innate immune cells decline to resemble adult-like immune cell distributions (Pérez-Cano et al., 2005; Pérez-Cano et al., 2007; Pérez-Cano et al., 2012).

The neonatal immune system is different from the adult immune system. Neonates have a typical Th2 dominant immune response during early life. This may be partly derived from the passive transfer of maternal immunity during gestation (Zaghouani et al., 2009). The Th2 bias promotes the preferential production of IL-4, IL-5 and IL-13 by CD4+ T cells. In addition to skewed Th2 immune responses, they also display underdeveloped counteracting Th1 responses during this period. A dominant Th2, suboptimal Th1 immune response and lack of immunological memories make infants more susceptible to infections and allergic diseases (Adkins et al., 2004). The role of DCs has been suggested in the lack of a strong Th1 response after birth (Zaghouani et al., 2009). In mouse pups younger than six days, the absence of IL-12-producing DC (CD8α+CD4- subset) was shown to be involved in the prevention of Th1 development and promotion of Th2 development (Zaghouani et al., 2009). However, the lack of strong Th1-type inflammatory response allows the infants to develop tolerance to self-antigens (in peripheral lymphoid organs) and harmless common environmental antigens without generating unnecessary inflammatory responses (Th1 cytokines, interferon (IFN)-γ and TNF-α) (Adkins et al., 2004). The imbalance in Th1 versus Th2 response also influences the T-cell-dependent activation of B cells and

subsequent immunoglobulin production. Therefore, a balance between Th1 and Th2 immune response is important for the development of neonatal immune system. Nonetheless, exposure to these antigens also promotes the development of adaptive immune cells (T cells and B cells) and the diversification of lymphocyte colonies with unique cell receptors (TCR and immunoglobulins).

The neonatal immune system is highly adaptive to environmental challenges and protective against self-antigens and non-self innocuous antigens (Tsafaras et al., 2020). Several aspects of adaptive immune responses in neonates are considered to be immature. Therefore, the innate immune cells are heavily involved in an immune response. Some reviews have highlighted the lower proportions and functionality of CD8+ T cells, CD4+ T cells that produce IL-2, IFN-γ and TNF-α, and T follicular cell that helps B-cell proliferation (Basha et al., 2014). The proportions of Treg cells are higher in neonates and explain the reduced inflammatory response by other T cells. To compensate for this, the functioning of innate immune cells, such as the neutrophils, NKs, monocytes and DCs, is vital for optimal protection. Compared to adults, the higher proportion of innate cells in neonates can protect against viral infection through phagocytosis and antibody-dependent cell-mediated cytotoxicity (Guilmot et al., 2011). However, some innate immune cells' functionality is suboptimal compared to adults (Georgountzou & Papadopoulos, 2017). The innate immune system also relies on mechanical and anatomical barriers to prevent the invasion of foreign antigens. As the neonatal gut begins to encounter food and other foreign antigens, the mucosal epithelial membrane goes through the maturation of tight junctions in the gut. This restricts the number of foreign antigens that can enter the GALT through a leaky gut (Georgountzou & Papadopoulos, 2017). A schematic of an essential component of innate immune system development during early life through adulthood is shown by Georgountzou and

Papadopoulos (2017). The period after birth is considered a particularly critical developmental window for the maturation of the immune system (Simon et al., 2015). Therefore, the dietary fats provided during this period have long-term effects on immune functions that can be considered beneficial for the optimal development of the immune system (Innis, 2011; Krauss-Etschmann et al., 2008; Palmer, 2011). For instance, the balance of omega-3 (n-3) and n-6 fatty acids in the diet can regulate the functioning of the immune system (Harbige, 2003; Simopoulos, 2002).

1.3. Essential fats: n-3 and n-6 fatty acid

The fat in our diet comprises saturated fats and unsaturated fats, which include monounsaturated and polyunsaturated fats. Polyunsaturated fatty acids (PUFA) comprise two families of fats, n-3 and n-6. The chemical structure of the n-3 family of fatty acids has characteristic double bonds starting at 3rd carbon on the fatty acid chain from the methyl end. Likewise, n-6 comprises fatty acid chains with double bonds starting at the 6th carbon from the methyl end. N-3 has myriad health benefits at different stages of life. The consumption of n-3 fats has been implicated in promoting health and preventing risk for certain diseases, such as cardiovascular disease, diabetes, cancer, allergies, and more (Shahidi & Ambigaipalan, 2018). A systematic review has concluded that n-3 can reduce the risk of coronary artery disease, arrhythmia, and other cardiovascular diseases (Abdelhamid et al., 2020). Similarly, n-6 have been described in the literature for their role, beyond providing energy, in gene expression, cellular communication and regulation of mechanist pathways through modulating cell membranes and generating lipid mediators (Saini & Keum, 2018).

1.3.1. Bioconversion of precursor PUFAs (ALA and LA) to long-chain PUFAs (LCPUFA)

The precursor n-3 PUFA, α-linolenic acid (ALA, 18:3 n-3), is essential for humans as it can not be synthesized in our body, just like n-6 PUFA precursor linoleic acid (LA, 18:4 n-6), and a dietary supply is their only source. The family of n-3 PUFA includes ALA (18:3 n-3), stearidonic acid (SDA, 18:4 n-3), eicosapentaenoic acid (EPA, 20:5 n-3), docosapentaenoic acid (DPA, 22:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3). Herein the fatty acid chain with 20 or more carbon is considered long-chain PUFAs (LCPUFAs). The oils extracted from plant seeds (flaxseed, soybean, and canola) are rich in ALA and SDA (Subedi et al., 2015). The n-3 LCPUFA (EPA and DHA) are commonly found in fatty fish (salmon, mackerel and whitefish), krill oil, seafood and algae sources, as well as fish oil supplements (Calder, 2016).

Although n-3 LCPUFAs can be biosynthesized from their precursor ALA, the conversion rate is very low. The conversion of ALA to EPA is estimated to be between 8 to 21%, and the conversion of ALA to DHA to be less than 1% in adults and about 1% in infants (Brenna et al., 2009; Burdge & Wootton, 2002). The bioconversion of DHA from SDA is considered more efficient than ALA, as it bypasses one of the rate-limiting steps in the LCPUFA biosynthesis pathways (James et al., 2003). SDA is not commonly found in the human diet. Nevertheless, plant seed (soybean (Harris, 2012) and flax seed (Lefort et al., 2016; Subedi et al., 2015)) have been genetically modified to increase the SDA composition of seed oils, making them an attractive choice to improve n-3 LCPUFA biosynthesis (Walker et al., 2013). SDA has also been identified as a sustainable choice for attaining n-3 PUFA intake due to its potential health benefits (Deckelbaum et al., 2012; Surette, 2013; Whelan, 2008).

The biosynthesis of arachidonic acid (ARA) from LA is highly restricted and shown to be about 0.5% in healthy young adults (Emken et al., 1994) (Adam et al., 2008). Therefore, low conversion rates, higher in men than women, are insufficient to meet the requirements of LCPUFA at different life stages (Burdge & Wootton, 2002). The metabolic conversion of ALA to EPA/DHA relies on the same rate-limiting enzymes, elongases (Δ6-elongase and Δ5-elongase) and desaturases (Δ6-desaturase, Δ5-desaturase, and Δ4-desaturase) that are shared between n-3 and n-6 PUFAs. These enzymes are also shared with the n-6 family for the metabolic conversion of LA to ARA. Since the n-3 and n-6 PUFAs share the same bioconversion pathways, the ratio and dosage of dietary LA and ALA can influence the efficiency of LCPUFA biosynthesis. Furthermore, some genetic factors also contribute to the discrepancies observed in conversion rates at population levels (Dyall et al., 2022; Gibson et al., 2011). Therefore, the only practical way to improve n-3 LCPUFA status is through dietary supply of EPA and DHA. Along with fatty fish and seafood, consuming foods fortified with EPA and DHA can also provide these LCPUFAs.

1.3.2. Dietary recommendations of essential fatty acids – ALA and LA

The dietary reference intakes (DRIs) for ALA and LA, as essential fatty acid intake, were published by the Food and Nutrition Board of the Institute of Medicine of the National Academy of Science in 2005 (Institute of Medicine, 2005). This provides DRI for people living in the United States of America and Canada. The adequate intake of ALA for adult men and women was set to be 1.6 g/day and 1.1 g/day, respectively. The adequate intake of LA for adult men and women was set to be 17 g/day and 12 g/day, respectively. For pregnant and breastfeeding women, the adequate intake for ALA and LA was set to be 1.3–1.4 g/day and 13 g/day, respectively. For infants, the adequate intake is set for total n-3 PUFAs and total n-6 PUFAs, not

just their precursor (ALA and LA, respectively). For infants between 0–6 months, n-3 and n-6 PUFAs were set to be 0.5 g/day and 4.4 g/day, respectively. For infants between 7–12 months, n-3 and n-6 PUFAs were set to be 0.5 g/day and 4.6 g/day, respectively.

The dietary fat intake determined by European Food and Safety Authority (EFSA) was described in 2010 (EFSA Panel on Dietetic Products & Allergies, 2010). The adequate intake for ALA and LA was set to be 0.5% and 4% of energy intake for the population residing in European countries. Regarding n-3 LCPUFAs, the adequate intake of EPA plus DHA was set to 250 mg/day in adults, considering the beneficial effects on cardiovascular outcomes. An additional 100 to 200 mg/day was set for pregnant and lactating women to compensate for the increasing maternal/fetal needs. Further, for infants 6–24 months of age, the adequate intake of DHA was set to be 100 mg/day considering their role in the development of visual functions. The dietary intake of 1 to 2 fatty fish servings (75g is one serving) can provide up to 250 mg/day of EPA+DHA and achieve dietary reference values.

1.3.3. Immunomodulatory properties of PUFA

The change in dietary PUFAs has long been known to influence immune system functions. The role of LCPUFA, through their immunomodulatory properties, can influence inflammatory processes. The n-3 PUFAs support anti-inflammatory and inflammation-resolving processes, whereas the n-6 PUFAs are known to promote inflammation processes. The mechanism of action behind these processes is a subject of ongoing research. However, there are several mechanisms and pathways thought to be involved. The PUFA actively participate in the formation of phospholipids present in the cellular membranes, and several of their action begins here.

LCPUFAs such as DHA and ARA are highly concentrated in the phospholipids of cell membranes (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and sphingomyelin). The cell membrane LCPUFAs can influence cell functions through the modulation of membrane fluidity and the formation of lipid rafts, which can subsequently influence the signalling kinetics. The fatty acids with a higher degree of desaturation (i.e., double-bond) will increase the membrane fluidity, whereas saturated fatty acid promotes rigidity to cell membranes (Calder et al., 1994; Fan et al., 2018; Los & Murata, 2004; Maulucci et al., 2016). The lipid raft is a subdomain in the cell membrane rich in sphingolipids, cholesterol, side chains of phospholipids, and intramembrane proteins. These structures provide an essential platform to facilitate the interaction of various signalling molecules. LCPUFAs influence cell function through receptors, metabolites, and gene expressions (Calder, 2012). For instance, the binding of G-protein couple receptor-120 (GPR120) found on macrophages and adipocytes with DHA can inhibit the transcription factor NF-κB. This gene involves the expression of various inflammatory cytokines (IL-6, TNF-α and IL-1β), adhesion molecules, nitric oxide synthase, etc. Further, DHA (and other PUFAs) can also influence another transcription factor PPAR-γ, involved in the regulation of inflammatory cytokine IL-6 and TNF-α (Calder, 2012; Kliewer et al., 1997; Zuo et al., 2006).

Membrane phospholipids also provide substrates for phospholipases (A1, A2, C and D). These enzymes liberate fatty acids (such as DHA or ARA) into the cell, where they can be further metabolized to form bioactive lipid mediators, which are known to be involved in various cellular functions. These lipid mediators, also known as oxylipins, are derived from the oxidation of PUFA. Eicosanoids are the most characterized series of oxylipins derived from ARA through the action of enzymes (cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450),

resulting in the formation of prostaglandin (PG), thromboxane (TX), thromboxane (LT). The lipid mediators derived from n-3 PUFAs have anti-inflammatory properties, and n-6 PUFAs have inflammatory properties, with some exceptions (Calder, 2009; Dyall et al., 2022; Bei Wang et al., 2021). The anti-inflammatory effects of EPA are mediated through 3-series PG, 3-series TX, 5-series LT and E-series resolvins. Similarly, the DHA-derived mediators are classified into D-series protectins, D-series resolvins and maresins. Several reviews have described the lipid mediators derived from the n-3 and n-6 PUFAs (Balestrieri et al., 2021; Calder, 2020; Philip C. Calder et al., 2010; Dyall et al., 2022; Gutiérrez et al., 2019; Patterson et al., 2012; Serhan et al., 2008). A comprehensive review by Gabbs et al. (2015) has the most comprehensive description of mediators and their functions that are derived from fatty acids such as ARA, LA, dihomo-γ-linolenic acid (20:3 n-6), adrenic acid, ALA, EPA and DHA. Further, the physiological importance of these fatty acid-derived lipid mediators in different cells and organs is also detailed (Patterson et al., 2012).

In the immune cells, the membrane phospholipid composition of LCPUFAs is predominantly dependent on their dietary supply. The n-3 and n-6 LCPUFAs are competitively incorporated in the membranes. For instance, providing high levels of DHA in the absence of ARA (or other n-6 LCPUFAs) may increase the DHA composition of membrane phospholipids but at the cost of suppressing ARA, resulting in an imbalance in n-6 to n-3 LCPUFA. The adverse implications of an imbalance in the ratio of n-6 to n-3 LCPUFA can hinder the optimal development of the immune system during early life. Findings from studies aimed at attaining n-3 and n-6 LCPUFA balance with DHA and ARA supplementation have shown modulations that are considered positive for the immune system (Fussbroich et al., 2020; Nettleton & Salem Jr., 2019; Weise et al., 2011a). (Field et al., 2000; Field et al., 2008a) showed that feeding infants with a formula

containing DHA and ARA (0.2-0.5 % of total fat each) was associated with a higher proportion of mature immune cells marked by CD45RO+ T cells in PBMC. Similarly, animal models supplemented with 0.9% DHA along with ARA showed a programming effect on the splenocytes of 6-week-old offspring. Specifically, the spleen contained a higher proportion of CD27+ cells (memory marker) and higher production of IFN-γ and IL-10 to T cell stimulation (Caroline Richard et al., 2016e; C. Richard et al., 2016). Additionally, LCPUFA supplementation in mice models of asthma also showed that the pro-resolving effect of DHA helps to reduce airway inflammation and promoted resolution (Fussbroich et al., 2020). However, studies evaluating physiologically relevant concentrations of dietary n-3 and n-6 LCPUFAs on immune system development are spares, especially in the context of food allergy.

1.3.4. Inadequate intake of n-3 LCPUFA during pregnancy, lactation, and early life

The deficiency of essential fatty acids has not been observed at population levels due to their presence in commonly consumed oils (canola and soybean) (Kris-Etherton et al., 2000). However, the low intake of essential fatty acids (ALA and LA) is associated with cardiovascular diseases, type 2 diabetes, impaired brain development, arthritis and other inflammatory diseases (Djuricic & Calder, 2021). It is important to note that the Institute of Medicine or EFSA has no specific recommendations for ARA, despite its essentiality in human health (Hadley et al., 2016; Caroline Richard et al., 2016a; Tounian et al., 2021). This is particularly important during prenatal and postnatal periods when the requirement of LCPUFA, such as ARA, EPA and DHA, is higher to support the growth of vital organs (Koletzko et al., 2014; Koletzko et al., 2008), including immune system (Miles et al., 2021; Shahidi & Ambigaipalan, 2018). A clinical trial by Carlson et al. (2021a) reported fewer preterm births with a DHA intake of 1000 mg/day in comparison to 200 mg/day (recommended by FAO/WHO) (World Health Organisation, 1995).

Furthermore, n-3 LCPUFA supplementation is associated with a small but significant increase in the gestation length and the birthweight of neonates in humans (Middleton et al., 2018). In terms of atopic conditions, epidemiological data suggest an association between n-3 LCPUFA and the prevention of allergies and asthma symptoms (Elghoudi & Narchi, 2022; Gunaratne et al., 2015; Hopkins et al., 2022; Sartorio et al., 2021). The anti-inflammatory properties of EPA and DHA are hypothesized to be involved in reducing the risk of inflammatory conditions, such as eczema, asthma, food allergies, etc. The n-3 LCPUFA supplementation during pregnancy and lacttaion reduces risk of IgE-mediated allergic condition in 24 months old infants (Furuhjelm et al., 2011b). A cochrane review evaluating n-3 LCPUFA status and allergy prevention indicated reduction in sensitization towards egg protein in 24-36 months old infants (Gunaratne et al., 2015). Epidemiological evidence suggested a reduction in the risk of asthma and wheeze in 12 months old infants was associated with PUFA (Stratakis et al., 2018). Specifically high n-3 LCPUFA status was associated with lower risk of asthma and wheeze (risk ratio 0.49 and 0.60, respectively). Therefore, the association between high n-3 LCPUFA status and risk of atopic conditions need to be further investigated.

The dietary recommendations for LCPUFA need to be determined in accordance with all the health aspects and not just selected outcomes (cardiovascular or visual functions). During pregnancy and lactation, the average nutrient requirement for DHA and ARA was estimated to be 200–1000 mg/day and 800 mg/day (Brenna & Lapillonne, 2009). Interestingly, the absorption of EPA, DHA and ARA by the fetus through placental transfer and later in neonates through breast milk occurs competitively. The presence of EPA and DHA in breast milk might require a higher dose of DHA. Further research is required to understand the nutrient requirement for optimal development of immune functions and how the nutrient-to-nutrient interactions can

influence the requirement of LCPUFA. Identifying and determining dietary recommendations for the LCPUFAs during gestation and early life is necessary. Optimal nutrition during early life, a critical window of opportunity, can dramatically reduce the burden of various diseases, such as food allergies, delayed cognitive and neuromotor development and cardiovascular diseases.

As described earlier, dietary intake of ALA is not a practical way of improving n-3 LCPUFA status. The supplementation of the maternal diet with DHA is necessary for improving DHA status in the infant and the mother (Brenna et al., 2009). This report also indicated that the bioconversion of DHA, unlike a linear increase in EPA, from dietary ALA only occurs in trace amounts, which is insufficient to meet the higher requirement of a growing infant. Several other RCTs have also shown that DHA supplementation during pregnancy and lactation improved the DHA statuses of mother and infant at birth (Connor et al., 1996; Krauss-Etschmann et al., 2007; Van Houwelingen et al., 1995). Therefore, dietary supplementation with n-3 LCPUFA is necessary to meet the recommended intake for EPA+DHA in pregnant and lactating women (Jia et al., 2015). This is particularly helpful when the breastmilk composition of DHA is highly variable and may not be high enough for optimal infant health (Brenna et al., 2007; Yuhas et al., 2006). On the other hand, the levels of ARA are more constant and do not show changes due to dietary factors. A comprehensive meta-analysis of breast milk composition reported means and SD of 0.32 ± 0.22% (range: 0.06–1.4%) for DHA and 0.47 ± 0.13% (range: 0.24–1.0%) for ARA (Brenna et al., 2007). In light of suboptimal LCPUFA levels in pregnant and lactating women, especially DHA, the supplementation of ARA and DHA in breastmilk substitute, infant formula, has been debated by experts. The rationale behind adding ARA and DHA in infant formula stems from their presence in breast milk. The World Health Organization guide of 2007 on infant formula made it mandatory to add ALA (50 mg/100 kilocalories) and LA (300 mg/100

kilocalories) at a ratio of LA/ALA between 5:1 to 15:1 but addition of DHA is optional at (0.5% of total fatty acids) (Codex Alimentarius Commission, 2007). This FAO/WHO guideline suggested that if DHA is added to the infant formula, the ARA content must match the DHA levels. Note 50 mg/100 kilocalories is approximately 1% of total fatty acids. The EFSA guideline suggested an addition of DHA up to 1% of total fatty acid in infant formula without adding ARA (EFSA Panel on Dietetic Products & Allergies, 2013). This allowed infant formals with 1% DHA and no ARA to be marketed in European countries. Knowing the essentiality of ARA for infant growth and development, such guidelines can have unknown consequences on the health of infant. Therefore, experts in the field have raised concerns and advocated for the addition of equal amount of ARA when DHA is present in the formula (Brenna, 2016; Crawford et al., 2015; Koletzko et al., 2019; Lien et al., 2018). Additionally, the optimal concentration of essential fatty acid, LA, in infant formula is also unresolved and the subject of ongoing research (Carlson et al., 2021b).

1.4. References

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Chapter 2. Research Plan

2.1. Rationale

Alpha-linolenic acid (ALA) and linoleic acid (LA) are considered essential nutrients as the human body can not synthesize them. Further, eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), docosahexaenoic acid (DHA) and arachidonic acid (ARA) are also considered conditionally essential since their bioconversion from their respective precursors is highly restricted (Brenna et al., 2009; Gibson et al., 2011). These LCPUFAs are critical for the growth and development of the brain, vision, cognition, and behaviour during early life (Colombo et al., 2013; Delgado-Noguera et al., 2015). Additionally, the immunomodulatory properties of these LCPUFA also make them essential for the development of the immune system. In general, the n-3 LCPUFA have anti-inflammatory properties, and n-6 LCPUFAs have pro-inflammatory properties, and the balance of these two is essential for the optimal development of the immune system during early life (Miles et al., 2021; Caroline Richard et al., 2016a). The growing influence of the western diet, high in vegetable oil, has skewed the balance toward relatively higher consumption of n-6 PUFA, thereby increasing the ratio of n-6 to n-3 dietary fat (Liput et al., 2021).

Human milk always contains DHA and ARA but their concentration depends on dietary sources of LCPUFA. The importance of DHA in growth and development during early life is widely recognized (S.M. Innis, 2014; Yuhas et al., 2006). Although much of the infant nutrition literature on immunity is focused on DHA, the role of ARA is also implicated in immune functions (Hadley et al., 2016; Hahn et al., 2020). Providing DHA in conjunction with ARA during early infancy (suckling and weaning) improves the immune system development in both

animal models (Hadley et al., 2017; C. Richard et al., 2016) and humans (Garcia-Larsen et al., 2018). However, based on current Canadian dietary recommendations, the addition of ARA and DHA in infant formula is only voluntary. Moreover, the European Food Safety Authority has recently concluded that adding ARA to term infant formula is not required, despite its presence in breast milk (Crawford et al., 2015). Such guidelines have been critically evaluated by experts in the field (Brenna, 2016; Lien et al., 2018; Tounian et al., 2021). Additionally, evidence suggests that providing DHA without ARA may be detrimental to development during early life (Colombo et al., 2017). Therefore, the current thesis research aims to evaluate the role of ARA in addition to DHA.

Current literature suggests an inverse relationship between higher n-3 LCPUFA status, especially DHA, and atopic conditions such as asthma, food allergies, atopic dermatitis, etc. (Sartorio et al., 2021). It has been demonstrated that consuming a diet high in n-3 LCPUFAs, such as EPA and DHA, can suppress some risk factors involved in developing food allergies and other atopic conditions (Miles & Calder, 2017). Further, during early life when the risk of developing food allergies is higher, the role of LCPUFAs (via breastmilk and weaning diet) is not fully elucidated (Calder, 2008; Philip C. Calder, 2013a). However, the specific mechanisms involved in this interaction remain unknown. The current literature has identified several potential mechanisms of action. First, LCPUFAs can influence cell function by influencing cell membrane fluidity and, by implication signalling pathways. The presence of LCPUFA can also influence the ligand-receptor interaction in the cell membrane. LCPUFAs can form lipid mediators through the action of lipoxygenase and cyclooxygenase. These lipid mediators can affect intracellular signalling and gene expression by inducing transcription factors. Some of the specific pathways of action have been highlighted for the lipid mediators derived from LCPUFA (Calder, 2010b; Dyall et al.,

2022; Saini & Keum, 2018; Serhan et al., 2008). However, more research is warranted to elucidate if these LCPUFA, particularly the DHA and ARA, can reduce the susceptibility to develop allergic disease and hence promote the development of oral tolerance.

2.2. Objective and hypotheses

The overall objective of the current doctoral thesis is to understand the essentiality of dietary LCPUFA sources in the development of the immune system and oral tolerance in healthy and allergy-prone conditions using pre-clinical rodent models during early life. Two main objectives and associated hypotheses were established to evaluate the overall objective.

Objective 1 was to determine the effect of feeding a stearidonic acid (SDA) enriched flaxseed oil, as a source of n-3 LCPUFA (EPA and DHA), on maternal and infant immune system development and infant OT development using a healthy rodent model. The oil source of SDA was derived from a genetically modified flaxseed. Further, the Sprague Dawley rats were used to model a healthy immune system as they are known to generate a balanced Th1 to Th2 response. We hypothesize that feeding SDA, through its conversion to EPA, DPA, and other n-3 LCPUFAs, would promote immune system maturation of the young rat offspring. Furthermore, these n-3 LCPUFAs would also be beneficial for the development of OT.

Objective 2 was to determine the effect of feeding DHA alongside ARA on the immune system and OT development in allergy-prone conditions during early life. In evaluating objective 2, three specific sub-objectives were established and are described below:

Objective 2.1 was identifying OT induction protocols and allergy-prone rodent models used for early-life dietary intervention studies. We hypothesized that the animal models with a

characteristic T helper type 2 (Th2) dominant immunity will be optimal for evaluating OT development in an infant with an allergy-prone condition.

Objective 2.2 used Brown Norway rat (identified in objective 2.1) offspring to study the effect of combined supplementation of DHA and ARA during early life (suckling and/or weaning period) on the following: (A) LCPUFA status in the offspring, (B) immune system development and functions, and (C) oral tolerance development. The suckling period (0 to 3 weeks) diet was supplemented with 0.8% of total fat as DHA and 0.45% of total fat as ARA. These concentrations were selected to match the highest amount of DHA identified in the lactating women and maintain the ratio of ARA to DHA as 1:2. The weaning diet (3 to 8 weeks) was supplemented with DHA and ARA at 0.5% of total fat each. This was estimated based on the 2015-2020 dietary guideline for Americans (Eighth edition) to match physiologically attainable with 2 to 3 servings of fish per week (US Department of Health and Human Services and US Department of Agriculture, 2015). Objective 2.2 evaluates the LCPUFA oils, ARASCO and DHASCO microbial oils derived from source single-cell organisms. These LCPUFA sources and concentrations have been used in our lab using healthy rat models. We hypothesized that DHA and ARA supplementation would (1) be beneficial for the Th2 bias Brown Norway offspring by promoting a Th1 response and early development of adaptive immune cells and (2) promote the development of OT towards orally introduced egg white protein, ovalbumin.

Objective 2.3 used BALB/c mouse (identified in objective 2.1) offspring to study the effect of plant-based DHA alongside ARA during early life (suckling and/or weaning period) on the immune system development of (A) 3-week offspring and (B) 6-week offspring as well as (C) OT development in 6-week offspring. In comparison to objective 2.2, objective 2.3 used a higher concentration of DHA and ARA for the suckling and weaning period (at 1% of total fat each).

The dietary source of DHA used in objective 2.3 was derived from a novel canola oil high in DHA. This plant-based dietary source of DHA is currently marketed in Canada, making it potentially easy to integrate into the diet for people with various dietary restrictions/preferences (i.e., vegans, vegetarians, etc.). Further, an allergy-prone mouse was selected for this experiment over an allergy-prone rat so that we could take advantage of easily available advance immunological assays and antibodies specific to the mice models. Although, limited tissue availability from the small size of mouse pups may be a limitation with mouse models. We hypothesized that feeding dams with DHA and ARA would increase the breastmilk composition of DHA, resulting in higher DHA composition of pups' splenocytes. The high DHA levels in splenocytes will promote immune system development through increasing Th1 cytokine response in the 3-week offspring. Further, it will promote beneficial changes in the immune function of BALB/c mouse offspring resulting in the suppression of antigen-specific response to induce OT.

2.3. Chapter formats

The objectives and hypotheses described earlier are tested in a series of experiments conducted using animal models. The studies are organized in the chapters of this thesis and have been either published or submitted for publication as a stand-alone manuscript. The list of chapters is as follows:

Chapter 1 of this thesis describes the scientific background relevant to the studies conducted to evaluate the main objectives. The research plan is described in chapter 2 of this thesis. This involves detailing the rationale for conducting the thesis research and specific objectives and hypotheses for planning the experiments.

Chapter 3 of the thesis examines the effect of feeding bioactive flaxseed oil (high in SDA) during the early life of Sprague-Dawley rat offspring. The results from the examination of objective 1 are described in this chapter. Briefly, feeding SDA led to the *in-vivo* biosynthesis of eicosapentaenoic acid (EPA) and docosapentaenoic acid (DPA) but not DHA while maintaining tissue concentrations of ARA. In the 3-week offspring, increasing n-3 LCPUFAs led to better growth and immune system maturation. In the 6-week offspring, the higher EPA and DPA had an anti-inflammatory effect, but unlike DHA supplementation, it did not alter the ability to develop OT. Therefore, we considered DHA important for OT development and used it in all the subsequent experiments.

This chapter reports findings from the examination of objective 2.1. Here we identified optimal rodent models (Brown Norway rats and BALB/c mouse) and OT induction protocol (low-dose continuous exposure of model antigen) to study the effects of dietary intervention on OT development during early infancy.

Chapter 4 of this thesis reports findings from the dietary intervention (ARA+DHA) experiments conducted in Brown Norway rat offspring. This chapter reports the effect of ARA+DHA during early life on LCPUFA status and immune system development in 8-wk offspring. The findings in this chapter address objective 2.2, parts (A) and (B).

Chapter 5 of this thesis reports findings from the dietary intervention experiment conducted in Brown Norway rat offspring. This chapter reports the effect of ARA+DHA during early life on the development of OT towards model egg protein, ovalbumin. The findings in this chapter address objective 2.2, part (C).

Chapter 6 of this thesis reports findings from the dietary intervention experiment conducted in BALB/c mouse offspring. This chapter reports the effect of high DHA canola oil supplementation during the suckling period on the immune system development of 3-week offspring. The findings in this chapter address objective 2.3 part (A).

Chapter 7 of this thesis reports findings from the dietary intervention experiment conducted in BALB/c mouse offspring. This chapter reports the effect of high DHA canola oil supplementation during the suckling and weaning period on the immune function and OT development in 6-week offspring. The findings in this chapter address objectives 2.3, part (B) and part (C).

Chapter 8 summarizes each objective's findings and their respective subparts. The overall discussion of key findings from this thesis aims to provide directions for future research.

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Chapter 3. Feeding a Bioactive Oil Enriched in Stearidonic Acid During Early Life Influences Immune System Maturation in Neonatal Sprague Dawley Rats[1]

3.1. Introduction

The effect of diet during early-stage development (critical window) has significant implications for both the immediate biological response of the young animal and the response later in life. The ability of T cells to respond appropriately to immune challenges develops early in life and is influenced by the availability of dietary long-chain PUFAs [LCPUFAs: n-3 (ω-3); reviewed by Field et al. (2008b) and Caroline Richard et al. (2016a). Oral tolerance (OT), which is the ability to distinguish between harmful and harmless dietary antigens (Garside & Mowat, 2001), also occurs early in life, and failure to develop OT results in atopic diseases such as food allergies and asthma (Garside et al., 1999).

Our research has demonstrated that, in healthy animals and humans, feeding DHA and arachidonic acid (ARA) improves immune function and OT in animals and infants (Field et al., 2010; Field et al., 2008b; Caroline Richard et al., 2016e, 2016f)). Clinical trials that supplemented n-3 LCPUFAs to lactating mothers or infants at high risk of allergy have resulted in less atopic symptoms in some (Blümer & Renz, 2007; D'Vaz et al., 2012; Furuhjelm et al., 2009) but not all (Anandan et al., 2009) studies [reviewed by Caroline Richard et al. (2016a)]. It has been established that the dose-dependent incorporation of n-3 LCPUFAs into plasma, RBCs,

[1] A version of this chapter has been published as: Patel D., Goruk S., Newell M., Chen G., Richard C., Field C.J. (2019) Feeding a Bioactive Oil Enriched in Stearidonic Acid during Early Life Influences Immune System Maturation in Neonatal Sprague-Dawley Rats. *The Journal of Nutrition*, 150(3): 606-615.

The content in this chapter was presented at (1) the Canadian Nutrition Society 2019 Annual Conference, *Niagara Falls*, ON, May 2019 and (2) Women and Children's Health Research Institute 11th annual research day, *Edmonton*, AB, October 2018.

and lymphocytes occurs with a corresponding reduction in ARA, reviewed Fritsche (2007). Studies have demonstrated that dietary interventions providing ARA and DHA in formula-fed infants (Field et al., 2008a) or suckled rodents (Caroline Richard et al., 2016f), compared with controls, benefitted parameters indicative of immune development.

The desaturation of α-linolenic acid (ALA, 18:3n-3) to stearidonic acid (SDA, 18:4n-3) is mediated by the rate-limiting Δ6 desaturases (Figure 3.1). We have previously shown that feeding an SDA-enriched diet to breast cancer tumour–bearing nu/nu mice increased the content of EPA and docosapentaenoic acid (DPA) but not DHA in tumour phosphatidylcholine [a major class of phospholipid (PL) in cells] (Subedi et al., 2015). The provision of SDA can bypass this rate-limiting step in converting to EPA, facilitating bioconversion to EPA (Figure 3.1) (James et al., 2003). There is a growing body of literature suggesting that EPA (Sierra et al., 2008) and DPA (Yazdi, 2014) also have effects on immune function in adults. However, such effects have not been extensively studied in infants. Increasing n-3 LCPUFAs could be important for infant OT development because women with the allergic disease have been found to have lower breast milk concentrations of EPA, DPA, and DHA (Johansson et al., 2011).

The objective of this study was to determine, during suckling and weaning, the immunomodulatory effects of feeding a bioactive oil enriched in SDA as a source of n-3 LCPUFAs on immune system maturation and the development of OT. The secondary objective of the study was to evaluate the ability of SDA to increase the total PL incorporation of n-3 LCPUFAs in neonates. We hypothesized that feeding a diet enriched in SDA, through its conversion to EPA, DPA, and other n-3 LCPUFAs, would be beneficial for the immune system maturation of the young rat pups. Furthermore, these n-3 LCPUFAs would also be beneficial for the development of OT.

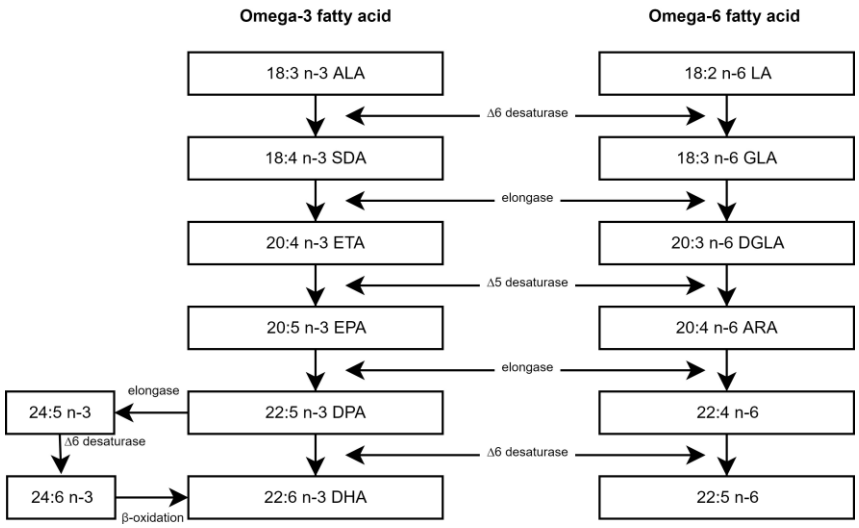

Figure 3.1. Fatty acid bioconversion pathways. Precursor n-3 and n-6 PUFAs compete for desaturases and elongases in the biosynthesis of long-chain PUFAs. Abbreviation: ARA, arachidonic acid; ALA, α-linolenic acid; DGLA, dihomo-γ-linolenic acid; DPA, docosapentaenoic acid; ETA, eicosatetraenoic acid; GLA; γ-linoleic acid; LA, linoleic acid; SDA, stearidonic acid.

3.3. Methods

3.2.1. Animals and diets

All animal care and experimental protocols were conducted in accordance with the Canadian Council of Animal Care and approved by the University of Alberta Animal Ethics Committee (AUP00000125). Primiparous Sprague-Dawley rats (n = 22) were obtained from Charles River Laboratories on day 14 of gestation and were individually housed in a temperature- and humidity-controlled environment with a 12/12-h reversed light cycle. The study design is illustrated in Figure 3.2. The experiments were conducted in 2 blocks with n = 6 and n = 5 per diet group over 2 consecutive summers, respectively. Pups were culled to have an equal number of males and females suckling with each dam. Dams were fed commercial standard rat unpurified diet (Lab diet 5001; PMI Nutrition International) during the

acclimatization period and then, 5 d prior to parturition, dams were randomly assigned to either of 2 nutritionally adequate diets: an SDA diet (3% of total fat as SDA, n = 11) or a control diet (0% SDA, n = 11). The litter was culled to 10 pups/dam Figure 3.2 and the dams consumed ad libitum during lactation and weaning. Offspring were kept with their mothers until the end of the 3-wk suckling period (day 21). At day 10, 4 pups from each dam were randomly assigned to OT treatment: placebo (sucrose, n = 2 pups/dam) or ovalbumin (Ova, n = 2 pups/dam). At the end of the 3-wk suckling period, tissues were collected from 1 pup/dam from each of the 4 diet-OT treatment groups. One pup per dam from each group was weaned to their respective maternal diet for an additional 3 wk (weaning period). At 6-wk of age, all the pups were killed by carbon dioxide asphyxiation and tissues were collected. When possible, male and female pups were equally distributed between groups.

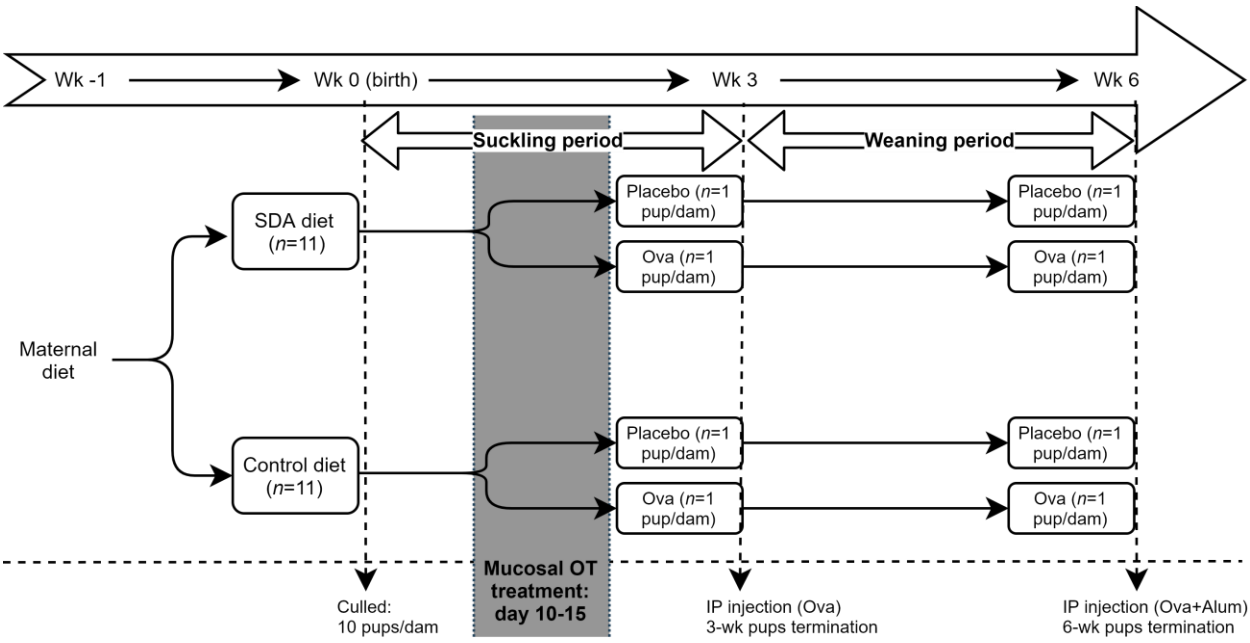

Figure 3.2. Animal study design. Sprague-Dawley rat dams were randomly assigned to either control (n = 11) or SDA (n = 11) diet 5 d prior to parturition and continued on the same diet through the suckling period (3 wk). After birth, the litter was culled to have equal numbers of males and females per dam. For OT treatment, 4 pups per dam were randomly allocated to either ovalbumin (Ova, n = 2 pups/dam) or placebo (sucrose, n = 2 pups/dam) for 5 consecutive days between days 10 and 15. Twenty-four hours prior to being killed the 3-week-old pups received an i.p. injection to induce systemic immunization. Pups were killed from each of the 4 diet-OT treatment groups (n = 1 pup/dam) and tissues were collected at 3 wk (suckling period) for analysis. The remaining pups (n = 1 pup/dam) were weaned to the same diets as their mother for an additional 3 wk. The i.p. injection, Ova with an adjuvant (alum), was administered 7 d (at week 5) prior to the pups being killed, to induce systemic immunization in 6-week-old pups. The dams were considered the experimental unit therefore the sample size of each group is equal to the number of dams (n = 11). The experiments were conducted in 2 blocks with n = 6 and n = 5 per diet group over 2 consecutive summers, respectively. OT, oral tolerance; Ova, ovalbumin; SDA, stearidonic acid; wk, week.

Both experimental diets were isocaloric and isonitrogenous (Table 3.1). The semi-purified basal diet has been previously described in detail (Lewis et al., 2016b). The fat mixture added (20 g/100 g diet) to the rodent diet was a blend of lard, canola oil, vegetable oil, and SDA-enriched flaxseed oil (Subedi et al., 2015). All the main fatty acids were matched closely except oleic acid, linoleic acid (LA), ALA, and SDA. Both diets met the essential fatty acid requirements of the rodent and had similar PUFA/SFA ratios. Note that the diets differed in the n-6 to n-3 ratio due to the difference in the content of SDA, ALA, and LA, but the diets were matched for total PUFA content. Diets were prepared weekly and stored at 4°C until fed; feed cups were replaced every 2–3 d to limit air exposure. Food intake and body weight were recorded every 2–3 d.

Table 3.1. Lipid composition of the experimental diets fed to Sprague-Dawley rat dams and weaning pups as determined by GLC[1]

Fatty acids	Control diet, g/100 g total fatty acids	SDA diet, g/100 g total fatty acids
14:0	1.2 ± 0.0	1.3 ± 0.0
16:0	22.0 ± 0.0	25.0 ± 0.2
16:1	1.9 ± 0.0	1.9 ± 0.0
18:0	12.0 ± 0.3	14.0 ± 0.1
18:1 n-9 (Oleic acid)	41.0 ± 0.1	35.0 ± 0.0
18:2 n-6 (LA)	17.0 ± 0.3	13.0 ± 0.1
18:3 n-3 (ALA)	1.9 ± 0.1	3.6 ± 0.0
18:4 n-3 (SDA)	0.0 ± 0.0	2.7 ± 0.0
20:0	0.1 ± 0.0	1.5 ± 0.0
20:3 n-6	0.3 ± 0.0	0.2 ± 0.0
Total SFA	36.0 ± 0.3	42.0 ± 0.0
Total PUFA	19.0 ± 0.5	20.0 ± 0.0
Ratio PUFA/SFA	0.5 ± 0.0	0.5 ± 0.0
Total n-6	17.0 ± 0.3	13.0 ± 0.1
Total n-3	2.0 ± 0.1	6.3 ± 0.1
Ratio n-6/n-3	8.8 ± 0.5	2.1 ± 0.0

[1]Values are presented in mean ± SEM (n=3). Fatty acids below 0.1 g/ 100 g of total fat are not included. LA, linoleic acid; SDA, stearidonic acid; ALA, α-linolenic acid; n, omega; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

3.2.2. Ovalbumin administration and immunization

Mucosal OT to Ova (Sigma-Aldrich) was induced by repeated oral exposure during the suckling period as previously described (Caroline Richard et al., 2016f). Briefly, 200 µL Ova (8 mg/mL in 8% wt:vol sucrose solution) or placebo (8% wt:vol sucrose in water) solution were delivered orally via syringe once daily for 5 d to 2 pups per litter. To induce systemic immunization 3-wk-old pups received an intraperitoneal injection of 10 µg Ova in 100 µL PBS 24 h before being killed, and 6-wk-old pups received an intraperitoneal injection of 10 µg Ova in 100 µL PBS combined with an adjuvant (1:1, Imject Alum Adjuvant; Thermo-Scientific) 7 d before being killed (Figure 3.2). Note, ovalbumin may contain very low concentrations of endotoxins that are important for activation of innate immune cells.

3.2.3. Tissue collection and immune cell isolation

Immediately after the pups were killed, blood was collected by cardiac puncture with a 5-mL syringe and stored in a K2 EDTA-containing tube. Within 1 h of collection, whole blood was analyzed on a hematology analyzer ADVIA 2120i (Siemens). Blood was then centrifuged (1734 × *g*; 10 min; 22°C), and plasma was removed and stored at −80°C until analysis. The spleens were collected aseptically, and immune cells were isolated as previously described (C. J. Field et al., 1990). Isolated live immune cells were counted on a hemocytometer using trypan blue exclusion (Sigma) and diluted to 1.25×10^6 cells/mL.

3.2.4. Immune cell phenotype analysis

Isolated immune cells from the spleen were identified by direct immunofluorescence assay as previously described (C. J. Field et al., 1990). Four-colour flow cytometry allowed the determination of the following surface molecule combinations: CD3/CD25/CD4/CD8,

CD28/CD152/CD4/CD8, CD4/CD25/FOXP3, OX62/CD25/OX6, CD68/CD284/CD11/CD45RA, OX12/OX6/CD80, CD27/OX12/OX6/CD45RA, and CD71/CD8/CD4. All antibodies were purchased from eBiosciences or BD Biosciences. (Note that the monoclonal antibody for detecting CD3+ cells binds to T cells in young rats at a lower-than-expected concentration; however, the addition of the identified CD4+ and CD8+ cells indicates an expected proportion of total T cells. CD28, a costimulatory T-cell marker, is expressed in all the T cells, and can be used to measure total T cells.) Cells were then washed and fixed in paraformaldehyde (10 g/L; Anachemia Science) in PBS. Within 72 h of isolating and fixing the stained cells, immune cells were analyzed by flow cytometry (FACSCalibur; Becton-Dickinson) according to the relative fluorescence intensity using Kaluza software (Beckman Coulter).

3.2.5. Ex vivo cytokine production by mitogen- or Ova-stimulated splenocytes and plasma Ova-specific IgG1 concentration

Cytokine production by stimulated splenocytes was measured as previously described (Blewett et al., 2009). Briefly, immune cells (1.25×10^6 cells/mL) were cultured for 48 h with or without the mitogen LPS (100 μg/mL; Sigma), a bacterial component that acts as a mitogen for immune cells, or the food protein Ova (150 μg/mL; Sigma). LPS stimulation was used to model an in vivo bacterial challenge. Commercial ELISA kits were used to measure the concentration of IL-1β, IL-2, IL-6, IL-10, TNF-α, and IFN-γ (R&D Systems) in the stimulated supernatants, according to the manufacturer's instructions. Detection limits for IL-1β, IL-2, TNF-α, and IL-10 were 16–4000 pg/mL. IL-6 and IFN-γ had a detection limit of 125–8000 pg/mL. Ovalbumin-specific IgG1 (Ova-IgG1) in the plasma was detected by an ELISA kit (Alpha Diagnostic International). Absorbance was read on a spectrophotometer, and concentrations were calculated

using a standard curve (SpectraMax 190; Molecular Devices). All measurements were conducted in duplicate with a CV <15%.

3.2.6. Fatty acid analysis

A modified Folch method was used to extract total lipids from cells and plasma as previously described (C. J. Field et al., 1988). Fatty acid methyl esters were prepared from the PL band and were separated and identified by automated GLC (GLC7890A; Agilent Technologies) on a 100-m CP-SIL 88 fused capillary column (100 m × 0.25 mm; Agilent) as described previously (Cruz-Hernandez et al., 2004).

3.2.7. Statistical analysis

Data are reported as mean ± SEM unless indicated otherwise. The study was powered to assess significant changes in immune function (as the primary outcome), and fatty acid changes (pups' plasma and splenocyte PL, as secondary outcomes). The sample size was based on a previous study from our group to assess differences in ex vivo cytokine production where $n = 6$ per group was found to be sufficient to detect a 20% (β value) difference at a significance level (α value) of 5%. Data were analyzed using the PROC MIXED procedure 2-factor ANOVA, with diet and OT treatment as the main effect in SAS software (Version 9.4; SAS Institute). The 2 × 2 study design allowed us to determine the effect of maternal diet (control compared with SDA), OT treatment (placebo compared with Ova), and the interaction between the 2 (diet × OT treatment). When the effect was found to be significant, we performed the LSMEANS procedure for post hoc statistical analysis comparing the individual 4 groups at either the 3- or 6-wk stage (control diet and placebo treatment, SDA diet and placebo treatment, control diet and Ova treatment, and SDA diet and Ova treatment). In the absence of a significant OT treatment effect (e.g., in

complete blood cell count or fatty acid analysis), the 2 diet groups were compared using an unpaired Student *t* test and mean and SEM were reported with the OT treatment group combined within a diet group. Differences at $P \leq 0.05$ (2-sided) were considered significant.

3.3. Results

3.3.1. Growth and hematological parameters

There was no significant difference in weekly food intake (47.1 ± 2.9 g/wk compared with 46.3 ± 2.7 g/wk) or final body weight (309 ± 9 g compared with 311 ± 10 g) between the dams fed the control diet compared with the SDA diet, respectively. At 3 wk there was no effect of OT treatment on the pups' body weight. However, the pups weaned to the SDA diet had a significantly higher body weight compared with pups weaned to the control diet starting from week 4 through week 6 (Figure 3.3). For 6-week-old pups, food intake did not differ between diet groups (24 ± 1.3 g/wk compared with 25 ± 1.2 g/wk). No significant differences were observed for complete blood cell count between diet groups or treatment groups for the dams or 6-week-old pups. Although the WBC count did not differ in the 3-wk-old pups, the SDA group had a significantly higher lymphocyte proportion of WBCs (reciprocally lower neutrophils) than the control-diet pups ($P < 0.01$; **Table 3.2**).

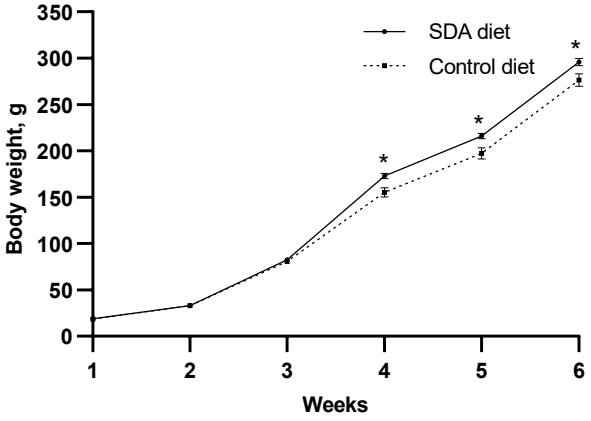

Figure 3.3. Effect of diet on the bodyweight of Sprague-Dawley rat pups from birth to 6 weeks. Values are mean ± SEM; n = 11 for each diet. Significant diet effect on pups' bodyweight is indicated by * different from Control diet, P<0.05. There was no effect of oral tolerance treatment on body weight therefore treatment groups within each diet group were combined. Abbreviation: SDA, stearidonic acid

Table 3.2. Effect of diet and oral tolerance treatment on hematologic parameters of 3-week-old Sprague Dawley rat pups[1]

Type of cells in blood	Control Diet (*n* = 11)	SDA Diet (*n* = 8)	*P*-Diet
WBC, 10^9 cells/L	6.0 ± 0.5	5.5 ± 0.3	0.60
Lymphocytes, % of WBC	80.6 ± 1.4	84.3 ± 0.9	0.03
Monocyte, % of WBC	3.7 ± 0.4	2.6 ± 0.2	0.69
Basophils, % of WBC	0.8 ± 0.1	0.5 ± 0.1	0.32
Eosinophil, % of WBC	2.9 ± 0.1	2.5 ± 0.3	0.14
Neutrophil, % of WBC	0.7 ± 0.2	0.6 ± 0.1	0.01

[1]Values are presented in mean ± SEM. P represents the probability for the main effect of diet on 3-week pups. As there was no significant effect of treatment nor a significant interaction the diet/treatment groups have been combined. Abbreviation; WBC, white blood cells, SDA, stearidonic acid

3.3.2. Plasma Ova-IgG1 concentrations

At 3 wk the SDA diet group had a significantly higher concentration of Ova-IgG1 in the plasma compared with the control group but there was no effect of OT treatment (Figure 3.4). We also observed a significant interaction between diet and OT treatment effects, in which Ova-tolerized

pups fed the SDA diet had plasma Ova-IgG1 concentrations significantly higher than Ova-tolerized pups fed the control diet ($P = 0.048$; Figure 3.4A). At 6 wk there was no significant diet effect on the Ova-IgG1 plasma concentrations but there was an OT treatment effect in that Ova-exposed pups had lower plasma Ova-IgG1 concentrations than the placebo group (Figure 3.4B; $P < 0.03$).

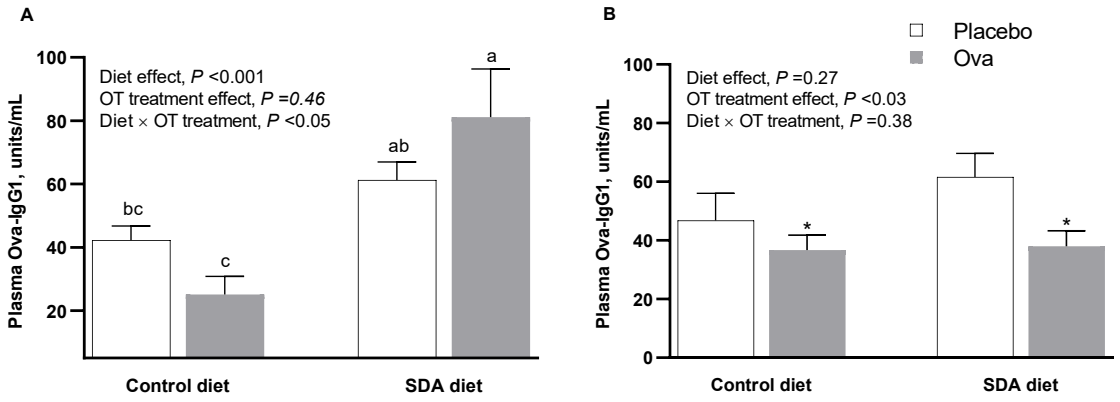

Figure 3.4. Plasma Ova-specific IgG1 levels were measured in Sprague-Dawley rat pups at (A) 3 weeks and (B) 6 weeks. Ovalbumin OT (Ova, $n = 6$) treatment groups are depicted by solid-coloured (grey) bars and placebo OT treatment groups (sucrose, $n = 6$) are depicted by open bars. Diet X OT treatment indicates the interaction between the main diet effect and the OT treatment effect. Labelled means without a common letter differ, $P<0.05$ based on post hoc analysis. Groups marked by * differ from the placebo OT treatment group, $P<0.05$. P values for the main effect of the diet, OT treatment and interaction between main effects were calculated using 2-way ANOVA (MIXED procedure, SAS). Samples from the first block of experiments were used for the analysis. Abbreviation: Ova, ovalbumin; SDA, stearidonic acid; OT, oral tolerance Ova-IgG1, ovalbumin specific immunoglobulinG1

3.3.3. Plasma PL fatty acid composition in 3- and 6-week-old pups

The total PL fatty acid composition of plasma collected from pups at 3 and 6 wk is reported in Table 3.3 and Table 3.4, respectively. Because there were no significant effects of OT treatment or any interaction only the diet effects are presented. At 3 wk, pups from the SDA group had a significantly higher proportion of SDA, eicosatetraenoic acid (ETA), EPA, DPA,

and DHA compared with control-group pups, resulting in 1.7 times more total n-3 LCPUFAs in plasma (Figure 3.5A). Feeding the SDA diet decreased the total n-6 PUFA PL composition by 10% due to significantly lower proportions of all the n-6 LCPUFAs, with the exception of ARA. The difference in total n-3 and n-6 PUFAs resulted in a lower (almost 50%) n-6/n-3 LCPUFA ratio in the SDA group compared with the control.

Table 3.3. Effect of diet on the total plasma phospholipid fatty acid composition of 3-week-old Sprague-Dawley rat pups measured using gas-liquid chromatography. [1]

Fatty acid, g/100g total fat	Control (*n* = 11)	SDA (*n* = 9)	*P*-Diet
16:0	21.07 ± 0.27	24.02 ± 0.67	0.002
18:0	28.45 ± 0.49	28.56 ± 0.57	0.84
18:1	6.23 ± 0.22	5.48 ± 0.13	0.005
18:2 n-6 (LA)	19.86 ± 0.87	16.17 ± 0.79	0.01
18:3 n-3 (ALA)	0.24 ± 0.01	0.26 ± 0.01	0.39
18:4 n-3 (SDA)	0.05 ± 0.01	0.07 ± 0.01	0.01
20:2 n-6	0.31 ± 0.01	0.28 ± 0.01	0.03
20:3 n-6	1.11 ± 0.04	1.57 ± 0.06	<0.001
20:4 n-6 (ARA)	15.00 ± 0.73	14.79 ± 0.70	0.91
20:4 n-3 (ETA)	0.16 ± 0.01	0.26 ± 0.01	<0.001
20:5 n-3 (EPA)	0.04 ± 0.01	0.63 ± 0.05	<0.001
22:4 n-6	0.23 ± 0.01	0.11 ± 0.01	<0.001
22:5 n-6	0.82 ± 0.09	0.09 ± 0.01	<0.001
22:5 n-3 (DPA)	0.32 ± 0.02	1.09 ± 0.07	<0.001
22:6 n-3 (DHA)	2.45 ± 0.16	3.15 ± 0.16	0.009
Total SFA	50.53 ± 0.37	53.72 ± 0.50	<0.001
Total MUFA	8.83 ± 0.28	7.64 ± 0.16	<0.001
Total PUFA	40.64 ± 0.25	38.64 ± 0.56	0.007
Total n-6	37.37 ± 0.24	33.19 ± 0.46	<0.001
Total n-3	3.27 ± 0.14	5.45 ± 0.23	<0.001
Ratio PUFA/SFA	0.18 ± 0.01	0.14 ± 0.01	0.003
Ratio n-6/n-3 LCPUFA	11.62 ± 0.54	6.18 ± 0.26	<0.001

[1]Values are presented in mean ± SEM. As there was no significant effect of treatment nor a significant interaction within the diet, treatment groups have been combined. *P*-Diet represents the probability for the main effect of maternal diet on 3-week pups. Abbreviations; LA, linoleic acid; SDA, stearidonic acid; ARA, arachidonic acid; ALA, α-linolenic acid; EPA, eicosapentaenoic acid; ETA, eicosatetraenoic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; MUFA, monounsaturated fatty acids; n, omega; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

Table 3.4. Effect of diet on the total plasma phospholipid fatty acid composition of 6-week-old Sprague Dawley rat pups measured using gas-liquid chromatography. [1]

Fatty acid, g/100 g total fat	Control (*n* = 11)	SDA (*n* = 10)	*P*-Diet
16:0	20.59 ± 0.31	21.85 ± 0.30	0.008
18:0	29.06 ± 0.36	29.87 ± 0.50	0.27
18:1	6.61 ± 0.19	5.62 ± 0.15	<0.001
18:2 n-6 (LA)	18.37 ± 0.48	17 ± 0.45	0.08
18:3 n-3 (ALA)	0.28 ± 0.01	0.29 ± 0.02	0.84
18:4 n-3 (SDA)	0.06 ± 0.01	0.07 ± 0.01	0.23
20:2 n-6	0.33 ± 0.01	0.33 ± 0.02	0.78
20:3 n-6	1.18 ± 0.05	1.67 ± 0.05	<0.001
20:4 n-6 (ARA)	16.11 ± 0.38	15.16 ± 0.59	0.17
20:4 n-3 (ETA)	0.21 ± 0.01	0.24 ± 0.02	0.14
20:5 n-3 (EPA)	0.04 ± 0.01	0.44 ± 0.03	<0.001
22:4 n-6	0.25 ± 0.01	0.13 ± 0.01	<0.001
22:5 n-6	0.33 ± 0.04	0.10 ± 0.02	<0.001
22:5 n-3 (DPA)	0.24 ± 0.02	0.82 ± 0.06	<0.001
22:6 n-3 (DHA)	2.16 ± 0.11	2.42 ± 0.19	0.47
Total SFA	50.78 ± 0.34	52.97 ± 0.69	0.02
Total MUFA	9.58 ± 0.23	8.13 ± 0.24	<0.001
Total PUFA	39.64 ± 0.33	38.90 ± 0.76	0.41
Total n-6	36.62 ± 0.32	34.63 ± 0.62	0.02
Total n-3	3.02 ± 0.09	3.97 ± 0.13	<0.001
Ratio PUFA/SFA	0.19 ± 0.01	0.15 ± 0.01	<0.001
Ratio n-6/n-3 LCPUFA	12.24 ± 0.39	8.31 ± 0.35	<0.001

[1]Values are presented in mean ± SEM. As there was no significant effect of treatment nor a significant interaction within the diet, treatment groups have been combined. *P*-Diet represents the probability for the main effect of a weaning diet on 6-week pups. Abbreviations; LA, linoleic acid; SDA, stearidonic acid; ARA, arachidonic acid; ALA, α-linolenic acid; EPA, eicosapentaenoic acid; ETA, eicosatetraenoic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; MUFA, monounsaturated fatty acids; n, omega; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

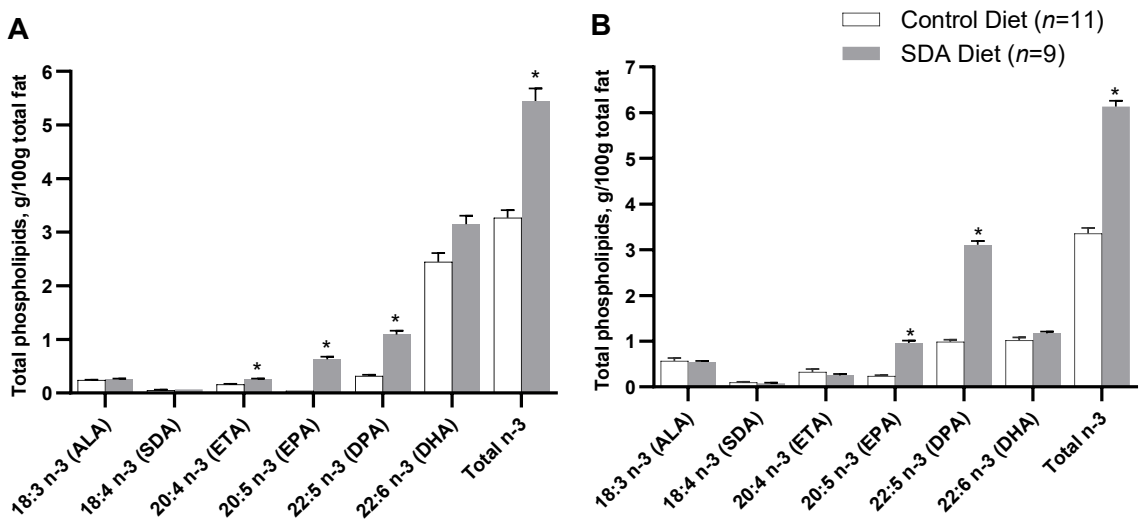

Figure 3.5. Effect of diet on the 3-week-old Sprague-Dawley rat pups' (A) plasma and (B) spleen total phospholipid fatty acid composition. There was no significant effect of OT treatment nor a significant interaction effect so the treatment groups within each diet were combined. Significant diet effect was calculated by unpaired student's t-test, * differ from Control diet, $P<0.05$. Outliers were excluded from the analysis resulting in $n<11$ per diet group. ALA, α-linolenic acid; SDA, stearidonic acid; ETA, eicosatetraenoic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; n, omega

At 6 wk of age, SDA-fed pups had a significantly higher plasma PL content of total n-3 PUFAs due to more EPA and DPA but not DHA (Figure 3.6A). Similar to 3-week-old pups, the SDA group had a significantly lower proportion of all n-6 fatty acids in plasma PL, but not ARA or LA, which did not differ between diet groups (Table 3.4). For both 3-week-old and 6-week-old pups, the plasma PL proportion of total SFAs was higher and the proportion of MUFAs was lower for the SDA group.

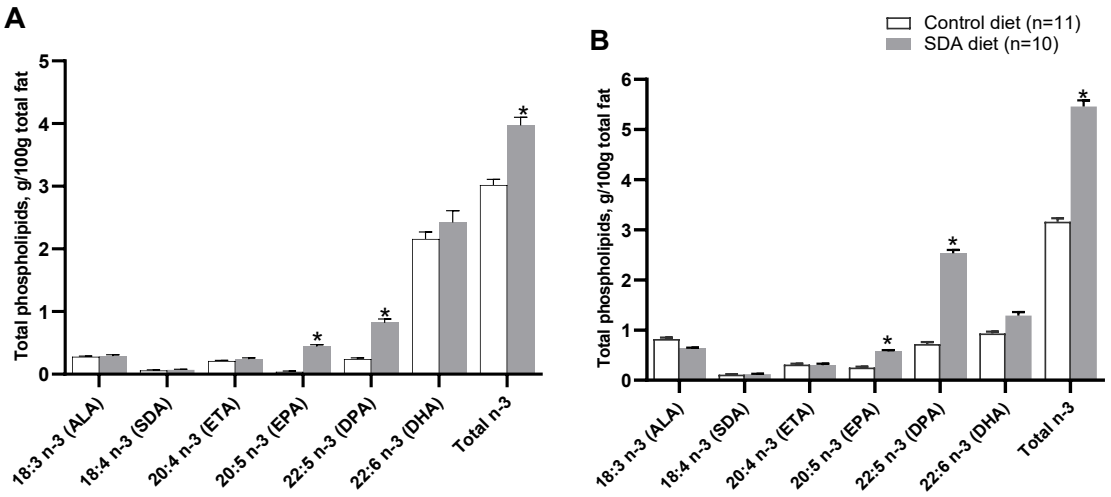

Figure 3.6. Effect of diet on the 6-week-old Sprague-Dawley rat pups' (A) plasma and (B) spleen total phospholipid fatty acid composition. There was no significant effect of OT treatment nor a significant interaction effect so the treatment groups within each diet were combined. Significant diet effect was calculated by unpaired student's t-test, * differ from Control diet, $P<0.05$. Outliers were excluded from the analysis resulting in $n<11$ per diet group. ALA, α-linolenic acid; SDA, stearidonic acid; ETA, eicosatetraenoic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; n, omega

3.3.4. PL fatty acid composition of the spleen in 3- and 6-wk-old pups

At 3 and 6 wk there were no OT treatment effects or interaction effects on PL fatty acid composition; diet effects are reported for 3-week-old (in Table 3.5) and for 6-week-old pups (in Table 3.6). In 3-wk-old pups, the total PL fatty acid composition of the splenocytes from the SDA group had a significantly higher proportion of total n-3 fatty acids largely due to the significantly higher proportion of the n-3 LCPUFAs (ETA, EPA, and DPA; Figure 3.5B). The relative proportion of DHA in PL did not differ between diet groups. Additionally, the proportion of total n-6 fatty acids was significantly lower in the SDA group compared with the control group, which resulted in a significantly lower n-6/n-3 LCPUFA ratio in the SDA group. No effect of the diet was observed on the proportion of total SFAs and MUFAs, or the ratio of

PUFAs to SFAs in 3-week-old pups. At 6 wk, despite no differences in the total n-6 fatty acid proportion (Table 3.6), the total n-3 fatty acid proportion of immune cell PL was significantly higher in pups that were weaned to the SDA diet, again due to a higher concentration of EPA and DPA (Figure 3.6B). This resulted in a lower n-6/n-3 LCPUFA ratio of spleen PL from rats fed the SDA diet when compared with the control diet (Table 3.6).

Table 3.5. Effect of diet on the fatty acid composition of total phospholipids in the spleen of 3-week-old Sprague Dawley rat pups measured using gas-liquid chromatography. [1]

Fatty acid, g/100 g total fat	Control (*n* = 10)	SDA (*n* = 9)	*P*-Diet
16:0	27.95 ± 0.46	28.51 ± 0.29	0.37
18:0	19.58 ± 0.31	20.14 ± 0.28	0.15
18:1	13.16 ± 0.19	12.25 ± 0.15	0.26
18:2 n-6 (LA)	7.66 ± 0.2	7.26 ± 0.16	0.17
18:3 n-3 (ALA)	0.57 ± 0.06	0.55 ± 0.02	0.054
18:4 n-3 (SDA)	0.10 ± 0.01	0.08 ± 0.01	0.10
20:2 n-6	0.71 ± 0.02	0.58 ± 0.01	<0.001
20:3 n-6	1.70 ± 0.05	2.28 ± 0.06	<0.001
20:4 n-6 (ARA)	16.30 ± 0.49	14.76 ± 0.5	0.86
20:4 n-3 (ETA)	0.33 ± 0.06	0.25 ± 0.03	0.13
20:5 n-3 (EPA)	0.24 ± 0.02	0.96 ± 0.05	0.002
22:4 n-6	0.66 ± 0.04	0.17 ± 0.02	0.98
22:5 n-6	0.45 ± 0.06	0.36 ± 0.04	0.31
22:5 n-3 (DPA)	0.99 ± 0.04	3.11 ± 0.08	0.04
22:6 n-3 (DHA)	1.02 ± 0.06	1.17 ± 0.04	0.44
Total SFA	50 ± 0.51	51.44 ± 0.46	0.27
Total MUFA	18.74 ± 0.26	16.32 ± 0.25	0.13
Total PUFA	31.04 ± 0.46	31.97 ± 0.49	0.76
Total n-6	27.68 ± 0.45	25.84 ± 0.37	0.003
Total n-3	3.36 ± 0.12	6.13 ± 0.13	0.004
Ratio PUFA/SFA	0.62 ± 0.02	0.62 ± 0.01	0.51
Ratio n-6/n-3 LCPUFA	8.40 ± 0.28	4.23 ± 0.05	<0.001

[1]Values are presented in mean ± SEM. As there was no significant effect of treatment nor a significant interaction within the diet, treatment groups have been combined. *P*-Diet represents the probability for the main effect of maternal diet on 3-week pups. Abbreviations; LA, linoleic acid; SDA, stearidonic acid; ARA, arachidonic acid; ALA, α-linolenic acid; EPA, eicosapentaenoic acid; ETA, eicosatetraenoic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; MUFA, monounsaturated fatty acids; n, omega; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

Table 3.6. Effect of diet on the fatty acid composition of total phospholipids in the spleen of 6-week-old Sprague Dawley rat pups measured using gas-liquid chromatography. [1]

Fatty acid, g/100 g total fat	Control (*n* = 11)	SDA (*n* = 10)	*P*-Diet
16:0	29.48 ± 0.69	30.66 ± 0.54	0.23
18:0	19.20 ± 0.33	19.25 ± 0.26	0.73
18:1	12.11 ± 0.19	12.04 ± 0.21	0.81
18:2 n-6 (LA)	6.49 ± 0.25	5.91 ± 0.23	0.43
18:3 n-3 (ALA)	0.82 ± 0.03	0.63 ± 0.02	0.40
18:4 n-3 (SDA)	0.11 ± 0.01	0.12 ± 0.01	0.76
20:2 n-6	0.74 ± 0.02	0.55 ± 0.02	0.09
20:3 n-6	1.81 ± 0.07	2.24 ± 0.05	<0.001
20:4 n-6 (ARA)	15.28 ± 0.58	13.54 ± 0.44	0.99
20:4 n-3 (ETA)	0.31 ± 0.02	0.30 ± 0.03	0.61
20:5 n-3 (EPA)	0.25 ± 0.02	0.58 ± 0.02	<0.001
22:4 n-6	0.55 ± 0.02	0.16 ± 0.02	<0.001
22:5 n-6	0.64 ± 0.05	0.63 ± 0.05	0.68
22:5 n-3 (DPA)	0.72 ± 0.04	2.53 ± 0.07	<0.001
22:6 n-3 (DHA)	0.93 ± 0.04	1.29 ± 0.07	0.07
Total SFA	51.54 ± 0.66	53.06 ± 0.65	0.39
Total MUFA	19.36 ± 0.28	18.24 ± 0.35	0.88
Total PUFA	28.76 ± 0.75	28.36 ± 0.65	0.74
Total n-6	25.60 ± 0.73	22.90 ± 0.62	0.93
Total n-3	3.16 ± 0.07	5.46 ± 0.12	<0.001
Ratio PUFA/SFA	0.56 ± 0.02	0.54 ± 0.02	0.42
Ratio n-6/n-3 LCPUFA	8.18 ± 0.25	4.23 ± 0.14	<0.001

[1]Values are presented in mean ± SEM. As there was no significant effect of treatment nor a significant interaction within the diet, treatment groups have been combined. *P*-Diet represents the probability for the main effect of a weaning diet on 6-week pups. Abbreviations; LA, linoleic acid; SDA, stearidonic acid; ARA, arachidonic acid; ALA, α-linolenic acid; EPA, eicosapentaenoic acid; ETA, eicosatetraenoic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; MUFA, monounsaturated fatty acids; n, omega; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

3.3.5. Ex vivo cytokine production by mitogen- and Ova-stimulated immune cells

Spleen

LPS stimulation: *Ex-vivo* cytokine (IL-1β, IL-6, IL-10, TNF-α, and IFN-γ) production by LPS-stimulated splenocytes from dams and 3-wk-old pups (Table 3.7) did not significantly differ with diet or OT treatment. However, at 6 wk there was significantly lower IL-6 and TNF-α production and significantly higher IL-10 production in stimulated splenocytes from SDA-fed pups (Figure 3.7A; $P < 0.05$). The production of IL-1β (an inflammatory cytokine) did not differ between diet groups (Figure 3.7A). Additionally, OT treatment had a significant effect on IFN-γ production by splenocytes after ex vivo stimulation with Ova. The Ova-exposed group produced significantly less IFN-γ compared with the placebo group (Table 3.8; $P = 0.03$).

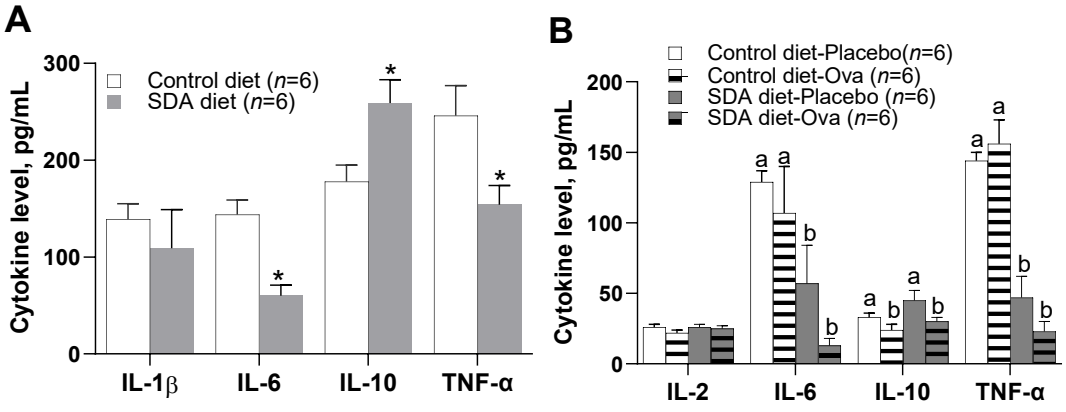

Figure 3.7. The effect of diet on *ex-vivo* cytokine production upon (A) LPS and (B) Ova stimulation response by splenocytes from 6-week-old Sprague-Dawley pups. No major OT treatment effect was observed in LPS-stimulated splenocyte cytokine production, therefore the means from Ova and placebo groups are combined for (A). Significant diet effect was calculated by unpaired student's t-test, * differ from Control diet, $P<0.05$. For Ova stimulated cytokine production, as there was an OT treatment effect, each diet group shows means from Ova and placebo treatment group separately. Labelled means without a common letter differ, $P<0.05$ based on post hoc analysis in 2-way ANOVA. Samples from the first block of experiments were used for the analysis. IL, interleukin; TNF-α, tumor necrosis factor-alpha; LPS, Lipopolysaccharide; Ova, Ovalbumin

Ova (dietary antigen) stimulation: There was no effect of diet on the cytokine response (IL-1β, IL-2, IL-6, IL-10, TNF-α) of splenocytes to the ex vivo Ova challenge in the 3-wk-old pups (Table 3.7). However, IL-10 showed a significant interaction effect between diet and OT treatment, with the Ova-exposed SDA-group pups producing significantly less than Ova-exposed control-group pups, but this was not different from placebo-exposed pups irrespective of the diet group. At 6 wk, pups from the SDA group produced significantly less IL-6 and TNF-α after incubation with Ova (Figure 3.7B; $P < 0.05$). A significant treatment effect was seen with IL-10 production, in which pups exposed to Ova produced less IL-10 than the placebo-exposed group irrespective of diet (Figure 3.7B; $P < 0.05$).

Table 3.7. Effect of diet and oral tolerance treatment on *ex-vivo* cytokine production by stimulated splenocytes from 3-week-old Sprague Dawley rat pups[1]

	Control diet		SDA diet				
	Placebo (*n*=5)	**Ova (*n*=5)**	**Placebo (*n*=4)**	**Ova (*n*=4)**	***P*-Diet[2]**	***P*-Treatment[3]**	***P*-Interaction[4]**
LPS							
IL-1β, pg/mL	228 ± 49	208 ± 46	208 ± 70	273 ± 41	0.69	0.69	0.46
IL-6, pg/mL	467 ± 182	397 ± 147	386 ± 166	352 ± 172	0.72	0.76	0.91
IL-10, pg/mL	228 ± 18	188 ± 45	205 ± 38	125 ± 44	0.27	0.13	0.59
TNF-α, pg/mL	589 ± 169	549 ± 152	515 ± 153	496 ± 166	0.70	0.86	0.95
Ova							
IL-1β, pg/mL	88 ± 15	179 ± 91	79 ± 31	69 ± 24	0.33	0.5	0.4
IL-2, pg/mL	8 ± 1	11 ± 2	5 ± 2	4 ± 3	0.09	0.56	0.32
IL-6, pg/mL	384 ± 108	354 ± 137	248 ± 90	399 ± 145	0.72	0.63	0.48
IL-10, pg/mL	39 ± 12[a]	95 ± 4[b]	48 ± 12[a]	39 ± 5[a]	0.12	0.12	0.04
TNF-α, pg/mL	459 ± 124	416 ± 127	395 ± 104	423 ± 92	0.81	0.95	0.76

[1]Values are presented in mean ± SEM. Labelled means in a row without a common superscript letter differ, $P < 0.05$.

[2]*P*-Diet represents the probability for the main effect of maternal diet on 3-week pups.

[3]*P*-Treatment represents the probability for the main effect of OT treatment on 3-week pups.

[4]*P*-Interaction represents the probability for the interaction of the main effects on 3-week pups.

Samples from only the first block of experiments were used for analysis resulting in *n* of less than 11 per diet group. Abbreviations; IL, interleukin; TNF-α, tumor necrosis factor-alpha; IFN-γ, Interferon-gamma; LPS, Lipopolysaccharide; OT, oral tolerance; Ova, ovalbumin

Table 3.8. Effect of diet and oral tolerance treatment on *ex-vivo* cytokine production by stimulated splenocytes from 6-week-old Sprague Dawley rat pups[1]

	Control diet		SDA diet				
	Placebo (*n*=6)	**Ova (*n*=6)**	**Placebo (*n*=6)**	**Ova (*n*=6)**	***P*-Diet[2]**	***P*-Treatment[3]**	***P*-Interaction[4]**
LPS							
IL-1β, pg/mL	140 ± 16	139 ± 31	154 ± 91	69 ± 21	0.49	0.29	0.30
IL-6, pg/mL	148 ± 24 [a]	140 ± 19 [a]	79 ± 19 [b]	42 ± 9 [b]	0.001	0.28	0.48
IL-10, pg/mL	188 ± 26 [a]	168 ± 24 [a]	292 ± 35 [b]	227 ± 28 [b]	0.01	0.16	0.44
TNF-α, pg/mL	205 ± 24 [a]	287 ± 54 [a]	154 ± 43 [b]	155 ± 8 [b]	0.03	0.29	0.31
IFN-γ, pg/mL	6 ± 4 [a]	4 ± 2 [b]	16 ± 4 [a]	4 ± 1 [b]	0.07	0.03	0.13
Ova							
IL-2, pg/mL	26 ± 2	22 ± 2	26 ± 2	25 ± 2	0.61	0.35	0.45
IL-6, pg/mL	129 ± 8 [a]	107 ± 33 [a]	57 ± 27 [b]	13 ± 5 [b]	0.002	0.17	0.63
IL-10, pg/mL	33 ± 3 [a]	24 ± 4 [b]	45 ± 7 [a]	30 ± 3 [b]	0.06	0.01	0.52
TNF-α, pg/mL	144 ± 6 [a]	156 ± 17 [a]	47 ± 15 [a]	23 ± 7 [a]	0.001	0.65	0.19

[1]Values are presented in mean ± SEM. Labelled means in a row without a common superscript letter differ, $P < 0.05$.

[2]*P*-Diet represents the probability for the main effect of a weaning diet on 6-week pups.

[3]*P*-Treatment represents the probability for the main effect of OT treatment on 6-week pups.

[4]*P*-Interaction represents the probability for the interaction of the main effects on 6-week pups.

Samples from only the first block of experiments were used for analysis resulting in *n* of less than 11 per diet group. Abbreviations; IL, interleukin; TNF-α, tumor necrosis factor-alpha; IFN-γ, Interferon-gamma; LPS, Lipopolysaccharide; OT, oral tolerance; Ova, ovalbumin

3.3.6. Effect of diet and OT treatment on immune cell phenotype

Spleen

There was no effect of diet on the proportion of different immune cell types in the spleen of dams. At 3 wk, splenocytes from the SDA group pups had a significantly higher proportion of helper T (Th) cells (CD3+CD4+), and a significantly lower proportion of NK cells (CD3−CD161+) and macrophages (CD11+) compared with pups fed the control diet (**Table 3.9**; $P < 0.01$). There were no OT treatment or interaction effects for any immune cell phenotype in the spleen from 3-week-old pups. In splenocytes from 6-week-old pups, there was a significantly lower proportion of CD28+ cells in Ova-exposed pups compared with the placebo group (**Table 3.10**; $P < 0.05$). In the pups that were fed the SDA diet, the proportion of CD4+CD25+ and total CD25+cells was higher compared with the control diet group (**Table 3.10**; $P < 0.05$).

Table 3.9. Effect of the diet on the immune cell phenotype isolated from the spleens of 3-week-old Sprague Dawley rat pups. [1]

	Control diet (*n*=6), % of total splenocytes	**SDA diet (*n*=6), % of total splenocytes**	***P*-Diet**
[2] Total CD3+	15.7 ± 0.7	17.7 ± 1.0	0.14
CD3+CD4+ (Th cells)	7.7 ± 0.4	9.5 ± 0.6	0.02
CD3+CD8+	7.2 ± 0.4	7.8 ± 0.5	0.38
[3] Total CD28+ (T cell co-receptor)	49.7 ± 1.6	45.5 ± 2.8	0.21
CD4+CD28+ (Th cells)	15.8 ± 0.6	17.7 ± 0.5	0.02
CD8+CD28+	10.8 ± 0.5	10.6 ± 0.8	0.82
Total CD25+	3.1 ± 0.2	3.4 ± 0.2	0.46
CD4+CD25+	3.1 ± 0.2	3.2 ± 0.4	0.91
CD8+CD25+	0.6 ± 0.1	0.8 ± 0.1	0.16
Total OX12+ (B cells)	17.4 ± 1.3	17.9 ± 1.1	0.8
Total OX6+ (APC)	41.5 ± 0.7	41.2 ± 1.0	0.86
Total OX62+ (DCs)	6.0 ± 0.4	4.9 ± 0.5	0.09
Total CD161+	10.1 ± 0.7	8.1 ± 0.6	0.02
CD3-CD161+ (NKs)	6.6 ± 0.4	4.8 ± 0.3	0.005
Total CD11+ (macrophages)	15.7 ± 0.5	12.6 ± 0.5	<0.001

[1] Values are presented as mean ± SEM; values are reported as percent of the total gated splenocytes (which includes a sum of lymphocytes, monocytes, macrophages, DCs, etc.) as determined by immunofluorescence.

[2] Monoclonal antibodies for CD3 (1F4, ThermoFisher Scientific) stains low in naïve T cells from young pups and it does not stain γδ T cells.

[3] CD28, a co-stimulatory T cell marker, is expressed in all the T cells and may be used for total T cells. Abbreviation: CD, cluster of differentiation; SDA, stearidonic acid; Th, T helper cells; APC, antigen-presenting cells.

Table 3.10. Effect of diet and oral tolerance treatment on immune cell phenotype isolated from the spleen of 6-week-old Sprague Dawley rat pups. [1]

	Control diet, % of total splenocytes		**SDA diet, % of total splenocytes**				
	Placebo (*n*=11)	Ova (*n*=11)	Placebo (*n*=10)	Ova (*n*=10)	***P*-Diet**	***P*-Treatment**	***P*-Interaction**
[2]Total CD3+	29.6 ± 1.5	29.9 ± 1.1	30.0 ± 1.5	29.2 ± 2.0	0.87	0.68	0.33
CD3+CD4+ (Th cells)	16.0 ± 0.8	15.7 ± 0.5	15.9 ± 1.0	15.2 ± 1.0	0.70	0.15	0.59
CD3+CD8+	12.8 ± 0.8	12.6 ± 0.6	12.0 ± 0.7	12.6 ± 1.0	0.69	0.72	0.57
CD3+CD4+CD25+FOXP3+ (Treg cells)	1.8 ± 0.2	1.9 ± 0.2	2.1 ± 0.3	1.9 ± 0.3	0.66	0.99	0.33
[3]Total CD28+ (T cell co-receptor)	42.1 ± 1.4[a]	41.2 ± 1.3[b]	40.9 ± 1.0[a]	38.5 ± 1.8[b]	0.49	0.04	0.33
CD4+CD28+ (mature Th)	17.6 ± 0.8	17.6 ± 0.7	18.1 ± 0.9	16.9 ± 1.0	0.85	0.09	0.09
CD8+CD28+ (mature CTL)	13.9 ± 0.8	13.6 ± 0.6	13.7 ± 1.0	12.9 ± 0.7	0.86	0.55	0.95
Total CD25+	4.6 ± 0.3 [a]	4.6 ± 0.2 [a]	5.6 ± 0.5 [b]	5.3 ± 0.4 [b]	0.05	0.53	0.60
CD4+CD25+	1.9 ± 0.2 [a]	1.8 ± 0.2 [a]	2.6 ± 0.2 [b]	2.2 ± 0.2 [b]	0.001	0.07	0.10
CD8+CD25+	0.8 ± 0.1	0.6 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.24	0.11	0.29
Total OX6+ (APC)	39.7 ± 2.4	40.4 ± 2.4	40.3 ± 2.6	38.3 ± 2.5	0.29	0.39	0.08
Total OX12+ (B cells)	36.7 ± 1.6	37.2 ± 1.9	37.3 ± 1.8	35.5 ± 1.9	0.54	0.64	0.44
Total OX62+ (DCs)	4.3 ± 0.2	4.0 ± 0.3	4.6 ± 0.2	4.9 ± 0.3	0.38	0.97	0.35
Total CD161+	13.1 ± 1.5	12.6 ± 1.2	13.2 ± 1.7	12.3 ± 1.6	0.73	0.10	0.67
CD3-CD161+ (NK cells)	8.8 ± 1.2	7.9 ± 0.8	8.5 ± 1.3	7.4 ± 1.0	0.29	0.39	0.08
Total CD11+ (macrophages)	10.5 ± 0.7	11.1 ± 0.9	10.1 ± 1	9.9 ± 0.8	0.15	0.68	0.41

[1] Values are presented as mean ± SEM; values are reported as percent of the total gated splenocytes (which includes an addition of lymphocytes, monocytes, macrophages, DCs, etc.) as determined by immunofluorescence.

[2] Monoclonal antibodies for CD3 (1F4, ThermoFisher Scientific) stains low in naïve T cells from young pups and it does not stain γδ T cells. This may have contributed to the lower CD3+ lymphocytes than what is previously reported.

[3] CD28, a co-stimulatory T cell marker, is expressed in all the T cells, which may be used to determine the total T cells.

Outliers were excluded from the analysis resulting in n<11 per diet group. P represents the main effect of diet or tolerance treatment in the mixed model on 6-week pups. Within a row, means without a common superscript letter are significantly different, P<0.05. CD, cluster of differentiation; SDA, stearidonic acid; APC, antigen-presenting cell; Th, T helper cells; Treg, T regulatory cells.

3.4. Discussion

We investigated the effect of feeding a diet containing a plant oil enriched in SDA (18:4n-3) on immune system development and the induction of OT to a food antigen (Ova) in neonatal rats. We examined the suckling diet effect in 3-week-old pups and the suckling + weaning diet effect in 6-week-old pups. Feeding SDA to dams resulted in a higher n-3 LCPUFA status (EPA, DPA, and DHA in plasma, and EPA and DPA, but not DHA, in splenocytes) in 3-week-old pups. These increases in n-3 LCPUFAs could have contributed to better overall growth, early maturation of adaptive immunity (markers of immune system development), and an improved in vivo humoral response (Ova-IgG1) to dietary food antigens. More importantly, higher total PL content of EPA and DPA in splenocytes of 6-wk-old pups was associated with better inflammation-resolving response when challenged ex vivo with bacterial antigen, characterized by lower amounts of inflammatory cytokines and higher amounts of the immunoregulatory cytokine, IL-10. Lastly, increasing EPA + DPA, unlike DHA, had no beneficial effect on the development of OT to dietary food antigen (Ova).

Feeding 3% of total fat as SDA to lactating dams resulted in significantly higher EPA, DPA, and DHA in the total plasma PL of the suckled pups. This suggests that dietary SDA is a substrate for EPA, DPA, and DHA biosynthesis in the dam and this likely gets transferred into breast milk. Previous studies in rodents (Valenzuela et al., 2004) and infants (Jensen et al., 1999; Lauritzen et al., 2004) have demonstrated that higher DHA content in breast milk increases infants' plasma DHA status, which might have contributed to the higher growth rate in the SDA offspring in the current study. Additional DHA during suckling has been shown to be beneficial for growth parameters: birthweight in infants (Muthayya et al., 2007; Smuts et al., 2003) and infant

development of the brain (Cao et al., 2009), immune function (Fritsche, 2007), neural tissue (Minns et al., 2010), and the respiratory system (Minns et al., 2010).

In the current study, we confirmed that feeding SDA to dams led to a more mature immune cell phenotype in pups at the end of suckling. More specifically, the spleen contained more adaptive immune cells (Th cells) and fewer innate immune cells (NK cells and macrophages), a pattern that more closely resembles adult rat splenocyte composition (Pérez-Cano et al., 2007). Consistent with this, SDA supplementation in the human diet (1 g/d SDA for 12 wk) resulted in a lower proportion of NK cells in the peripheral blood mononuclear cell (PBMC) population (E Miles et al., 2004), another indicator of maturation. Despite the changes in immune cell phenotypes, there was no significant effect of the SDA diet on the splenocytes' ex vivo response to LPS, a mitogen that stimulates B cells, DCs, and macrophages. The comparison between the effect of increasing DHA (with DHA supplementation) and EPA + DPA (with SDA supplementation) on the immune cell function of 3-week-old, suckled pups suggests a different effect of these LCPUFAs on immune development.

At 3 wk, splenocyte PLs from SDA pups had a higher EPA and DPA content and this might facilitate adaptive immune cell maturation (Liu et al., 2003; Sierra et al., 2008). Consistent with this, feeding the SDA diet led to signs of early maturation of the humoral response (B cell) characterized by a higher in vivo plasma concentration of Ova-IgG1, after an intraperitoneal challenge with only Ova. B-cell maturation begins in the bone marrow and involves isotype switching from primary Igs (IgM and IgA) to secondary Igs (IgG, IgD, and IgE) in the periphery, resulting in enhanced antigen-specific humoral immunity (Ova-IgG1). The greater Ova-IgG1 concentration without any corresponding increase in the proportion of total B cells (total $OX12^+$ and $CD45^+$ cells) in the spleen might suggest a higher antigen-specific adaptive

immunity. n-3 LCPUFAs have been previously reported to affect B-cell function (Gurzell et al., 2013; Teague et al., 2014), therefore a higher proportion of EPA and DPA in splenocyte PLs might favour B-cell maturation. Consistent with the current study, a fish-oil supplementation study that increased total n-3 LCPUFAs (including EPA and DPA but also DHA) was shown to improve immunoglobulin production after an immune challenge (Gurzell et al., 2013). In a previous study involving supplementation of the maternal diet with DHA, there was no effect of increasing DHA (without any changes in EPA or DPA) in splenocyte PLs on plasma IgG concentration (Caroline Richard et al., 2016c). This suggests that EPA + DPA and DHA could have different effects on the B-cell function in young animals, similar to what has been reported in adults (Teague et al., 2014). Although the clinical significance of higher antigen-specific IgG1 concentrations in plasma is unknown, low concentrations of plasma IgG1 and total IgG are associated with a high rate of respiratory infections (de la Torre et al., 2016). Further studies are required to understand the functional importance of altering the neonatal status of n-3 LCPUFAs on plasma immunoglobulin concentrations.

At 6 wk, Ova- or LPS-stimulated splenocytes from the SDA group produced lower amounts of proinflammatory cytokines (IL-6 and TNF-α). They also produced a stronger immunoregulatory cytokine response (IL-10) but only with LPS stimulation. Splenocytes from SDA-fed pups had a greater proportion of total CD25+ (activation marker) and CD4+CD25+ (activated Th cells) cells. Taken together, these observations suggest that the higher content of EPA and DPA in splenocytes supports a greater anti-inflammatory response (by increasing IL-10) in neonatal rat splenocytes after stimulation. Similarly, a study in which a higher SDA diet (10% w/w of total fat) was fed, reported significantly lower ex vivo TNF-α production by whole blood stimulated with LPS, and a trend toward lower TNF-α production by splenocytes when stimulated with LPS

(Ishihara et al., 2002). However, in this same study, the proportions of DPA and DHA in the total lipid composition of splenocytes were higher in pups fed the SDA diet, but there was also a significantly reduced ARA content in these splenocytes (Ishihara et al., 2002). Therefore, the anti-inflammatory effect might have been due to both an increase in the DPA and DHA content, and a decrease in the ARA content of the splenocytes. Similar anti-inflammatory effects were also seen in PBMCs from a study in humans supplemented with 9.7 mL/d of 0, 5, 10, or 17% SDA (Lefort et al., 2017). This study reported a linear dose-related increase in plasma and PBMC proportion of EPA (with no change in DHA), which was associated with a significantly higher production of IL-10 with LPS stimulation of PBMC (Lefort et al., 2017). In comparison, an earlier study that supplemented the diet of dams and pups with DHA reported that an increase in DHA (but not ARA) was associated with a lower proinflammatory cytokine response (IL-1β and IL-6) by LPS-stimulated splenocytes from 6-week-old pups (C. Richard et al., 2016). It is well established that the n-3 LCPUFAs have an anti-inflammatory effect on the immune response (Calder, 2006), and our results suggest that a dietary intervention that increases EPA + DPA has an anti-inflammatory effect similar to that observed in previous studies where diets were supplemented with DHA (C. Richard et al., 2016). Further studies directly comparing the effect of dietary interventions that increase EPA + DPA and/or DHA are needed to determine specific mechanisms of the different n-3 LCPUFAs in immune function.

Our previous findings suggest that increasing DHA in cells by DHA supplementation lowers inflammatory cytokine (IL-1β and IL-6) production after mitogen stimulation (Caroline Richard et al., 2016f), whereas the current study suggests that increasing EPA + DPA might have a regulatory effect by increasing IL-10 production. It does not appear that a reduction in the ARA content of tissues is required for the anti-inflammatory effects of these n-3 LCPUFAs. Results

from the current study and others (Lefort et al., 2017; E Miles et al., 2004) support the hypothesis that SDA is an efficacious substrate for EPA or DPA biosynthesis. Additionally, others have shown that at higher doses SDA could be a precursor for DHA (Ishihara et al., 2002).

OT was induced in 6-week-old pups previously exposed to Ova in both diet groups. Upon systemic immunization, pups from the Ova OT treatment group had a significantly lower plasma concentration of Ova-IgG1 ($P < 0.03$) compared with the placebo-exposed group. This is further supported by results from in vitro analysis. Consistent with other studies (C. Richard et al., 2016; Silva et al., 2010), we showed that there was a reduction in IL-10 production by splenocytes stimulated with Ova in the Ova-exposed OT group compared with the placebo-exposed group. This is particularly important because IL-10 enhances the IgE-mediated mast cell response in allergic reactions to food antigens (Ova) (Polukort et al., 2016), therefore a lower IL-10 response to Ova would support OT induction. Additionally, there was a lower proportion of CD28+ cells in the splenocyte population from the Ova-exposed OT group (in both diets) compared with the placebo group. This finding might indicate the suppression of antigen-specific T cells in the development of OT (van Wijk et al., 2007) in our model using multiple low-dose exposures to a food antigen. The interaction of the B7 molecule of DCs with either the costimulatory CD28 or coinhibitory cytotoxic T lymphocyte-associated protein 4 (CTLA4) of naïve T cells determines T-cell response (Bour-Jordan & Bluestone, 2002). Providing Ova (orally) early in life might have led to fewer CD28+ cells in the spleen compared with the placebo group, allowing B7 to interact more with CTLA4 on T cells, thus promoting tolerance by inducing the antigen-specific T cell to undergo clonal suppression (Boussiotis et al., 1994). It is important to note that there was no beneficial effect at 6 wk of feeding the SDA weaning diet on OT induction (plasma Ova-IgG1 concentrations), despite increased EPA + DPA content in splenocytes. This contrasts with

our previous experiments where we fed DHA and found it beneficially affected the OT induction but also increased the DHA content of splenocyte PLs (C. Richard et al., 2016).

There are limitations to the current study. First, the study was conducted in 2 blocks. Although we used a sensitive statistical model to control for the effects of random errors on the independent variable (diet), it was not entirely possible to correct for some block differences and this also prevented us from reporting data from 2 blocks combined in some cases. Second, we calculated the sample size based on previous studies that have been conducted to study the diet effect on neonatal development in females. This prevented us from reporting on the effect of sex on the results. It has been shown that the immune system develops differently in males and females (Kleina et al., 2015) and there is a difference in their ability to elongate and desaturate n-3 PUFAs (Lin et al., 2016; Walker et al., 2014), which could change their risk of developing allergies (a breach in OT) (Uekert et al., 2006). Finally, this study used healthy rodents. Further investigation could include employing a more allergy-sensitive model to determine if the changes in maturation observed in the current study impact the development of food allergy.

In conclusion, feeding SDA led to the in vivo biosynthesis of EPA and DPA but not DHA while maintaining tissue concentrations of ARA. At 3 wk, increasing n-3 LCPUFAs led to better growth and immune system maturation. At 6 wk, EPA + DPA had an anti-inflammatory effect, but unlike DHA supplementation, it did not alter the ability to develop OT.

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Chapter 4. Long-chain polyunsaturated fatty acids docosahexaenoic acid and arachidonic acid supplementation in the suckling and the post-weaning diet influences the immune system development of T helper type-2 bias Brown Norway rat offspring[1].

4.1. Introduction

Docosahexaenoic acid (DHA), an omega-3 (n-3/ω-3) long-chain polyunsaturated fatty acid (LCPUFA) and arachidonic acid (ARA), an n-6 LCPUFA, are essential fatty acids needed for infant neural and vision development (S.M. Innis, 2014; Jensen et al., 1999). However, their role in the development of the immune system, particularly in those at risk for atopic conditions due to T helper type-2 (Th2) dominant immunity, has been sparsely studied. Studies have shown the beneficial effects of DHA in populations predisposed to developing conditions associated with Th2 dominance (Noakes et al., 2012; Palmer et al., 2012). However, often these studies were conducted using a diet containing no ARA or a very high level of DHA. Such dietary conditions have anti-inflammatory and immunosuppressive effects (Akhtar Khan, 2010; Kim et al., 2010) and may result in a lower concentration of ARA in the immune cells (Calder, 2008; Fritsche, 2007).

The essentiality of ARA in the formula for development in a term infant has been debated, despite its presence in human milk (Hosea Blewett et al., 2008; Yuhas et al., 2006). The importance of ARA supplementation, alongside DHA, on infant immune system development

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has been shown in healthy-term infants by our group (Foiles et al., 2016). Previous studies from our lab with DHA and ARA supplementation, conducted in infants and Sprague-Dawley rats, have shown beneficial effects of ARA+DHA on the immune response and oral tolerance development (Field et al., 2008a; Caroline Richard et al., 2016d, 2016e, 2016f) yet to date, no studies have assessed the combination of dietary ARA+DHA in a Th2 biased preclinical model.

Passive immunity in neonates arises during pregnancy and lactation, contributing to the initial immune response against pathogens (Laouar, 2020). This changes as an infant's immune organs develop and the adaptive immune system matures. In the first few months after birth, neonates rely heavily on the innate immune system, consisting of granulocytes, antigen-presenting cells (APCs), macrophages and natural killer (NK) cells to protect against pathogens until the adaptive immune response, through T cells and B cells, reaches a more adult level of maturation (Basha et al., 2014). The T cells and B cells first enter the bloodstream from the primary lymphoid organs (thymus and bone marrow respectively) in a naïve state and are unable to mount an effective response toward pathogens. Therefore, they undergo additional education in the secondary lymphoid organs (such as the spleen and lymph nodes) to become effector cells that are able to mount an adult level of immune response to pathogens (Hunter et al., 2016). Similarly, gut-associated lymphoid tissue (GALT) including Peyer's patches (PP) and mesenteric lymph nodes (MLN), form germinal centers and result in rapid changes in adaptive immune cell populations post-weaning when the intestine is exposed to solid-food-derived antigens (Hara et al., 2019; Manzano et al., 2002). Therefore, the development of the spleen, lymph nodes and GALT become critical for the maturation of adaptive immunity (Spahn & Kucharzik, 2004). Furthermore, the nutrition provided during these periods is a source of nutrients and antigens necessary for adaptive immune cell maturation (Calder et al., 2006b).

The immune response during early infancy is skewed towards a prenatally derived Th2 cytokine pattern, such as IL-4, IL-5 and IL-13, making them more susceptible to infection and allergic reactions (Basha et al., 2014; Wilson & Kollmann, 2008; Zaghouani et al., 2009). As infants are introduced to solid food, between 6 and 12 months of age, they rely less on maternally derived passive immunity and develop antigen-specific adaptive immunity which is associated with an increase in Th1 cytokines (IL-2, IFNγ, TNF-α) and cell-mediated immune responses (Raphael et al., 2015). If the switch from Th2 (characterized by a high level of Th2 cytokines and low level of Th1 cytokines) to Th1 does not occur, it can lead to conditions such as food allergies and atopic disorders (Van Der Velden et al., 2001). The Th2 dominant environment of Brown Norway rats makes them highly prone to developing food allergies with high levels of Immunoglobulin-E (IgE) and IgG1 antibodies (Th2-related antibodies) (Fournie et al., 2001). More specifically, they have higher circulating $CD45RC^{low}CD4+$ T cells and lower circulating CD8+ T cells resulting in fewer Th1 cytokines and more Th2 cytokines (IL-4 and IL-13), compared to other rodent models upon *ex-vivo* stimulation with T cell mitogens (Fournie et al., 2001; Knippels et al., 1999). This dominant Th2 environment provides a unique condition, similar to infants with genetic predispositions to allergies, to study the immunomodulatory properties of LCPUFA on immune system development (Campbell et al., 2015). However, LCPUFA supplementation during early infancy on immune system development has not been assessed. Therefore, the primary objective of the current study is to determine the effect of feeding ARA+DHA during the suckling period (through the maternal diet), and in the post-weaning period on (1) LCPUFA status and (2) immune system development and function in Brown Norway offspring at 8 weeks. We hypothesized that the addition of ARA and DHA to

their diet would be beneficial for the Th2 bias Brown Norway offspring by promoting a Th1 response and early development of adaptive immune cells.

4.2. Materials and methods

4.2.1. Study design and diet

Experimental diets were isocaloric, isonitrogenous and nutritionally adequate. The macronutrient and micronutrient composition of the semi-purified basal diet (fat omitted) used has been previously described in detail (Lewis et al., 2016a). The fat mixture added to the experimental diets (20g/100g diet) was obtained by blending lard, olive oil, Mazola canola oil, Mazola corn oil, ARAsco and DHAsco (DSM, Nutritional Products, Columbia, MD, USA). The experimental diets were closely matched to have similar PUFA to saturated fatty acid (SFA) ratios and n-6 to n-3 ratios (Table 4.1). The diets were prepared biweekly and stored at 4°C until used to prevent exposure to air.

Table 4.1. Total fatty acid composition of the experimental maternal diets fed during the suckling period and post-weaning diets fed to Brown Norway offspring.[1]

Fatty acid (g/100g total fatty acids)	Control maternal diet	ARA+DHA maternal diet	***P*-maternal diet[3]**	Control post-weaning diet	ARA+DHA post-weaning diet	***P*-post-weaning diet[4]**
14:0	0.9±0.1	1.2±0.1	0.11	0.9±0.1	1.0±0.1	0.50
16:0	19.5±0.2	20.7±0.2	0.03	19.5±0.2	19.9±0.6	0.52
18:0	10.6±0.1	11.3±0.2	0.07	10.6±0.1	11.0±0.4	0.36
16:1n-7	1.6±0.1	1.7±0.1	0.38	1.6±0.1	1.6±0.1	0.95
18:1n-9	43.9±1.0	39.4±1.5	0.08	44.0±1.0	42.7±2.6	0.62
18:2n-6 LA	19.9±0.3	21.3±0.5	0.07	19.9±0.3	19.7±0.7	0.78
20:4n-6 ARA	0.0±0.0	0.5±0.1	<0.001	0.0±0.0	0.5±0.0	<0.001
18:3n-3 ALA	2.5±0.0	1.9±0.0	0.001	2.5±0.0	2.1±0.1	0.01
22:6n-3 DHA	0.0±0.0	0.8±0.0	<0.001	0.0±0.0	0.6±0.1	<0.001
Total[2] SFA	31.1±0.1	33.2±0.1	0.002	31.1±0.1	31.9±1.2	0.40
Total PUFA	22.4±0.3	24.5±0.6	0.04	22.3±0.3	22.8±0.9	0.63
Total MUFA	45.6±0.9	41.1±1.4	0.07	45.5±0.9	44.3±2.5	0.61
Total n-6	19.9±0.3	21.8±0.5	0.04	19.9±0.3	20.7±0.7	0.70
Total n-3	2.5±0.0	2.7±0.0	0.03	2.5±0.0	2.6±0.2	0.36

Ratio n-6/n-3	8.1±0.0	8.1±0.1	0.65	8.1±0.1	7.8±0.2	0.18

[1] Fatty acid analysis of diet was done using gas-liquid chromatography. Data are presented as the mean ± SEM of 2-3 batches of oil mix used for diet. Abbreviations: ALA, α-linolenic acid; LA, linoleic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; PUFA, polyunsaturated fatty acid; MUFA, monounsaturated fatty acid; SFA, saturated fatty acid.

[2] Some fatty acids that were traced were not presented as they were very small or not relevant, this may lead to a mismatch in total proportions of fatty acids such as total SFA, PUFA, MUFA, n-6 and n-3.

[3] Indicates *P* value for a T-test for the difference between maternal diets.

[4] Indicates *P* value for a T-test for the difference between post-weaning diets.

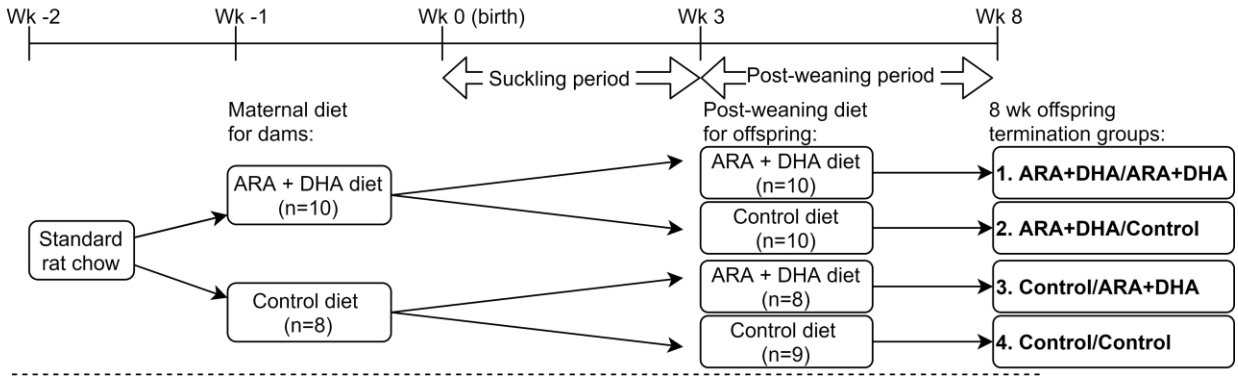

Figure 4.1. Animal study design. Pregnant Brown Norway dams were assigned to either ARA+DHA supplemented maternal diet (n = 10) or control diet (n = 8) 5-7 d before parturition and continued the same diet throughout the suckling period (3 weeks). Male and female offspring were culled between the same diet group of dams to get an approximate sex ratio match between the 4 diet groups. Offspring breastfed to their dam during the suckling period. At the end of the suckling period, offspring from each dam consumed either ARA+DHA post-weaning diet or control post-weaning diet until 8 weeks (post-weaning period) from birth. Dams were killed at the end of the suckling period and offspring at the end of the post-weaning period. The animal experiments were conducted in serial blocks to achieve n = 8 offspring per diet group.

Animal care and experiments were conducted as per guidelines stipulated by the Canadian Council on Animal Care and approved by the University of Alberta Animal Ethics Committee (AUP00000125). Timed-pregnant Brown Norway rats (Charles River Laboratories, Laval, Quebec, Canada; n = 18) were obtained on day 7 of gestation and housed in a temperature and humidity-controlled environment with a 12/12 h reverse light cycle. Dams were fed standard rat chow purified diet (Lab diet 5001; PMI Nutrition International, St Louis, MO, USA) for 1 week acclimatization period (Figure 4.1). One week prior to parturition, dams were randomized to consume one of the experimental diets: ARA+DHA (0.45% ARA, 0.8% DHA w/w of total fat; n = 10) or control (0% ARA, 0% DHA; n = 8) diet (Table 4.1). The DHA concentration for the maternal diet was selected to achieve the upper end (0.8% of total fat as DHA) of breastmilk reported in human populations (Brenna et al., 2007). Subsequently, the ARA concentration was selected to attain an ARA/DHA ratio of 1:2, as previous experiments from our lab found this ratio effective in increasing DHA levels without affecting the ARA levels in immune cell phospholipids. The litters were culled, or the pups were cross-fostered to ensure an equal number of offspring per dam (3-5 pups/dam) to avoid weight differences in the offspring. Dams were fed experimental diets *ad libitum* throughout the suckling period (3 weeks). At the age of 3 weeks, offspring from each dam were randomized to either the ARA+DHA post-weaning diet (0.5% DHA and 0.5% ARA w/w of total fat, n = 18) or control post-weaning diet (0% ARA and 0%DHA, n = 19) until 8 weeks of age. The DHA concentration of 0.5% of total diet fat was calculated based on physiologically achievable levels of DHA with 2 to 3 servings of fish per week, as outlined in the 2015-2020 dietary guideline for Americans (Eighth edition) (US Department of Health and Human Services and US Department of Agriculture, 2015) and the ARA concentration of 0.5% was selected to obtain a 1:1 ratio for ARA/DHA. Male and female

offspring were equally distributed between post-weaning diet groups to enable the studying of sex effects. The offspring had unrestricted access to food and water. Food cups were changed every 2-3 days to limit air exposure. Bodyweight and food intake were regularly recorded. Dams were killed at the end of the 3-week suckling period and all the offspring were euthanized at 8 weeks (end of post-weaning period) and tissues were collected.

4.2.2. Tissue collection: Spleen, MLN, PP, blood and plasma, mammary tissue

Following euthanization, blood was collected by cardiac puncture, centrifuged (1734 x g; 10 min; 22°C), plasma aliquoted and stored at -80°C until further analysis. The spleen, MLN and PP from the offspring and the mammary gland from the dams were aseptically removed. Immune cells from the spleen, MLN and PP were isolated as previously described (C J Field et al., 1990). Briefly, tissues were pushed through a nylon mesh screen to obtain a single-cell suspension. Erythrocytes were lysed with ammonium chloride lysis buffer (155 mM NH_4Cl, 0.1 mM EDTA and 10 mM KHCO3; Fisher Scientific, Alberta, Canada), cells were washed and resuspended in complete cell culture media (RPMI 1640 media supplemented with 5% v/v fetal calf serum, 2.5 mM 2-mercaptoethanol and 1% antibiotic / antimycotic; Thermo Fisher Scientific, Mississauga, Ontario, Canada). Cell concentration was determined using cell viability trypan blue dye (Sigma-Aldrich) and hemocytometer and diluted to 1.25×10^6 cells/mL. Note, we were not able to collect cells at the required concentration from MLN and PP for some 8-week offspring. Therefore, the final group size assessed for fatty acid analysis and immune cell phenotyping differs from the original group size and is detailed in the results.

4.2.3. *Ex-vivo* immune cell stimulation and cytokine measurement

Ex-vivo cytokine production by stimulated immune cells was measured as previously described (Blewett et al., 2009). Briefly, spleen or MLN immune cells (1.25×10^6 cells/mL) were

incubated in the presence or absence of phorbol-myristate-acetate and ionomycin (PMAi, 2µl/mL, Cell Stimulation Cocktail, Thermo Fisher Scientific, Mississauga, Ontario, Canada), lipopolysaccharide (LPS, 2µl/mL, Thermo Fisher Scientific, Mississauga, Ontario, Canada) for 72 h at 37 °C and 5% CO_2. Cells were then centrifuged, supernatant collected and stored at -80°C. Commercial ELISA kits were used to measure cytokine concentration (range of detection in brackets) of IL-1β (15.6-4000 pg/mL), IL-2 (250-4000 pg/mL), IL-6 (31.3-4000 pg/mL), IL-10 (15.6-4000 pg/mL) and TNF-α (31.3-4000 pg/mL) (R&D Systems, Minneapolis, MN, USA), IFN-γ (8-1024 pg/mL, U-CyTech Bioscience, Cedarlane, Burlington, Ontario, Canada), TGF-β1 (31.3-500 pg/mL, BioLegend, San Diego, CA, USA) and IL-4 (0.1-4500 pg/mL) and IL-13 (0.1-3200 pg/mL) (Mesoscale Discovery, U-Plex, Rockville, MD, USA) as per the manufacturer's instruction. Absorbance was read on a spectrophotometer, and concentrations were calculated using a standard curve (SpectraMax 190 Microplate Reader, Molecular Devices, San Jose, CA, USA). All measurements were conducted in duplicate with a CV<15%.

4.2.4. Fatty acid analysis of phospholipid and total lipids

Total lipids from the diet, mammary gland, splenocytes, MLN, and plasma were extracted by modified Folch as previously described (C J Field et al., 1988b; Caroline Richard et al., 2016d). Total phospholipids from splenocytes, MLN and plasma were further isolated by thin layer chromatography on silica G plates and fatty acids were separated by automated gas-liquid chromatography (Agilent Technologies, Ontario, Canada) on a 100 m CP-Sil 88 fused capillary column as previously described (Cruz-Hernandez et al., 2013). They were quantified as relative percentage of total phospholipid fatty acid content.

4.2.5. Immune cell phenotype

The phenotype of isolated immune cells from the spleen, MLN and PP were assessed by direct immunofluorescence assay as previously described (Field et al., 2000). Four-colour flow cytometry was used to identify cell surface markers in combinations. The fluorochrome of monoclonal antibodies (mAb) was selected in a way to minimize or avoid spectra overlap, and compensation controls and unstained controls for each fluorochrome were used to guide the gating strategy. For splenocytes, the following combination of mAb was used: CD3/CD25/CD4/CD8, CD25/CD3/FoxP3/CD4, TCRαβ/CD27/CD8/CD4, CD4/CD152/CD8/CD28, CD25/OX62/OX6/CD11, CD284/CD68/CD11/CD45RA, OX12/CD86/OX6/CD45RA, CD3/CD161, IgE/CD45RA/IgG and IgA/CD45RA. For immune cells of MLN, the following combinations of monoclonal antibodies were used: CD3/CD25CD4/CD8, CD25/CD3/FoxP3/CD4, TCRαβ/CD27/CD8/CD4, CD4/CD152/CD8/CD28, OX12/CD27/OX6/CD45RA, IgA/CD45RA/IgG and IgE/CD45RA. For immune cells of PP, the following combinations of monoclonal antibodies were used: CD3/CD25CD4/CD8, CD284/CD68/CD11/CD45RA, OX62/CD86/OX6/CD45RA, IgA/CD45RA/IgG and IgE/CD45RA. Note, CD152 identifies CTLA4 on T cells, OX62 marker identifies the integrin molecule on DCs, OX6 identifies MHC-II on APCs, CD68 identifies tissue-resident macrophages, OX12 identifies Ig-κ chain on splenic B cells. All antibodies were purchased from Biolegend (San Diego, CA, USA)or BD Biosciences (Mississauga, Ontario, Canada). Briefly, immune cells (200,000) were incubated for 30 min at 4°C with cell surface monoclonal antibodies. Subsequently, for intracellular staining of FoxP3, cells were fixed, permeabilized, and stained with fluorophore-conjugated FoxP3 antibody for 20 min. Then the cells were washed, fixed in paraformaldehyde (10g/L; Thermo Fisher Scientific, Mississauga, Ontario, Canada) and acquired within 72 h by flow cytometry (FACSCalibur; Becton-Dickinson,

San Diego, CA, USA) according to the relative fluorescence intensity and analyzed using FlowJo software. The gating strategies for major cell types are described in Figure 4.2.

4.2.6. Statistical analysis

Data are presented as mean ± SEM unless stated otherwise. The current study was powered to assess the primary objective of immune function and the secondary objective of changes in the fatty acid composition of various tissues in the Th2-biased Brown Norway rats. The sample size of $n = 8$ per group was calculated based on experiments conducted in our lab using healthy Sprague-Dawley rats, in which a difference of 20% (β value) could be identified at a significance level of 5% (α value). Data were checked for normality and homogeneity. When the assumptions criteria were not met, the data were transformed, and assumptions were rechecked before conducting statistical analysis. Data were analyzed using the PROC MIXED procedure 3-factor ANOVA with suckling (maternal) diet, post-weaning diet and sex as the main effects (SAS 9.4 software, Cary, NC, USA). However, only a 2-factor ANOVA was pursued when sex or an interaction effect involving sex was not statistically significant. This allowed us to use a 2x2 study design to study the effect of the suckling diet, post-weaning diet, and suckling diet × post-weaning diet interaction. Note, when a significant interaction between the suckling diet × post-weaning diet was observed, a post hoc analysis (PROC LSMEANS) was conducted to compare the means of the four groups (ARA+DHA/ARA+DHA, ARA+DHA/Control, Control/ARA+DHA and Control/Control). Furthermore, when there was a trend toward significance, the four groups based on diet were reported for better visualization. When the post-weaning diet variable was absent (fatty acid analysis of diet and mammary gland for dams), an unpaired Student's *t*-test was conducted for 2 group comparisons. Differences were assumed to be significant at $P \leq 0.05$ (2-tailed).

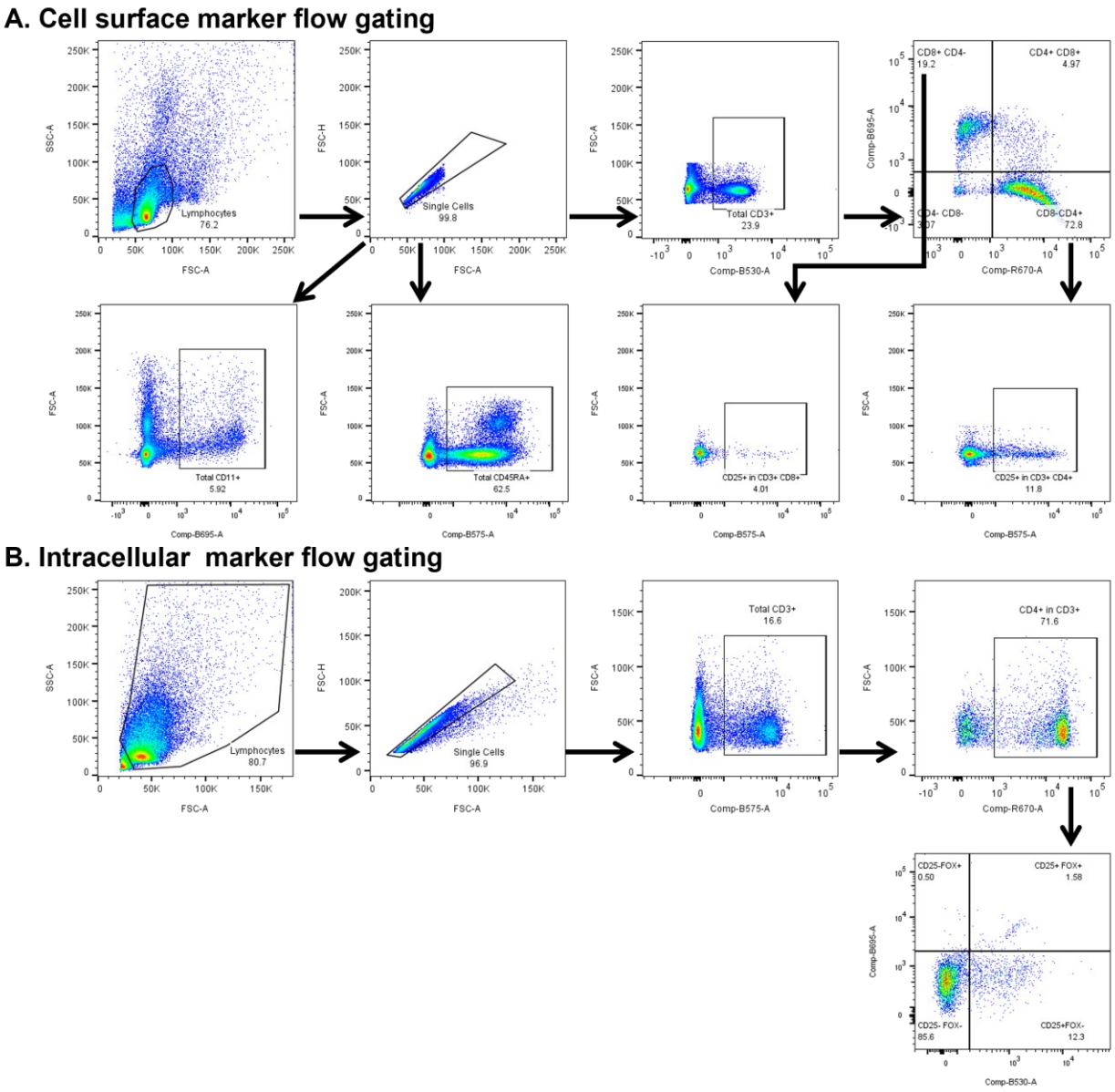

Figure 4.2. Panel A shows representative plots of the gating strategy used to identify subtypes of T cells, CD45RA+ cells and CD11+ cells based on cell surface markers on splenocytes from 8-week-old Brown Norway rat offspring. The forward-scatter area (FSC-A) versus side-scatter area (SSC-A) plot was used to draw the size gate to delimitate the lymphocytes region followed by an FSC-A versus forward-scatter height (FSA-H) for doublet exclusions (single cells). Gates were drawn for single fluorescence positive cells such as CD3+ (total T cells), CD11+ (macrophages), and CD45RA+ (total B cells). Further, a 2-dimension CD8 versus CD4 graph was plotted on CD3+ T cells to identify CD8+CD4- (cytotoxic T cells) and CD8-CD4+ (helper T cells) populations. Subsequently, single stain CD25 was plotted on CD8+ T cells and CD4+ T cells to identify CD25+ on CD8+CD3+ (activated CTL) and CD25+ on CD4+CD3+ (activated Th) cells as a percent of their parent generation. Panel B shows representative plots of the gating strategy used to identify T regulatory cells based on cell surface marker (CD3, CD4 and CD25) and intra-

cellular marker (FoxheadP3, FoxP3) on splenocytes from 8-wk Brown Norway rat offspring. The FSC-A versus SSC-A plot was used to draw the size gate to delimitate the lymphocytes region followed by an FSC-A versus FSA-H for doublet exclusions (single cells). Gates were drawn for single fluorescence CD3+ cells followed by CD4+ cells. Then, a 2-dimension FoxP3 versus CD25 graph was plotted to identify FoxP3+CD25+ on CD4+CD3+ cells (regulatory T cells). Numbers for the gated cell population represent the percent of positive cells for the stated marker in the parent population. The cell marker combination and the associated cell population identified are shown in Table 4.2.

Table 4.2. Description of immune cells phenotype used to identify immune cell subpopulations from lymphoid tissues collected from 8-week-old Brown Norway rat offspring.

Phenotype	Cell types	Tissues
T cells		
CD3+	Total T cells	Spleen, MLN and PP
TCRαβ	T cells with αβ-TCR	Spleen and MLN
CD3+CD8+	Naïve CD8+ T cells	Spleen, MLN and PP
CD3+CD8+CD25+	Activated CD8+ T cells	Spleen, MLN and PP
CD3+CD4+	Th cells	Spleen, MLN and PP
CD3+CD4+CD25+	Activated Th cells	Spleen, MLN and PP
CD3+CD4+CD25+FoxP3+	Treg cells	Spleen and MLN
CD8+CD4-CD152+	CTLA4 on CTL	Spleen and MLN
CD8+CD4-CD28+	Co-stimulatory marker on CTL	Spleen and MLN
CD4+CD8-CD152+	CTLA4 on Th	Spleen and MLN
CD4+CD8-CD28+	Co-stimulatory marker on Th	Spleen and MLN
CD27+	Total memory lymphocytes	Spleen and MLN
B cells		
CD45RA+	Total B cells and naïve leukocytes	Spleen, MLN and PP
OX12+	B cell subtype with	Spleen and MLN
CD45RA+IgG+	B cell subtype with IgG	Spleen, MLN and PP
CD45RA+IgE+	B cell subtype with IgE	Spleen, MLN and PP
CD45RA+IgA+	B cell subtype with IgA	Spleen, MLN and PP
Innate immune cells		
CD3-CD161+	NKs	Spleen
CD3+CD161+	NKT cells	Spleen
OX6+	APC	Spleen, MLN and PP
OX62+	DC	Spleen, MLN and PP
CD68+	Tissue Macrophages	Spleen and PP
CD11+	Macrophages and granulocytes	Spleen and PP
CD284+	TLR4 on macrophage, DC and B cells	Spleen and PP

Abbreviation: MLN, mesenteric lymph nodes; PP, peyer's patches; αβ-TCR, αβ-T cell receptors; Th, Helper T cells; Treg, regulatory T cells; APCs, antigen-presenting cells; CTLA4, Cytotoxic T-Lymphocyte Associated Protein 4; TLR4-4, toll-like receptor-4; DC, dendritic cells; NK, natural killer cells; NKT cells, natural killer T cells.

4.3. Results

4.3.1. Growth parameters

There were no significant effects of the suckling diet or post-weaning diet on the body weight (mean 156±5 grams), liver weight (mean 6.4±0.2 grams), spleen weight (mean 0.4±0.0 grams), and splenocyte count (mean 154±8 x10^6 cells) at 8 weeks of age (Table 4.3).

4.3.2. Fatty acid composition of breastmilk (mammary gland) and plasma of dams

Breastmilk from dams fed the ARA+DHA maternal diet resulted in significantly higher DHA content (4 times, $P < 0.0001$), total n-3 fatty acids, total n-6 fatty acids, total PUFA and PUFA/SFA ratio when compared to the control diet (P's < 0.05). Further, ARA+DHA maternal diet resulted in significantly lower ARA/DHA ratio, total monounsaturated fatty acids (MUFA), α-linolenic acid (ALA) and C22:5n-6 content in breastmilk when compared to dams that were fed the control maternal diet ($P < 0.05$). However, supplementing the maternal diet with ARA+DHA did not alter ARA, n-6/n-3 ratio and total SFA content of breastmilk total lipids (Table 4.4). Note, feeding ARA+DHA maternal diet to breastfeeding dams also resulted in significantly higher plasma total phospholipid content of DHA (1.5±0.2 vs 0.9±0.1, $P = 0.04$) when compared to the maternal control diet ($P = 0.04$) without affecting the ARA content (Table 4.5)

Table 4.3. Anthropometric measurements of 8-week offspring based on suckling diet and post-weaning diet.[1]

Suckling diet	Control	Control	ARA+DHA	ARA+DHA			
Post-weaning diet	Control	ARA+DHA	Control	ARA+DHA			
	Mean ± SEM (n = 9)	Mean ± SEM (n = 8)	Mean ± SEM (n = 10)	Mean ± SEM (n = 10)	*P*-SD[2]	*P*-WD[3]	*P*-SD × WD[4]
Body weight (g)	153±10	149±12	158±8	164±9	0.23	0.69	0.79
Animal length (cm)	18.0±0.3	17.7±0.5	18.2±0.3	18.6±0.5	0.53	0.95	0.33
Liver weight (g)	6.3±0.4	6.1±0.4	6.4±0.4	6.6±0.3	0.17	0.87	0.41
Spleen weight (g)	0.4±0.0	0.4±0.0	0.4±0.0	0.4±0.0	0.22	0.86	0.27
Total splenocytes (x10^6 cells)	152±17	134±14	175±17	151±18	0.38	0.35	0.72

[1] Data is presented in mean ± standard error of the mean. Abbreviations: SD, suckling diet; WD, post-weaning diet ARA; arachidonic acid; DHA, docosahexaenoic acid,

[2] Indicates P-value for the main effect of the SD in a mixed model.

[3] Indicates P-value for the main effect of the WD in a mixed model.

[4] Indicates interaction between maternal SD and offspring WD.

Table 4.4. Fatty acid composition of total lipids in breastmilk from the mammary gland of Brown Norway dams at the end of the suckling period.[1]

Fatty acid (g/100g total fatty acids)	Control diet (n = 4)	ARA+DHA diet (n = 6)	P-maternal diet
14:0	1.4±0.1	1.3±0.0	0.27
16:0	20.8±0.4	20.1±0.5	0.36
18:0	10.0±0.5	11.6±0.8	0.16
20:0	0.1±0.0	0.1±0.0	0.88
24:0	0.1±0.0	0.2±0.0	0.29
16:1n-9	1.9±0.2	1.5±0.1	0.05
18:1n-9 Oleic acid	43.0±0.8	38.5±0.9	0.008
24:1n-9	0.1±0.0	0.1±0.0	0.43
18:2n-6 LA	16.9±0.4	19.1±0.3	0.001
20:2n-6	0.4±0.0	0.5±0.0	0.03
20:3n-6	0.2±0.0	0.3±0.0	0.33
20:4n-6 ARA	1.6±0.1	2.2±0.3	0.12
22:4n-6	0.2±0.0	0.2±0.0	0.98
22:5n-6	0.07±0.0	0.1±0.0	<0.001
18:3n-3 ALA	1.7±0.1	1.5±0.1	0.02
20:5n-3 EPA	0.0±0.0	0.0±0.0	0.96
22:5n-3 DPA	0.1±0.0	0.1±0.0	0.66
22:6n-3 DHA	0.2±0.0	0.8±0.0	<0.0001
Total[2] SFA	33.6±0.6	35.0±0.7	0.21
Total MUFA	44.9±0.8	40.1±0.9	0.008
Total PUFA	21.5±0.6	24.9±0.3	0.001
Total n-6	19.5±0.5	22.4±0.3	0.001
Total n-3	2.0±0.1	2.5±0.1	0.002
Ratio n-6/n-3	9.6±0.1	9.1±0.3	0.31
Ratio PUFA/SFA	0.6±0.0	0.7±0.0	0.015
Ratio ARA/DHA	9.0±0.9	2.9±0.27	0.001

[1] Data is presented in mean ± standard error of the mean. Abbreviations: ALA, α-linolenic acid; LA, linoleic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; PUFA, polyunsaturated fatty acid; MUFA, monounsaturated fatty acid; SFA, saturated fatty acid.

[2] Some fatty acids that were traced were not presented as they were very small or not relevant, which may not add up to the total amount such as total SFA, PUFA, MUFA, n-6 and n-3.

Table 4.5. Total fatty acid composition of plasma phospholipids from dams at the end of suckling period [1]

Fatty acid (g/100g total fatty acids)	Control diet (n = 4)	ARA+DHA diet (n = 6)	P-SD
14:0	2.9±0.5	3.6±0.3	0.19
16:0	24.9±1.1	25.4±0.5	0.66
18:0	36.1±1.1	34.5±0.7	0.22
20:0	0.9±0.3	0.9±0.2	0.91
24:0	0.9±0.1	0.9±0.0	0.54
16:1n-9	0.6±0.2	0.3±0.1	0.13
18:1n-9 Oleic acid	6.3±0.3	5.8±0.2	0.14
24:1n-9	0.9±0.2	0.8±0.1	0.94
18:2n-6 LA	13.7±0.8	13.2±0.7	0.67
20:2n-6	0.5±0.1	0.7±0.1	0.35
20:3n-6	0.7±0.1	0.8±0.2	0.80
20:4n-6 ARA	6.5±0.6	7.2±0.6	0.44
22:5n-6	0.6±0.1	0.7±0.1	0.40
18:3n-3 ALA	0.9±0.2	0.7±0.1	0.55
20:5n-3 EPA	0.4±0.1	0.4±0.1	0.93
22:5n-3 DPA	0.5±0.1	0.5±0.1	0.88
22:6n-3 DHA	0.9±0.1	1.5±0.2	0.04
Total[2] SFA	66.4±1.1	66.2±0.7	0.88
Total MUFA	9.0±0.4	8.1±0.2	0.045
Total PUFA	24.6±0.8	25.7±0.6	0.29
Total n-6	21.4±1.1	21.9±0.8	0.71
Total n-3	2.6±0.4	3.1±0.2	0.29
Ratio n-6/n-3	9.1±1.6	7.4±0.7	0.31

[1] Data is presented in mean ± standard error of the mean. Abbreviations: ALA, α-linolenic acid; LA, linoleic acid; ARA, arachidonic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PUFA, polyunsaturated fatty acid; MUFA, monounsaturated fatty acid; SFA, saturated fatty acid; SD, suckling diet. Outliers were removed from the analysis resulting in an n value less than the total used for the experiment.

[2] Some fatty acids that were traced were not presented as they were very small or not relevant, which may not add up to the total amount such as total SFA, PUFA, MUFA, n-6 and n-3.

4.3.3. Fatty acid composition of total phospholipids in tissues from 8-wk offspring

Regardless of the suckling diet, the ARA+DHA post-weaning diet resulted in 50% higher DHA (1.2±0.1 vs 0.8±0.0, $P = 0.02$) in plasma phospholipids compared to the control post-weaning diet (Figure 4.4. A). The suckling or post-weaning diets had no effects on ARA, LA, eicosatetraenoic acid (ETA), docosapentaenoic acid (DPA), ALA, total SFA, total MUFA, total PUFA and total n-6 content of plasma phospholipids. Splenocyte phospholipid fatty acid content of 8-wk old offspring was not significantly altered by the suckling diet. However, in offspring that were fed an ARA+DHA post-weaning diet (regardless of suckling diet), the splenocyte phospholipid content of DHA was 2 times higher (1.65±0.04 vs 0.85±0.02; $P < 0.001$), total n-3 was higher (3.6±0.1 vs 2.9±0.0; $P = 0.003$) and the n-6/n-3 ratio was lower (10.8±0.1 vs 8.7±0.1; $P < 0.001$) compared to offspring fed the control post-weaning diet. The ARA content was not significantly different across the diet groups (Figure 4.4. B and Table 4.6).

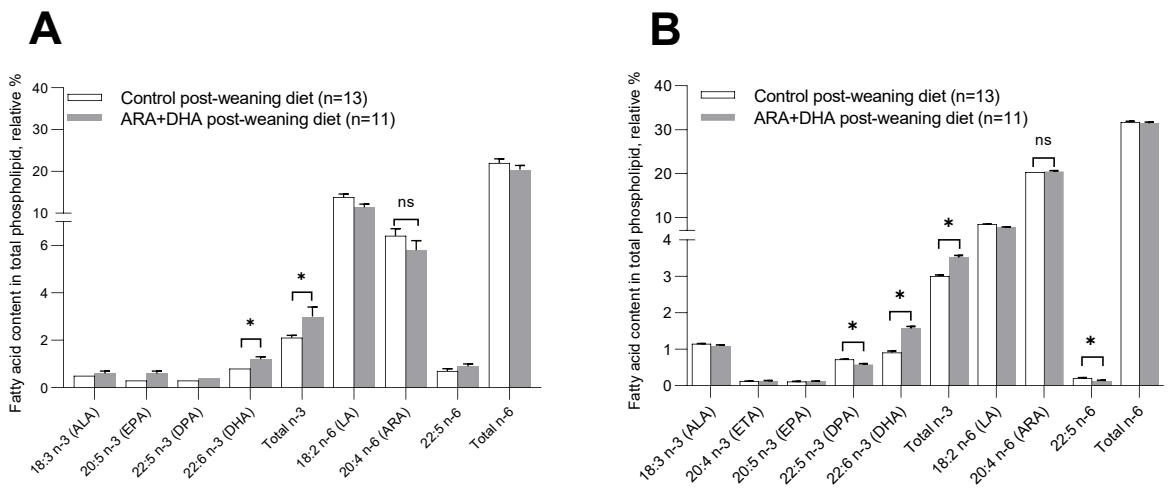

Figure 4.3. Effect of post-weaning diet on the relative % of fatty acid content in total phospholipids in 8-week Brown Norway offspring' (A) plasma and (B) splenocytes. All values are presented in mean ± SE. At 8 weeks, there was no significant effect of the suckling period diet (0-3 weeks) or the suckling diet × post-weaning diet interaction. Therefore, the suckling diet groups were combined, and the differences observed by significant post-weaning diet were calculated by unpaired Student *t*-test. Differences were considered significant at $P < 0.05$ marked by *. Abbreviations used: α-linolenic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; LA, linoleic acid; ARA, arachidonic acid.

Table 4.6. The effect of supplementing suckling diet and post-weaning diet on the total fatty acid composition in phospholipids of splenocytes of pups at 8 wk [1]

Suckling diet	Control	Control	ARA+DHA	ARA+DHA			
Post-weaning diet	Control	ARA+DHA	Control	ARA+DHA			
Fatty acid (g/100g total fatty acids)	Mean ± SEM (n = 8)	Mean ± SEM (n = 7)	Mean ± SEM (n = 8)	Mean ± SEM (n = 9)	P-SD[2]	P-WD[3]	P-SD × WD[4]
14:0	0.3±0.1	0.4±0.1	0.4±0.1	0.4±0.1	0.99	0.77	0.86
16:0	24.3±0.5	24.5±0.3	24.5±0.3	24.2±0.2	0.87	0.72	0.37
18:0	20.7±0.3	20.6±0.3	21.2±0.3	21.3±0.5	0.32	0.83	0.05
20:0	0.3±0.0	0.3±0.0	0.3±0.0	0.4±0.0	0.11	0.43	0.14
24:0	0.6±0.0	0.6±0.0	0.7±0.0	0.7±0.0	0.15	0.57	0.36
16:1n-9	0.9±0.0 [ab]	1.0±0.0 [b]	0.9±0.0 [b]	0.8±0.0 [a]	0.29	0.81	0.001
18:1n-9 Oleic acid	10.9±0.1	10.6±0.1	10.7±0.1	10.5±0.2	0.75	0.23	0.42
24:1n-9	3.6±0.1	3.4±0.1	3.6±0.1	3.5±0.1	0.98	0.11	0.41
18:2n-6 LA	8.3±0.1 [ab]	8.1±0.2 [b]	8.5±0.2 [b]	7.8±0.3 [a]	0.48	0.23	0.008
20:2n-6	1.0±0.0	1.0±0.0	1.0±0.0	0.9±0.0	0.49	0.18	0.14
20:3n-6	1.7±0.0	1.7±0.0	1.7±0.0	1.7±0.0	0.57	0.29	0.30
20:4n-6 ARA	20.5±0.4	20.7±0.1	19.8±0.3	20.7±0.3	0.25	0.14	0.16
22:4n-6	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.58	0.97	0.28
22:5n-6	0.2±0.0	0.1±0.0	0.2±0.0	0.1±0.0	0.17	<0.001	0.07
18:3n-3 ALA	1.1±0.0	1.1±0.0	1.1±0.0	1.1±0.0	0.47	0.41	0.69
20:4n-3 ETA	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.25	0.95	0.50
20:5n-3 EPA	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.80	0.46	0.14
22:5n-3 DPA	0.7±0.0	0.6±0.0	0.7±0.0	0.6±0.1	0.51	0.01	0.64
22:6n-3 DHA	0.8±0.0	1.7±0.0	0.8±0.0	1.6±0.1	0.84	<0.001	0.21
Total SFA	46.7±0.5	46.8±0.2	47.5±0.5	47.3±0.6	0.46	0.88	0.83
Total MUFA	18.4±0.1	17.8±0.0	18.2±0.0	17.6±0.1	0.46	0.10	0.54
Total PUFA	34.7±0.5	35.3±0.2	34.2±0.4	34.9±0.5	0.58	0.25	0.81
Total n-6	31.8±0.4	31.6±0.2	31.2±0.4	31.3±0.4	0.52	0.84	0.80

Suckling diet	Control	Control	ARA+DHA	ARA+DHA			
Post-weaning diet	Control	ARA+DHA	Control	ARA+DHA			
Fatty acid (g/100g total fatty acids)	Mean ± SEM (n = 8)	Mean ± SEM (n = 7)	Mean ± SEM (n = 8)	Mean ± SEM (n = 9)	P-SD[2]	P-WD[3]	P-SD × WD[4]
Total n-3	2.9±0.1	3.6±0.1	2.9±0.0	3.6±0.1	0.98	0.003	0.56
Ratio n-6/n-3	10.7±0.2	8.7±0.2	10.6±0.1	8.6±0.1	0.20	<0.001	0.41
Ratio PUFA/SFA	1.9±0.0	2.0±0.0	1.9±0.0	2.0±0.0	0.85	0.02	0.94

[1] Data is presented in mean ± standard error of the mean. Means within a row without a common superscript letter are significantly different, $P < 0.05$. Abbreviations; ALA, α-linolenic acid; LA, linoleic acid; ARA, arachidonic acid; ETA, eicosatetraenoic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; PUFA, polyunsaturated fatty acid; MUFA, monounsaturated fatty acid; SFA, saturated fatty acid; SD, suckling diet; WD, post-weaning diet

[2] Indicates P-value for the main effect of the SD in a mixed model.

[3] Indicates P-value for the main effect of the WD in a mixed model.

[4] Indicates interaction between maternal SD and pups' WD

Table 4.7. The effect of supplementing suckling diet and post-weaning diet on the total fatty acid composition in phospholipids of immune cells isolated from mesenteric lymph nodes (MLN) of offspring at 8 weeks [1]

Suckling diet	Control	Control	ARA+DHA	ARA+DHA			
Post-weaning diet	Control	ARA+DHA	Control	ARA+DHA			
Fatty acid (g/100g total fatty acids)	Mean ± SEM (n = 7)	Mean ± SEM (n = 3)	Mean ± SEM (n = 6)	Mean ± SEM (n = 7)	P-SD[2]	P-WD[3]	P-SD × WD[4]
14:0	1.2±0.2	1.2±0.3	0.9±0.2	1.0±0.2	0.03	0.38	0.99
16:0	32.7±2.1	34.7±1.3	27.8±2.0	26.2±2.3	0.12	0.86	0.36
18:0	23.4±1.7	29.3±4.8	25.4±3.5	32.8±5.1	0.58	0.15	0.85
20:0	0.9±0.2	0.8±0.0	0.9±0.1	0.8±0.2	0.35	0.78	0.60
24:0	0.5±0.0	0.3±0.0	0.5±0.0	0.4±0.1	0.93	0.17	0.60
16:1n-9	1.0±0.0	0.8±0.0	1.2±0.1	0.8±0.0	0.52	0.10	0.38
18:1n-9 Oleic acid	16.0±3.3	8.6±1.0	14.8±2.9	15.2±3.8	0.47	0.28	0.39
24:1n-9	0.7±0.1	1.1±0.3	0.9±0.2	0.5±0.1	0.15	0.79	0.45
18:2n-6 LA	7.5±1.2	5.5±1.2	10.3±1.7	9.6±1.9	0.01	0.26	0.58
20:2n-6	0.7±0.1	0.9±0.1	0.9±0.1	0.7±0.1	0.53	0.90	0.08
20:3n-6	0.7±0.1	0.8±0.1	0.8±0.1	0.6±0.1	0.72	0.55	0.22
20:4n-6 ARA	6.3±1.0	7.8±2.2	7.7±1.3	4.9±1.2	0.74	0.48	0.18
22:5n-6	1.3±0.3	1.1±0.2	1.1±0.2	1.0±0.3	0.54	0.35	0.97
18:3n-3 ALA	1.3±0.1	1.2±0.2	1.2±0.1	1.1±0.1	0.68	0.42	0.83
20:5n-3 EPA	0.6±0.2	0.5±0.1	0.6±0.2	0.3±0.1	0.28	0.58	0.89
22:5n-3 DPA	0.4±0.1 [ab]	0.5±0.1 [b]	0.5±0.1 [ab]	0.3±0.1 [a]	0.03	0.95	0.01
22:6n-3 DHA	0.8±0.2	1.0±0.1	0.7±0.1	0.6±0.2	0.28	0.87	0.31
Total SFA	60.3±4.1	67.7±5.4	56.7±2.4	62.1±4.7	0.52	0.27	0.88
Total MUFA	19.2±3.3	12.1±1.7	18.5±2.8	18.0±4.0	0.76	0.35	0.70
Total PUFA	19.9±1.9	20.6±2.7	24.0±1.6	19.4±2.1	0.17	0.21	0.33
Total n-6	16.8±1.8	17.5±2.6	21.0±1.5	17.0±2.2	0.07	0.30	0.23
Total n-3	3.1±0.5	3.1±0.3	3.0±0.4	2.4±0.2	0.23	0.64	0.75
Ratio n-6/n-3	6.2±1.3	5.5±0.7	7.7±1.1	7.9±1.4	0.04	0.67	0.91

Ratio ARA/DHA	11.4±3.8	8.6±2.1	13.3±3.0	10.0±2.1	0.64	0.39	0.95

[1] Data is presented in mean ± standard error of the mean. Means within a row without a common superscript letter are significantly different, $P < 0.05$. Abbreviation; ALA, α-linolenic acid; LA, linoleic acid; ARA, arachidonic acid; ETA, eicosatetraenoic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; PUFA, polyunsaturated fatty acid; MUFA, monounsaturated fatty acid; SFA, saturated fatty acid; SD, suckling diet; WD, post-weaning diet. Outliers were removed from the analysis resulting in an n value less than the total used for the experiment.

[2] Indicates *P*-value for the main effect of the SD in a mixed model.

[3] Indicates *P*-value for the main effect of the WD in a mixed model.

[4] Indicates interaction between maternal SD and offspring WD.

A significant suckling diet effect in the fatty acid content of MLN was observed in three instances with lower 14:0, higher LA and a higher n-6/n-3 ratio for 8-week-old offspring from dams fed the ARA+DHA diet compared to the control diet. There were no significant post-weaning diet effects observed in the MLN fatty acid composition (Table 4.7). The composition of total n-3, ALA, EPA and DHA showed no significant suckling diet effect or post-weaning diet effect (Table 4.7). However, a suckling diet × post-weaning diet interaction was observed for the proportion of DPA in MLN phospholipids, with a lower proportion of DPA in offspring that received ARA+DHA during both suckling and post-weaning period compared to the group that received control for the suckling period and ARA+DHA for the post-weaning period (0.3±0.1 vs 0.5±0.1, $P = 0.01$; Table 4.7).

4.3.4. Ex vivo cytokine response to mitogens by immune cells

Cytokine (IL1-β, TGF-β, TNF-α, IL-6, IL-10 and IFN-γ) production by splenocytes stimulated with LPS showed no suckling diet effect, post-weaning diet effect or interactions (Table 4.8). Production of IL-2 (as a surrogate marker of cell proliferation), TGF-β, IL-10, IL-4 and IL-13 with PMAi stimulation of splenocytes did not differ between groups. TNF-α and IFN-γ production by PMAi stimulated splenocytes was higher in offspring when they were weaned to the ARA+DHA diet compared to control offspring regardless of their suckling diet (30% higher, $P = 0.02$ and 60% higher, $P = 0.04$, respectively; Figure 4.4 A). A significant interaction between the suckling diet and the post-weaning diet occurred in two instances. Specifically, PMAi stimulated splenocytes from offspring who received the control diet during suckling and post-weaning produced lower IL-6 while those who received ARA+DHA during the suckling period and then the control diet during the post-weaning period had the highest IL-6 production (73±9 vs 183±42, $P <0.01$ Figure 4.4 B). With respect to IL-10 production, unstimulated

splenocytes from offspring that received ARA+DHA during the suckling and post-weaning period had the lowest levels of IL-10 production compared to all other diet group offspring (P = 0.01, Table 4.8).

In LPS-stimulated MLN cells, there was a significant suckling diet × post-weaning diet interaction effect regarding IL-10 production, where the offspring that received the control diet during suckling and post-weaning period produced significantly higher IL-10 compared to the group that received ARA+DHA diet (either during suckling period or post-weaning period) or the group that received ARA+DHA throughout suckling and post-weaning (Table 4.9). The IL-2, TGF-β, IL-10 and TNF-α production by PMAi-stimulated MLN cells did not differ among diet groups (Table 4.9).

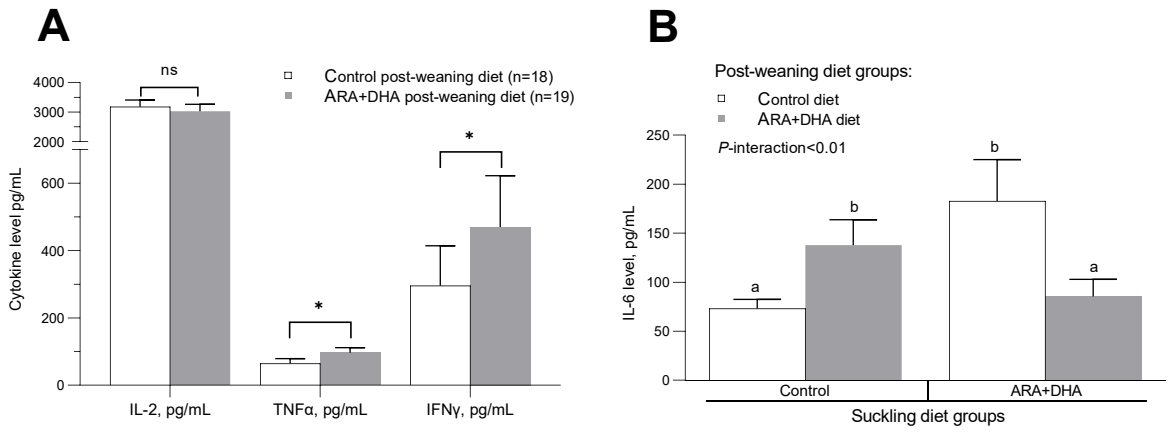

Figure 4.4. The effect of post-weaning diet on the *ex-vivo* cytokine production after phorbol-myristate-acetate and ionomycin (PMAi) stimulation of splenocytes from 8-week offspring. Values are presented in mean ± SEM. Figure A shows the post-weaning diet group comparison for IL-2 (P = 0.55), TNF-α (P = 0.02) and IFNγ (P = 0.04). The interaction of the suckling diet and post-weaning diet (P-interaction<0.01) on *ex-vivo* IL-6 production to PMAi stimulation is shown in figure B. The P values for the suckling diet and post-weaning diet are P = 0.81 and P = 0.92. Post-hoc differences are calculated using LSMEANS in a mixed model. The groups that do not share the same letter are significantly different.

Table 4.8. The effect of supplementing suckling diet and post-weaning diet on *ex-vivo* cytokine production by splenocytes upon stimulation with mitogens [1]

Suckling diet	Control	Control	ARA+DHA	ARA+DHA			
Post-weaning diet	Control	ARA+DHA	Control	ARA+DHA			
	Mean ± SEM (n = 9)	Mean ± SEM (n = 8)	Mean ± SEM (n = 10)	Mean ± SEM (n = 10)	P-SD[2]	P-WD[3]	P-SD × WD[4]
Lipopolysaccharide (pg/ml)							
IL-1β	38±4	27±2	28±3	27±2	0.77	0.06	0.08
TGF-β	314±19	321±17	322±13	311±25	0.65	0.86	0.57
TNF-α	89±17	80±18	98±20	113±24	0.60	0.89	0.83
IL-6	419±41	499±48	449±56	414±51	0.59	0.59	0.09
IL-10	249±19	258±35	239±30	240±14	0.70	0.84	0.86
IFN-γ	343±78	757±227	603±158	319±62	1.00	0.97	0.06
PMAi (pg/ml)							
IL-2	3063±397	3031±417	3282±263	3023±284	0.53	0.55	0.68
IL-6	73±9 [a]	138±26 [b]	183±42 [b]	86±17 [a]	0.81	0.92	<0.01
TGF-β	250±18	199±15	224±31	232±14	0.98	0.32	0.09
TNF-α	49±20	102±22	78±21	92±22	0.41	0.02	0.16
IL-10	57±12	96±32	69±12	91±10	0.27	0.19	0.74
IL-4	5.2±1.1	8.4±2.4	8.7±2.1	11.3±3.0	0.07	0.12	0.60
IL-13	9.4±1.9	13.9±2.5	12.9±3.2	9.8±1.3	0.67	0.35	0.18
IFN-γ	270±215	612±300	321±119	346±122	0.07	0.04	0.23
Unstimulated (pg/ml)							
TNF-α	17±1	18±2	25±5	21±2	0.58	0.70	0.87
IL-10	42±6 [b]	59±11 [b]	52±10 [b]	24±3 [a]	0.07	0.05	<0.01

[1] Data is presented in mean ± standard error of the mean. Means within a row without a common superscript letter are significantly different, $P < 0.05$. Abbreviations: SD, suckling diet; WD, post-weaning diet; ARA, arachidonic acid; DHA, docosahexaenoic acid; PMAi, phorbol-myristate-acetate and ionomycin; IL, interleukin; TGF-β, transforming growth factor-β; TNF-α, tumour necrosis factor-α; IFN-γ, interferon-γ; SEM, standard error of mean; SD, suckling diet; WD, post-weaning diet.

[2] Indicates P-value for the main effect of the SD in a mixed model.

[3] Indicates P-value for the main effect of the WD in a mixed model.

[4] Indicates interaction between maternal SD and offspring WD.

Table 4.9. The effect of supplementing suckling diet and post-weaning diet on *ex-vivo* cytokine production by mesenteric lymph node cells after stimulation with mitogens [1]

Suckling diet	Control	Control	ARA+DHA	ARA+DHA			
Post-weaning diet	Control	ARA+DHA	Control	ARA+DHA			
	Mean ± SEM (*n* = 6)	Mean ± SEM (*n* = 5)	Mean ± SEM (*n* = 8)	Mean ± SEM (*n* = 7)	*P*-SD[2]	*P*-WD[3]	*P*-SD × WD[4]
Lipopolysaccharide (pg/ml)							
IL-10	69±22 [a]	29±9 [b]	23±4 [b]	25±7 [b]	0.21	0.29	0.03
PMAi (pg/ml)							
TGF-β	229±10	244±12	193±41	292±50	0.99	0.16	0.25
IL-2	3412±1708	4807±1823	3897±1666	3078±1400	1.00	1.00	0.23
IL-10	56±21	80±28	52±19	57±26	0.70	0.42	0.32
TNF-α	149±73	158±71	126±43	134±41	0.37	0.31	0.46

[1] Data is presented in mean ± standard error of the mean. Means within a row without a common superscript letter are significantly different, $P < 0.05$. Abbreviations: ARA, arachidonic acid; DHA, docosahexaenoic acid; SEM, standard error of mean; SD, suckling diet; WD, post-weaning diet; PMAi, phorbol-myristate-acetate and ionomycin; IL, interleukin; TGF-β, transforming growth factor-β; TNF-α, tumour necrosis factor-α.

[2] Indicates *P*-value for the main effect of the SD in a mixed model.

[3] Indicates *P*-value for the main effect of the WD in a mixed model.

[4] Indicates interaction between maternal SD and offspring WD.

4.3.5. Immune cell phenotype of spleen

Splenocyte proportion of total Th cells and CD8+ T cells, DCs (OX62+), APCs (OX6+), macrophage (CD68+), NK cells (CD3- CD161+), NKT cells (CD3+ CD161+), and total TCRαβ+ (αβ T cells) did not differ among groups. Note, the cell surface marker combinations and the associated cell populations are shown in Table 4.2. However, significant effects of the suckling diet were observed. The splenocyte proportion of T regulatory cells (Treg, CD25+FOXp3+ on CD3+CD4+, $P = 0.04$) was higher in the ARA+DHA suckling diet group compared to those who were suckled from control diet-fed dams (Figure 4.5 A). While there were no differences in total naïve leukocytes (CD45RA+), there was a higher proportion of activated B cells (OX12+, $P < 0.001$) in splenocytes in the ARA+DHA suckling diet group compared to the control suckling diet (36.6±1.5 vs 27.0±0.9, $P < 0.001$), however, this was only observed when the offspring were weaned to control post-weaning diet, as revealed by a significant suckling diet X post-weaning diet interaction (Table 4.10). Furthermore, at 8 weeks of age, there was a higher proportion of total IgE+ splenocytes ($P = 0.02$) and no changes in IgG+ or IgA+ cells in offspring suckled on the ARA+DHA diet compared to control suckling diet group offspring (Table 4.10).

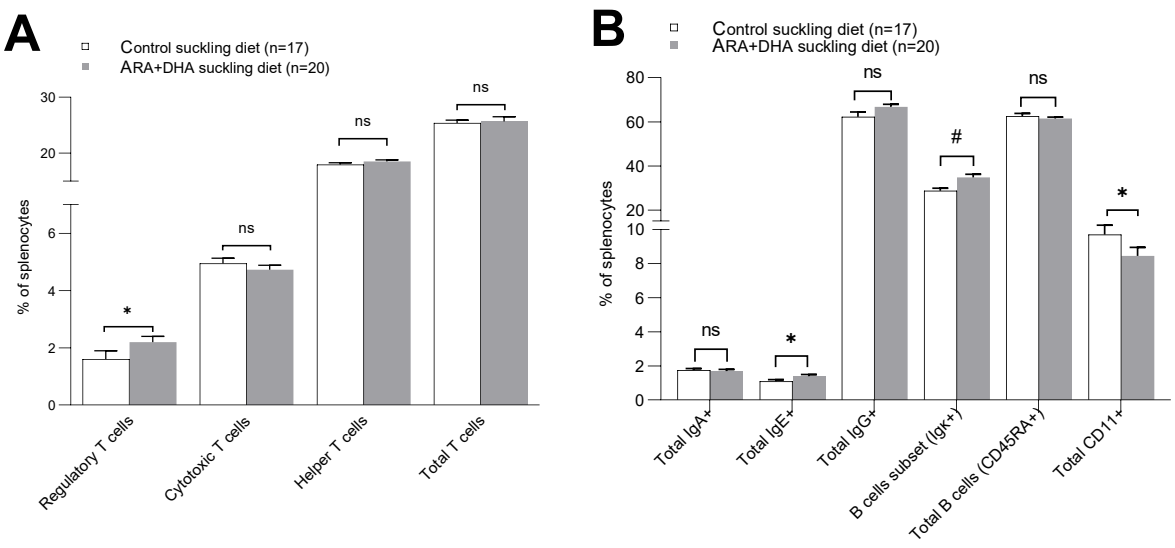

Figure 4.5. The effect of suckling diet on the immune cell phenotypes of spleen from 8-week offspring. The major proportion of T cells is shown (A) and other major immune cell phenotypes (B). The values are shown in mean ± SE for each suckling diet group. The *P*-value for the main effect of the suckling diet (Regulatory T cells: $P = 0.04$, Helper T cell: $P = 0.77$, Cytotoxic T cells: $P = 0.78$, Total T cells: $P = 0.83$, IgA: $P = 0.36$, IgE: $P = 0.02$, IgG: $P = 0.37$, B cell subset: $P = 0.001$, Total B cells (CD45RA+): $P = 0.35$ and monocytes/macrophages (CD11+): $P = 0.03$) are also presented in Table 4. The significant suckling diet effect is shown by an asterisk. The suckling diet and post-weaning diet interaction effect on the B cell subset are shown by a number sign (#), *P* interaction = 0.02.

Table 4.10. The effect of supplementing suckling diet and post-weaning diet on the composition of immune cell types isolated from the spleen of 8 weeks Brown Norway offspring [1]

Suckling diet Post-weaning diet	Control Control Mean ± SEM (*n* = 9)	Control ARA+DHA Mean ± SEM (*n* = 8)	ARA+DHA Control Mean ± SEM (*n* = 10)	ARA+DHA ARA+DHA Mean ± SEM (*n* = 10)	*P*-SD[2]	*P*-WD[3]	*P*-SD × WD[4]
T cells subset marker							
Total CD3+	25.2±0.7	26.2±0.6	25.0±0.5	26.3±1.0	0.83	0.28	0.49
Total TCRαβ+ (Tαβ cells)	23.4±0.5	23.8±0.6	24.0±0.5	24.4±0.8	0.99	0.35	0.91
Total CD4+	21.6±0.4	21.7±0.5	21.8±0.6	22.5±0.9	0.91	0.43	0.67
Total CD8+	8.4±0.2	8.4±0.1	8.2±0.1	8.5±0.1	0.84	0.30	0.47
FOXp3+ in CD3+CD4+CD25+ (Treg)	1.5±0.2	1.7±0.3	2.1±0.2	2.2±0.2	0.04	0.62	0.94
CD4+ in CD3+ (Th cells)	70.1±1.3	71.4±1.4	71.8±1.0	72.4±1.2	0.77	0.25	0.20
CD25+ in CD3+ CD4+ (activated Th cell)	12.0±1.7	10.1±0.4	11.6±1.3	9.6±0.4	0.99	0.13	0.65
CD8+ in CD3+	19.0±0.6	20.0±0.9	18.4±0.5	18.3±0.6	0.78	0.37	0.25
CD25+ in CD3+ CD8+ (activated CTL)	3.6±0.2	3.6±0.2	4.4±0.5	3.7±0.2	0.59	0.99	0.18
CD152+ in CD4+	3.2±1.4	1.5±0.3	2.8±1.1	1.6±0.4	0.99	0.46	0.91
CD152+ in CD8+	3.0±0.8	1.5±0.4	1.9±0.7	1.0±0.3	0.21	0.05	0.84
B cell subset marker							
Total CD45RA+	61.1±1.1	63.9±1.6	62.1±0.6	60.8±0.8	0.35	0.49	0.07
Total OX12+ (B cell subtype)	27.0±0.9 [c]	30.5±1.5 [bc]	36.6±1.5 [a]	33.0±1.4 [ab]	0.001	0.96	0.02
Total IgE+	1.1±0.1	1.0±0.0	1.4±0.1	1.4±0.1	0.02	0.90	0.48
Innate immune cell subset marker							
Total CD11+	10.1±0.6	9.3±0.4	8.6±0.6	8.3±0.4	0.03	0.35	0.68
Total OX6+ (APC – MHC-II)	50.8±1.8	56.2±1.7	56.1±1.5	56.3±1.3	0.31	0.09	0.12
Total OX62+ (dendritic cell)	4.9±0.3	4.6±0.2	4.8±0.2	4.9±0.4	0.39	0.39	0.33
Total CD68+ (macrophage)	0.9±0.1	1.0±0.2	1.2±0.2	1.2±0.3	0.07	0.88	0.37
Total CD284+ (TLR4)	1.0±0.1	0.8±0.1	1.2±0.2	1.2±0.1	0.03	0.49	0.30
Total CD86+ (CD28 ligand)	13.5±0.8	14.2±0.7	17.3±0.8	17.6±1.2	0.09	0.57	0.83
CD3- CD161+ (NK cell)	7.5±0.4	7.1±0.3	7.1±0.2	7.5±0.3	0.60	0.74	0.25

[1] Values are presented as mean ± SEM; values are reported as % of the total gated splenocytes. Means within a row without a common superscript letter are significantly different, $P < 0.05$. Abbreviations: SD, suckling diet; WD, post-weaning diet; APC, antigen-presenting cell; Th, helper T; CTL, cytotoxic T lymphocytes; NKT, natural killer T cells; NK, natural killer; CTLA4, cytotoxic T lymphocyte-associated protein-4; IL2Rα, Interleukin2 receptor α-chain; TCRαβ, T cell with αβ receptor; TLR4, toll-like receptor-4; TCR, T cell receptor; Treg, T regulatory cell; SD, suckling diet; WD, post-weaning diet; MFI, median fluorescence intensity.
[2] Indicates *P*-value for the main effect of the SD in a mixed model.
[3] Indicates *P*-value for the main effect of the WD in a mixed model.
[4] Indicates interaction between maternal SD and offspring' WD.
Note: Some immune cell markers had no significant effect on diets or sex therefore they were not included in the table. This includes total FOXp3+ cells, total CD27+ (memory marker) cells, CD4+ in CD27+ cells, CD8+ in CD27+ cells, Total CD28+ (T cell co-receptor) cells, CD28+ in CD8+ CD4- cells, CD28+ in CD8- CD4+ cells, CD4+ in TCR+ cells, CD8+ in TCR+ cells, total IgA+ cells, total IgG+ cells, total CD152+ (CTLA4+) cells, total CD25+ (IL2Rα) cells, total CD161+ cells, CD3+ CD161+ (NKT) cells, CD25+ in OX6+ (activated APC) cells, OX62+ in OX6+ cells, CD11+ in CD45RA+ cells, CD68+ in CD45RA+ cells, CD284+ in CD45RA+ cells and CD68+ in CD284+ cells.

The proportion of TLR-4+ (total CD284+, shown in Table 4.10.) cells was significantly higher and the proportion of monocytes (total CD11+, shown in Figure 4.5 B) was significantly lower in splenocytes from the ARA+DHA suckling diet group compared to control suckling diet fed offspring ($P < 0.05$). At 8 weeks, a significant post-weaning diet effect was observed in only one instance, showing a 50% lower proportion of CD8+CD4-CD152+ in total splenocytes from the ARA+DHA post-weaning compared to control diet group ($P = 0.05$, Table 4.10). Note, the gating strategy for CD152+ CD8+ was conducted on CD4- cells to ensure only T cells were gated as T cell-specific antibodies could not be used due to limitations with the number of antibodies used in combination.

4.3.6. Immune cell phenotype of MLN

The major immune cell populations in MLN, T cells (CD3+), αβ T cell receptor (TCRαβ+), naïve CD8+ T cells, Th cells (CD3+CD4+), APC (OX6+), B cells (OX12+), IgA+ cells, IgG+ cells and IgE+ cells did not differ amongst groups. However, at 8 weeks, the proportion of naïve leukocyte marker (CD45RA+) was 10% lower ($P = 0.002$) and total CD152+ cells in MLN were 4 times higher in offspring from ARA+DHA suckling diet group compared to control group offspring (Table 4.11). The post-weaning diet supplementation of ARA+DHA resulted in a significantly lower proportion of CD3+CD8+CD25+ (17%, $P = 0.05$) and total CD8+ cells (12%, $P = 0.04$), with no differences amongst post-weaning groups in MLN proportions of total CD4+ cell ($P = 0.15$) or activated Th cell (CD3+CD4+CD25+, $P = 0.44$) compared to control post-weaning diet group (Table 4.11). Further, there was no significant interaction between suckling and post-weaning diets on different immune cell populations of MLN.

4.3.7. Immune cell phenotype of PP

PP of 8-wk old offspring showed that the proportion of major immune cells subsets, such as T cells (CD3+), Th cells (CD3+CD4+), naïve CD8+ T cells, APC (OX6+) and naive leukocytes (CD45RA+) did not differ amongst groups (Table 4.12). A significant suckling diet effect was observed in one instance with a 30% higher proportion of total CD4+ cells in offspring that received the ARA+DHA diet during suckling compared to the control suckling diet at the end of 8 weeks ($P = 0.001$). Next, a significant post-weaning diet effect revealed that the total IgG+ cells in 8-week offspring that received ARA+DHA post-weaning diet were approximately 30% higher compared to control offspring ($P = 0.004$). Finally, a suckling diet × post-weaning diet interaction was observed for total IgE+ cells with a significantly lower proportion in offspring who received the ARA+DHA suckling diet and control post-weaning diet group compared to ARA+DHA suckling diet and ARA+DHA post-weaning diet ($P = 0.04$, Table 4.12).

Table 4.11. The effect of supplementing suckling diet and post-weaning diet on the composition of immune cell types isolated from the mesenteric lymph nodes of 8-week Brown Norway offspring [1]

Suckling diet Post-weaning diet	Control Control Mean ± SEM (n = 8)	Control ARA+DHA Mean ± SEM (n = 6)	ARA+DHA Control Mean ± SEM (n = 9)	ARA+DHA ARA+DHA Mean ± SEM (n = 9)	P-SD[2]	P-WD[3]	P-SD × WD[4]
T cell subset markers							
Total CD3+	54.3±2.0	49.7±2.3	54.8±1.9	53.2±0.8	0.39	0.25	0.41
Total TCRαβ+ (αβ T cells)	52.5±2.0	51.7±1.2	59.7±2.8	58.9±3.3	0.15	0.76	0.88
Total CD4+	50.8±1.8	46.0±1.6	52.1±1.9	50.8±1.1	0.30	0.15	0.24
Total CD8+	7.0±0.5	6.1±0.3	7.3±0.4	6.5±0.1	0.16	0.04	0.71
CD4+ in CD3+ (Th cell)	80.1±1.5	79.6±1.9	80.3±1.1	79.8±2.5	0.96	0.85	0.78
CD25+ in CD3+ CD4+ (activated Th cell)	7.7±0.6	7.0±0.5	6.6±0.3	6.6±0.3	0.18	0.44	0.30
CD8+ in CD3+ (naïve CD8+ t cells)	8.2±0.8	7.7±0.5	7.7±0.3	7.5±0.2	0.63	0.63	0.98
CD25+ in CD3+ CD8+	10.1±0.9	9.0±1.0	10.7±1.6	8.3±1.1	0.15	0.048	0.44
B cell subset markers							
Total CD45RA+	46.0±1.4	47.5±1.3	42.0±1.5	40.7±1.6	0.002	0.94	0.38
Total OX12+ (B cells subtype)	18.6±3.2	19.6±2.3	15.6±2.8	13.7±2.5	0.18	0.99	0.26
Total IgA+	4.0±0.5	4.6±1	3.9±0.5	2.6±0.3	0.11	0.36	0.15
Total IgG+	38.6±3.6	44.2±1.7	38.9±1.7	39.7±1.5	0.26	0.33	0.45
Total IgE+	4.9±0.5	5.9±1	3.7±0.6	5.2±0.9	0.30	0.10	0.86
Innate immune cell subset markers							
Total CD28+ (T cell co-receptor)	49.1±1.4	46.2±1.4	56.7±3.9	52.3±1.1	0.18	0.17	0.44
Total CD152+ (CTLA4)	0.1±0.0	0.1±0.0	0.6±0.2	0.5±0.2	0.03	0.93	0.53
Total OX6+ (APC – MHC-II)	20.5±3.3	20.9±2.7	14.2±1.4	13.0±1.4	0.16	0.67	0.35
Total CD27+	72.6±4.2	72.6±3.8	76.2±3.2	79.0±3.3	0.09	0.35	0.41

[1] Values are presented as mean ± SEM; values are reported as % of the total gated immune cells. Abbreviations: SD, suckling diet; WD, post-weaning diet; APC, antigen-presenting cell; Th, helper T; CTLA4, cytotoxic T lymphocyte-associated protein-4; IL2Rα,

Interleukin2 receptor α-chain; Tαβ, T cell with αβ receptor; TLR4, toll-like receptor-4; TCR, T cell receptor; SD, suckling diet; WD, post-weaning diet

[2] Indicates *P*-value for the main effect of the SD in a mixed model.

[3] Indicates *P*-value for the main effect of the WD in a mixed model.

[4] Indicates interaction between maternal SD and offspring' WD.

Note: Some immune cell markers had no significant effect on diets or sex, therefore they were not included in the table. This includes CD4+ in TCRαβ+ cells, CD8+ in TCRαβ+ cells, CD27+ in CD4+ TCR+ cells, CD27+ in CD8+ TCR+ cells and total CD25+ (IL2Rα) cells.

Table 4.12. The effect of supplementing suckling diet and post-weaning diet on the composition of immune cell types isolated from the Peyer's patches of 8-week Brown Norway offspring.[1]

Suckling diet Post-weaning diet	Control Control Mean ± SEM (n = 8)	Control ARA+DHA Mean ± SEM (n = 7)	ARA+DHA Control Mean ± SEM (n = 9)	ARA+DHA ARA+DHA Mean ± SEM (n = 9)	P-SD[2]	P-WD[3]	P-SD × WD[4]
T cell subset marker:							
Total CD3+ (T cells)	43.0±4.9	38.4±2.9	43.2±3.4	39.3±2.9	0.94	0.40	0.41
Total CD4+	20.1±4.2	21.6±4.7	27.9±4.1	26.5±4.1	0.001	0.87	0.38
Total CD8+	10.2±1.5	9.2±2.5	9.9±1.5	8.6±2.1	0.94	0.39	0.53
CD4+ in CD3+ (Th cells)	41.5±8.7	47.3±9.0	43.6±6.7	44.1±5.1	0.33	0.35	0.21
CD8+ in CD3+ (naïve CD8+ T cell)	18.6±2.5	17.5±3.6	18±2.4	17.4±2.8	0.63	0.44	0.64
CD25+ in CD3+CD4+ (activated Th cells)	20.5±3.6	22.8±5.3	23.6±3.7	19.9±3.4	0.39	0.74	0.08
CD25+ in CD3+ CD8+	53.4±3.3	52.9±4.7	47.2±5.3	42.7±5.1	0.40	0.54	0.68
B cell subset marker:							
Total CD45RA+	52.1±4.4	58.7±3.5	50.4±2.1	60.6±2.5	0.56	0.14	0.39
Total IgA+	6.3±1.1	3.5±0.9	7.5±1.0	5.0±1.2	0.45	0.27	0.44
Total IgG+	57.9±5.4	69.2±4.7	46.7±5.3	65.4±3.1	0.13	0.004	0.45
Total IgE+	4.2±1.1 a	3.7±1.2 a	7.2±0.7 b	4.9±1.0 a	0.07	0.09	0.04
Innate immune cell subset markers							
Total CD25+ (IL2Rα)	11.6±1.6	8.4±2.0	14.4±1.7	11.1±2.4	0.65	0.06	0.67
Total OX62+	11.9±2.1	17.8±3.1	16.6±2.4	15.8±2.8	0.92	0.20	0.06
Total CD86+	16.4±3.4	14.4±1.9	19.4±2.7	21.2±3.1	0.28	0.68	0.83
Total OX6+ (APC – MHC-II)	64.5±3.9	64.5±4.0	53.5±3.3	59.5±4.9	0.46	0.43	0.32

[1] Values are presented as mean ± SEM; values are reported as % of the total gated immune cells. Abbreviations: SD, suckling diet; WD, post-weaning diet; APC, antigen-presenting cell; Th, helper T; CTL, cytotoxic T lymphocytes

[2] Indicates *P*-value for the main effect of the SD in a mixed model.

[3] Indicates *P*-value for the main effect of the WD in a mixed model.

[4] Indicates interaction between maternal SD and offspring' WD.

4.3.8. Sex differences in offspring at 8-week

All parameters described here for the 8 weeks old offspring were first assessed for differences by sex. We found in the MLN, total PUFA phospholipid content was higher in males versus females (21.5±1.9 vs 16.9±1.9, $P = 0.007$), however, the composition of ARA or DHA did not differ. Additionally, a significant sex effect was observed in Peyer's patches (PP) with CD8+ cells being significantly higher in the PP of female offspring compared to male (11.6±1.7 vs 8.3±1.0, $P < 0.05$). No other significant differences due to sex were observed for any growth parameters, immune cell *ex-vivo* cytokine production, immune cell phenotype or fatty acid phospholipid composition. Therefore, the data below was pooled for the sexes, and it is described as per their diet groups only.

4.4. Discussion

Using a Th2 bias Brown-Norway rodent model, we investigated the effect of supplementing the diet with ARA and DHA during critical immune development periods. The maternal diet supplementation of ARA+DHA resulted in higher DHA composition in the breastmilk total lipids and plasma phospholipids of dams, however, there was no change in the proportion of ARA. ARA+DHA supplementation through the suckling period (0-3 weeks) showed no lasting effects on the phospholipid composition of DHA or ARA in splenocytes or MLN in offspring at 8 weeks. Despite this, suckling period supplementation resulted in a programming effect on the types of immune cells in the spleen (Treg and B cell), MLN (naïve lymphocytes) and PP (IgG+ B cells), in that there were more mature immune cells observed at the end of the post-weaning period in offspring from dams fed ARA+DHA suckling diet. More importantly, the post-weaning period (3-8 weeks) ARA+DHA supplementation was associated with higher Th1 cytokines, such as TNF-α and IFN-γ, and lower IL-6 (which can be considered Th2 cytokine)

after mitogen stimulation, indicating a shift from Th2 to Th1 immune response (Figure 4.6). Furthermore, differences due to weaning diet on immune cell types or proliferation (based on IL-2 production by splenocytes) involved in Th1 cytokine production were absent.

4.4.1. Effect of suckling period and post-weaning period supplementation on fatty acid composition of 8-wk old offspring

Offspring from ARA+DHA fed dams received, through breastmilk, significantly more DHA (from 0.2% to 0.8% of total fatty acid) and similar ARA (approximately 2% of total fatty acids). As expected, this effect of the 3-week suckling period diet supplementation on fatty acid composition was no longer present at the end of 8 weeks, where the fatty acid composition was influenced by the post-weaning diet. Consistent with other studies, growth parameters, such as body weight and length, were not different amongst dietary groups (Field et al., 2000; Caroline Richard et al., 2016d). Offspring fed the ARA+DHA post-weaning diet, regardless of the suckling diet, had a higher DHA composition, without affecting the ARA, in plasma and splenocytes phospholipids at 8 weeks. Although the increase in DHA breastmilk levels with dietary supplementation is dose-dependent, ARA level in breastmilk is mainly dependent on maternal stores (Brenna et al., 2007; Del Prado et al., 2001). In the current study, dietary supplementation of ARA along with DHA was able to maintain ARA while increasing the breastmilk DHA composition in dams. Previous experiments from our lab with Sprague-Dawley rats have also reported similar changes at the end of the post-weaning period (Caroline Richard et al., 2016e). Contrary to our hypothesis, at the current dietary concentration (0.5% DHA w/w of total fat), post-weaning diet supplementation did not appear to be sufficient to induce changes in the total phospholipid fatty acid composition of the immune cells from MLN. LCPUFA metabolism in different lymphoid tissue may vary depending on the dominant immune cell type

and function which may affect the tissue composition of fatty acids (Gutiérrez et al., 2019). Clinical trials have also reported tissue-specific changes in fatty acid composition (Westcott et al., 2005).

Diet-induced changes in fatty acids composition	**Programming effect of suckling period ARA+DHA diet**	**Effect of post-weaning period ARA+DHA diet on the immune system**
ARA + DHA in maternal diet → ↑ DHA, no change in ARA in dams' breast milk	In spleen: ↑ Treg, ↑ B cell, ↓CD11+ and ↑ TLR4+	In spleen: ↓ Co-inhibitory receptors on CD8+
ARA + DHA suckling diet → no changes in DHA or ARA in offspring splenocytes and plasma phospholipids at 8 weeks	In MLN: ↓ CD45RA+ leukocytes In PP: ↑ Total CD4+ cells	In MLN: ↓ Activated CD8+ T cells and ↓ total CD8+ cells In PP: ↑ IgG+ and ↓ IgE+ cells
ARA + DHA post-weaning diet → ↑ DHA in splenocytes, no change in ARA in offspring' splenocytes and plasma phospholipids at 8-week	No change in splenocyte cytokine response to LPS or PMAi stimulation	↑ Th1 cytokines (IFNγ, TNF-α) by splenocytes to PMAi stimulation ↓ IL-6 (only in ARA+DHA suckling diet groups)

Figure 4.6. Summary of significant effects of supplementing ARA+DHA in infant diets on the immune system development of Brown Norway offspring. ↑, increase; ↓, decrease; ARA, arachidonic acid; DHA, docosahexaenoic acid; Treg, T regulatory cell, TLR4, toll-like-receptor 4; LPS, lipopolysaccharide; MLN, mesenteric lymph node; PP, Peyer's patches.

4.4.2. Programming effect of suckling period supplementation on immune cell phenotype in the spleen, MLN and PP

Dietary supplementation during the critical window of development in early infancy can result in changes, referred to as a programming effect, that can be observed later in life (P. C. Calder et al., 2010; Lucas, 2005). Studies have shown that n-3 supplementation can also have a

programming effect using an animal model (Caroline Richard et al., 2016d) as well as in clinical trials (Laouar, 2020; Lauritzen et al., 2005; Warstedt et al., 2009). Our data suggest that providing ARA and DHA during the suckling period had a programming effect on the maturation status of immune cell populations (summarized in Figure 4.6). The population of adaptive immune cells in the spleen increases during early infancy to match adult levels as they mature. Specifically, splenocyte proportions of B cells, T cells and NK cells increase during the suckling period (Pérez-Cano et al., 2012) and additional immune cell maturity is achieved as food components are introduced during a post-weaning period (Da Silva Menezes, 2003; Garcia et al., 2003). Our findings, in a Th2 bias Brown Norway rat are consistent with other LCPUFA supplementation studies showing higher B cells and T cell memory marker (CD27) in Sprague-Dawley rat offspring at the end of post-weaning (Caroline Richard et al., 2016e). Furthermore, clinical trials have shown that 4 weeks of LCPUFA supplementation of formula during the suckling period in infants also resulted in higher adaptive cells such as T cells and CD4+CD28+ cells in peripheral blood mononuclear cells (Field et al., 2000). Next, we found that ARA+DHA suckling period supplemented offspring had a lower proportion of innate immune cell marker (CD11) that is generally expressed on granulocytes, DCs, and monocytes, and higher TLR-4+ splenocytes at the end of 8 weeks irrespective of the post-weaning diet. LPS, a bacterial component known to trigger APCs, B cells and macrophages, can bind to TLR4 and induce activation of the innate immune response (Akira et al., 2006). However, no differences were observed in the *ex-vivo* cytokine response to LPS with ARA+DHA supplementation at the end of post-weaning. This suggests that while suckling period supplementation (0-3 weeks) of ARA and DHA can promote higher adaptive immune cells, it is not associated with any changes in their

ability to produce Th1 or Th2 response to *ex-vivo* mitogen challenges after the post-weaning period.

Consistent with our findings in the spleen, suckling period ARA+DHA supplementation did not alter the proportion of the total T cells, B cells, NK cells or APCs in the MLN at 8 weeks, however, CD45RA+ cells were significantly lower in the supplemented group offspring. As the lymphocytes circulate in the lymph node and encounter an antigen, it transitions from naïve to mature phenotype, which is marked by switching of CD45 isoform from CD45RA to CD45RO (Berard & Tough, 2002; Hathcock et al., 1992; Osugi et al., 2008). Although we did not analyze the CD45RO cell surface marker in the current study, a decrease in the CD45RA surface marker on lymphocytes is generally associated with an increase in CD45RO markers in circulating lymphocytes as the immune system matures (Cossarizza et al., 1996). Hence, lower naïve lymphocytes in MLN observed in the ARA+DHA group offspring can be perceived as more mature lymphocytes. Furthermore, we found immune cells of PP consisted of more CD4+ cells in ARA+DHA-supplemented suckling diet group offspring, regardless of the post-weaning diet at 8 weeks. The PP also showed a higher proportion of IgG+ cells and a lower proportion of IgE+ cells (only the ARA+DHA suckling group) due to post-weaning period ARA+DHA supplementation with no differences in IgA+ cells or total B cells. Specialized pro-resolving mediators synthesized from DHA can enhance B cell differentiation and antibody production (S. Ramon et al., 2012), however, DHA has also been shown to reduce IgE class-switching and production (Weise et al., 2011b). Our data indicates there were tissue-specific changes in the phenotype of the immune cells, showing a more mature phenotype in the spleen, MLN and PP. However, unlike other reports that showed changes in *ex-vivo* cytokine production with stimulation; such as higher IL-10 responses by T cells (Caroline Richard et al., 2016e), Th1-type

cytokines to phytohaemagglutinin (D'Vaz et al., 2012; Field et al., 2008a), and food antigens (Field et al., 2008b), we observed no functional changes in the splenocytes *ex-vivo* cytokine response by adaptive immunity (PMAi stimulation) or innate immunity (LPS stimulation) for the suckling period ARA+DHA supplementation in 8 week old Brown Norway offspring.

4.4.3. Post-weaning period ARA and DHA supplementation, regardless of the suckling diet, is associated with higher Th1 cytokine response.

Production of higher Th1 cytokines can be an indicator of a reduction in Th2 response which is considered a more mature immune response in weaned infants (Basha et al., 2014). We have demonstrated for the first time in a Th2-biased model, that ARA+DHA diet supplementation in the post-weaning period, irrespective of suckling diet, resulted in higher Th1 cytokines (IFN-γ and TNF-α) upon *ex-vivo* lymphocyte stimulation with PMAi. Interaction of the B7 molecule of APC with a co-stimulatory (CD28) and co-inhibitory (CD152 as known as CTLA4) molecule of naïve T cell determines its overall effect (Bour-Jordan & Bluestone, 2002). We showed splenocytes with lower co-inhibitory receptors (CD8+CD152+) in ARA+DHA post-weaning offspring, with no differences in co-stimulatory marker (CD4+CD28+ or CD8+CD28+). This may also promote the Th1 cytokine response, as the CD152 marker has been reported to affect T cell polarization (Ubaldi et al., 2003). Our findings are consistent with those reported by a clinical study where DHA+EPA (fish oil) supplementation in Th2-biased allergy-prone infants showed increased Th1 cytokines (IFN-γ and TNF-α) and reduced Th2 cytokine (IL-13) to PHA (mitogen stimulating T cells) (D'Vaz et al., 2012). Furthermore, these immunomodulatory properties were shown to be protective against food allergies later in life (D'Vaz et al., 2012).

The anti-inflammatory effects of n-3 LCPUFA on the immune system (due to increased Th2 cytokines, IL-4, IL-5 and IL-13, along with immunoregulatory IL-10 cytokine) are widely reported (Calder, 2010a) especially with higher dietary intakes of EPA and/or DHA. It is hypothesized that ARA (and other n-6 LCPUFA) counters the anti-inflammatory effects of EPA and DHA, which may be responsible for reducing the Th1 response by splenocytes in rodents (Soni et al., 2017). In addition, higher Th1 cytokines are also known to suppress Th2 cytokine production. However, the current study found no differences in key Th2 cytokines; IL-4, IL-13 or TGF-β to PMAi due to ARA+DHA post-weaning diet supplementation. These findings once again demonstrate that a more mature immune response is associated with post-weaning period ARA+DHA supplementation where a higher Th1 cytokine was observed without any changes in Th2 cytokines or lymphocyte proliferation marker (IL-2 was used as an indirect measure of proliferation as it plays a vital role in lymphocyte proliferation). Lastly, we reported a lower IL-6 production with PMAi stimulation with ARA+DHA post-weaning diet compared to the control group (when the suckling period was also supplemented). This is also consistent with other DHA supplementation studies in rodents (Caroline Richard et al., 2016e; Yaqoob & Calder, 1995) and human feeding trials (Haghiac et al., 2015; Wallace et al., 2003). The anti-inflammatory effects of DHA have been hypothesized to be operating through the inhibition of NF-κB, which may mediate its beneficial role in various inflammatory diseases (Philip C. Calder, 2013b).

The current study has some limitations. Due to the small litter size of the Brown Norway dams, we had to conduct our animal experiments in 4 blocks. Statistical analysis was conducted to address random errors in the main effects due to differences in a block; however, it was not entirely possible to control the block effect for some parameters. Second, the *ex-vivo* cytokine response to the mitogens was measured at a one-time point (72 hours post-incubation) and does

not allow us to distinguish the early cytokine response from the later response. It is known that some cytokines such as IFN-γ from macrophages are produced early on (within 24 hours) as compared to cytokines released by lymphocytes take longer stimulation. We included male and female offspring in our study to determine if there were sex differences. However, we did not observe major sex differences, apart from differences in the total PUFA composition of MLN and the proportion of CD8+ immune cells of PP. As it has not been studied before, our study may not have been appropriately powered or designed to assess the sex effect in Th2 bias Brown Norway offspring. Therefore, the sex effect on neonatal immune system development needs to be further studied.

4.5. Conclusion

Our findings suggest that providing more DHA during the suckling period through maternal diet ARA+DHA supplementation programmed the development of the Brown Norway offspring's Th2-biased immune system. We demonstrated that suckling period supplementation had a beneficial effect on the maturation status of the immune cell population of the spleen, MLN, and PP regardless of the post-weaning diet. However, these changes were not associated with any functional differences in the ability of mature immune cells from the spleen to respond to polyclonal mitogens at 8 weeks. Providing ARA+DHA in post-weaning diet results in a higher Th1 cytokine response by the splenocytes without affecting the Th2 cytokine response. Overall, the current study shows the importance of LCPUFA's immunomodulatory properties, when provided during the suckling and post-weaning periods, resulting in a higher Th1 response by immune cells. This may be important for preventing allergies in infants with a genetic predisposition to develop allergic diseases.

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Chapter 5. Combined supplementation of arachidonic and docosahexaenoic acids in T helper type-2 skewed Brown Norway rat offspring is beneficial in the induction of oral tolerance towards ovalbumin and immune system development[1]

5.1. Introduction

Food allergy prevalence is increasing globally, particularly during early childhood (Prescott et al., 2013). There is an increasing risk of developing allergic diseases during the first two years of life as the infant's immune system is still immature (Hill & Hosking, 1995; Saavedra & Dattilo, 2017). This involves switching from a dominant innate immune response to a more robust and long-lasting adaptive immune response (Simon et al., 2015). During this period, the naïve immune system is exposed to antigens which can result in the development of oral tolerance (OT) (Pabst & Mowat, 2012; Sackesen et al., 2019). OT is defined as the specific suppression of immune response towards an orally exposed food antigen (Garside & Mowat, 2001; Pabst & Mowat, 2012). Although different mechanisms are involved in the induction of OT, when a low-dose repetitive feeding is used, OT development occurs through antigen-specific immune suppression mediated by T regulatory cells (Treg) (Pabst & Mowat, 2012). The breakdown in OT towards food antigens results in the development of a food allergy (Garside et al., 1999; Satitsuksanoa et al., 2018), which is associated with the naïve T helper cells differentiating into effector T helper type 2 (Th2) cells in the presence of interleukin (IL) -4 and IL-13 and promoting the production of antigen-specific immunoglobulin-E (IgE) (Georas et al., 2005).

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At birth, infants also have a Th2-skewed immune response due to the Th2-dominant maternal immune response (Björkstén, 2000; Wenjuan Wang et al., 2020). As the immune system starts to mature a robust Th1 immune response is expected to develop in the first two years of life. In the presence of interferon-γ (IFN-γ) and IL-12, naïve CD4+ T cells differentiate into effector Th1 cells, which is important to generate an efficient Th1 response and counteract the Th2 skewed immune system of the neonate. However, a strong and prolonged Th2 cytokine profile is observed in infants at high risk of developing allergies (Van Der Velden et al., 2001). Similarly, animal models such as Brown Norway rats that have a dominant Th2 immune response are useful in understanding the development of OT in preventing food allergy (Zhu et al., 2021).

The immunoregulatory properties of n-3 long-chain polyunsaturated fatty acid (LCPUFA) and n-6 LCPUFA differently influence immune cell function (Kim et al., 2010). For instance, prostaglandin E2 that is produced from arachidonic acid (ARA, n-6 LCPUFA) results in the suppression of Th1 response towards bacterial and viral infection and promotes the Th2 skewed allergic response (Hilkens et al., 1995). On the contrary, docosahexaenoic acid (DHA, n-3 LCPUFA) and eicosapentaenoic acid (EPA, n-3 LCPUFA) can reduce the production of prostaglandin E2 and promote the Th1 response (L. D. Peterson et al., 1998). The immune-modulating effect of DHA has been shown to be beneficial for infants with atopic conditions by preventing asthma and food allergy (Schubert et al., 2009). Fish oil (which includes EPA and DHA) supplementation has been reported to be beneficial for children with a genetic predisposition to developing allergies by lowering Th2-dominant response (Alm et al., 2009; Furuhjelm et al., 2011a; Kull et al., 2006; Nagakura et al., 2000). Despite the importance of LCPUFA in immune cell function, the increased consumption of n-6 PUFA in the western diet is

thought to be involved with the development of food allergies during early life (van den Elsen et al., 2015).

When DHA is provided in combination with ARA, in early infancy, it has been shown to promote immune system maturation (Field et al., 2001). DHA, as an n-3 LCPUFA, promotes anti-inflammatory or inflammation-resolving properties whereas ARA, as an n-6 LCPUFA, promotes inflammatory properties, both of which are required in the functioning of the immune system (Philip C. Calder, 2013a; P. C. Calder et al., 2010). Additionally, the current recommendation for infant formula required the addition of ARA at least at the same levels as DHA, when DHA is added (Codex Alimentarius Commission, 2007). Limited studies have been conducted to study ARA + DHA supplementation on OT development in allergy-prone Th2 skewed rodent models. Some studies have looked at ARA and DHA together in allergy-prone Th2 models but in the context of allergen-induced dermatitis (Weise et al., 2011a). Clinical trials with ARA and DHA have shown reduced incidence of common allergic diseases in 3 years old children (Birch et al., 2010). Furthermore, clinical trials from our lab have shown the importance of ARA and DHA supplementation in pre-term (Field et al., 2000) and full-term neonates (Field et al., 2008b). Despite the essentiality of ARA and DHA during early infancy for immune system development (Caroline Richard et al., 2016b), several studies were conducted either in the absence of ARA or very high doses of DHA which resulted in less than ideal conditions for infant immune system development. The current study aimed to understand the role of combined supplementation of ARA + DHA on the development of OT and immune system maturation in allergy-prone Brown Norway offspring. The specific objectives involved determining the programming effect of ARA+DHA supplementation during the suckling period (to resemble breastfeeding) and the weaning period (to resemble the introduction of weaning food) in Brown

Norway offspring. Immune function was assessed using *ex-vivo* cytokine production by splenocytes to different mitogens and OT was assessed using *in-vivo* plasma levels of ovalbumin (Ova, model food antigen) specific-immunoglobulins to systemic immunization.

5.2. Materials and methods

5.2.1. Study design and diets

The animal experiments and protocol were conducted as per the Canadian Council on Animal Care and approved by the University of Alberta Animal Care and Use Committee under AUP125. All the timed pregnant Brown Norway rats (by the 7th day of gestation) and additional breeder rats were purchased from Charles Rivers Laboratories (Montreal, Quebec, Canada) and housed in a temperature and humidity-controlled environment with a 12/12-h reverse light cycle. During the acclimatization period, dams were fed the standard rat chow (Lab diet 5001; PMI Nutrition International, Brentwood, Missouri, USA), until the estimated 14th day of gestation. Five days prior to giving birth, dams were randomly assigned to consume either of the two experimental maternal suckling period diets (SPD); ARA+DHA (0.45% ARA, 0.8% DHA w/w of total fat; n = 10) or control (0% ARA, 0% DHA w/w of total fat; n = 8) (Table 5.1) for 3 weeks (Figure 5.1). To ensure an equal number of offspring and sex ratio, offspring were cross-fostered within a diet group. Diets were fed *ad libitum* to dams and the offspring consumed their mother's breastmilk. Offspring from each maternal SPD group were randomly assigned to one of the two weaning diets (WD); ARA+DHA (0.5% ARA, 0.5% DHA w/w of total fat) or control (0% ARA, 0% DHA w/w of total fat), in a cross-over design (Figure 5.1). The ARA and DHA concentrations in SPD were selected to model the higher end of the range found in the human population (Brenna et al., 2018; Jackson & Harris, 2016), whereas the weaning diet ARA and DHA concentrations were based on the recommendations for infant formulas by Codex

Alimentarius (World Health Organization/Food and Agriculture Organization of the United Nations) (Codex Alimentarius Commission, 2007). This study design allowed us to study the SPD as well as WD effect, independently as well as in combination, by comparing offspring from four resulting diet group combinations (i.e., control SPD to control WD, control SPD to ARA+DHA WD, ARA+DHA SPD to control WD and ARA+DHA SPD to ARA+DHA WD). All the offspring were terminated at 8 weeks of age by CO_2 asphyxiation followed by cervical dislocation, and relevant tissues were collected for further analysis.

The non-fat composition of experimental diets used has been previously described (Lewis et al., 2016a). All the diets (SPD and WD) were isocaloric and isonitrogenous, and the nutrient content was identical except for fatty acid composition (Table 5.1). The fatty acid composition was obtained by mixing commercially available lard, olive oil, canola oil, corn oil, ARAsco and DHAsco (ARA and DHA were provided by DSM, Nutritional Products, Maryland, USA), and were aimed to match the PUFA to SFA ratio and n-6 to n-3 PUFA ratio. To limit oxidation only small batches of diet were prepared and stored at 4°C until used. Bodyweight and food intake of the dams and the offspring were monitored twice weekly throughout the intervention.

Table 5.1. Fatty acid composition of the experimental SPD fed to dams during the suckling period and WD fed to offspring during the weaning period. [1]

Suckling period diet fatty acid (g/100g total fatty acids)	**Control SPD**	**ARA+DHA SPD**
16:0	19.5±0.18	20.7±0.23
16:1n-7	1.57±0.05	1.67±0.10
18:0	10.6±0.13	11.3±0.24
18:1n-9	44.0±1.00	39.4±1.53
18:2n-6 LA	19.9±0.28	21.3±0.51
18:3n-3 ALA	2.46±0.03	1.87±0.04
20:4n-6 ARA	ND	0.45±0.05
22:6n-3 DHA	ND	0.81±0.01
Total SFA	31.1±0.13	33.2±0.13
Total PUFA	22.4±0.31	24.5±0.60
Total MUFA	45.5±0.94	41.1±1.42
Total n-6	19.9±0.28	21.8±0.55
Total n-3	2.46±0.03	2.68±0.04
Ratio n-6/n-3 PUFA	8.09±0.05	8.14±0.07
Ratio PUFA/SFA	0.72±0.01	0.74±0.02
Weaning diet fatty acid	**Control WD**	**ARA+DHA WD**
16:0	19.5±0.18	19.9±0.63
16:1n-7	1.57±0.05	1.57±0.07
18:0	10.6±0.13	11.0±0.44
18:1n-9	44.0±1.00	42.7±2.62
18:2n-6 LA	19.9±0.28	19.7±0.75
18:3n-3 ALA	2.46±0.03	2.01±0.08
20:4n-6 ARA	ND	0.48±0.02
22:6n-3 DHA	ND	0.59±0.09
Total SFA	31.1±0.13	31.9±1.17
Total PUFA	22.4±0.31	22.8±0.9
Total MUFA	45.6±0.94	44.3±2.54
Total n-6	19.9±0.28	20.2±0.73
Total n-3	2.46±0.03	2.60±0.17
Ratio n-6/n-3 PUFA	8.09±0.05	7.77±0.23
Ratio PUFA/SFA	0.72±0.01	0.71±0.00

[1] Values mean ± SEM of 3 batches of oil mix used for diet. Fatty acid concentrations < 0.1 g/100g of total fat were not included. Abbreviation; SPD, suckling period diet; WD, weaning diet; ND, not detectable; ALA, α-linolenic acid; LA, linoleic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; PUFA, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; SFA, saturated fatty acids

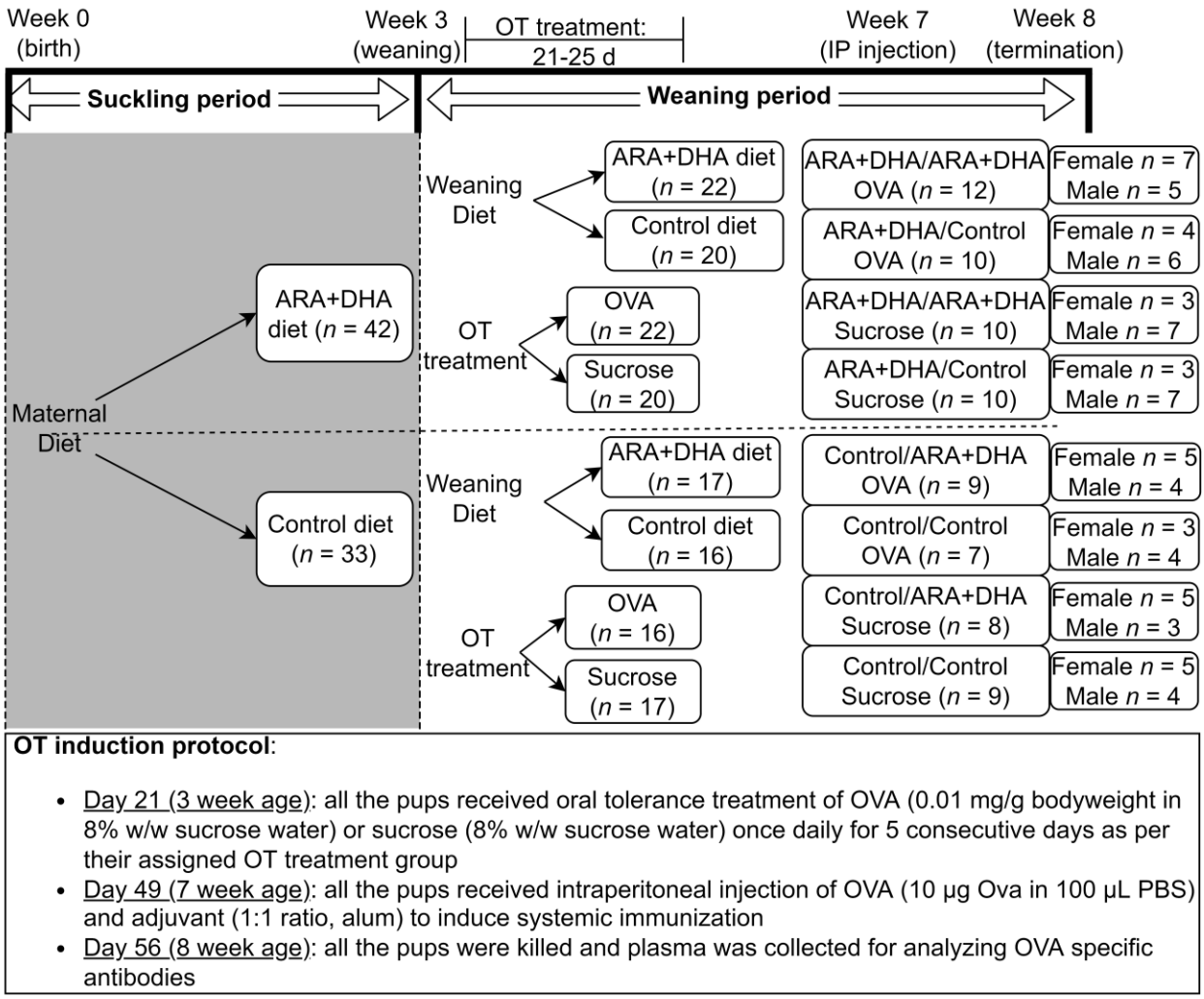

Figure 5.1. Study design. Brown Norway dams were randomized to consume either the maternal suckling period diet (SPD); the control diet or the ARA+DHA diet, starting from 5-7 d prior to parturition until the end of 3 weeks of lactation. During this time offspring consumed their mother's breastmilk referred to as SPD for offspring. At 3 weeks, offspring from each SPD group were randomly assigned to either a control weaning diet (WD) or ARA+DHA WD. For oral tolerance (OT) treatment, half of the offspring from each of the four diet groups received Ovalbumin or sucrose between 21 – 25 d. On week 7, all the offspring received an IP injection of Ovalbumin along with an adjuvant to induce systemic immunization. All offspring were killed at 8-week and relevant tissues were collected. Abbreviation: SPD, suckling period diet; WD, weaning diet; OT, oral tolerance; ARA, arachidonic acid; DHA, docosahexaenoic acid.

5.2.2. Fatty acid analysis of phospholipid and total fat

We used the modified Folch method to extract total lipids from the diet and splenocytes (C J Field et al., 1988a; Caroline Richard et al., 2016d). For splenocytes, total phospholipids were separated on silica G plates for further analysis. Methyl esters were prepared from the scraped phospholipid band on silica G plates. Followed by their methylation and separation using automated gas-liquid chromatography (Agilent Technologies) using a 100 m CP-Sil 88 fused capillary column (Kramer et al., 2004). The methylated fatty acids were quantified as the relative percentage of total fatty acid content in the phospholipids.

5.2.3. OT development and analysis: feeding Ova and immunization.

Three-week-old offspring were administered OT treatment to induce tolerance towards Ova. Approximately, half of the offspring from each of the four diet groups (described above) were fed Ova in sucrose water (0.01 mg / g body weight in 8% w/w sucrose water referred to as Ova OT treatment group) through oral gavage once daily between 21-25 days. The other half were fed sucrose water (8% w/w sucrose water referred to as sucrose OT treatment group) orally once daily between 21-25 days. On day 49 (7-week age), all the offspring received intraperitoneal injection of Ova (10 μg Ova in 100 μL PBS) combined with an adjuvant (1:1, Imject Alum Adjuvant; Thermo-Scientific, Mississauga, Ontario, Canada) to induce systemic immunization. Blood was collected in a K2 EDTA-containing tube using a 5 mL syringe by cardiac puncture. Within 1 hour of blood collection, tubes were centrifuged, and plasma was collected and stored at -80 °C for further analysis. Commercial ELISA kits were used to analyze Ova-specific IgG1 (Ova-IgG1) and Ova-IgE in the plasma (Alpha Diagnostic International, San Antonio, Texas, USA).

5.2.4. Splenocyte isolation and *ex-vivo* stimulation for cytokine production to mitogens or Ova

The spleen from 8-week-old offspring was aseptically collected and immune cells were isolated as previously described (C J Field et al., 1990). Briefly, spleen tissues were pushed through nylon mess to obtain single cell suspension and lysed with ammonium chloride lysis buffer (155 mM NH_4Cl, 0.1 mM EDTA and 10 mM $KHCO_3$; Fisher Scientific, Alberta, Canada) to remove red blood cells. Cells were washed and resuspended in complete cell culture media (RPMI 1640 media supplemented with 5% v/v fetal calf serum, 2.5 mM 2-mercaptoethanol and 1% antibiotic / antimycotic; Invitrogen, Burlington, Ontario, Canada). Splenocytes were then counted using trypan blue dye exclusion and concentrated to 1.25×10^6 cells/mL for further analysis.

Splenocyte function was analyzed by *ex-vivo* cytokine production with mitogen stimulation as previously described (Blewett et al., 2009). Briefly, splenocytes (1.25×10^6 cells/mL) were incubated for 72 hours in 5% CO_2 and 37 °C either in the presence or absence of phorbol-myristate-acetate and ionomycin (PMAi, 2µL/mL, Cell Stimulation Cocktail, Thermo Fisher Scientific, Mississauga, Ontario, Canada), lipopolysaccharide (LPS, 2µL/mL, Thermo Fisher Scientific, Mississauga, Ontario, Canada) or Ova (200 µg/mL, Sigma) a food antigen. Note, splenocyte stimulation of PMAi (lymphocyte stimulant) and LPS (bacterial challenge) was used to study the cytokine response from the adaptive immune cell and innate immune cell, respectively. Cells were then centrifuged, and the supernatant was collected and stored at -80 °C. ELISA kits from R&D systems (Minneapolis, Minnesota, USA) were used to measure the cytokine concentration of IL-1β (15.6-4000 pg/mL), IL-2 (250-4000 pg/mL), IL-6 (31.3-4000 pg/mL), IL-10 (15.6-4000 pg/mL), TGF-β and TNF-α (31.3-4000 pg/mL). The IFN-γ had a detection range of 4-1024 pg/mL (U-CyTech, Utrecht, Netherlands) and TGF-β had a detection range of 31.3-500 pg/mL (BioLegend, San Diego, California, USA). Additionally, the MSD U-plex kit was used to measure IL-4 (0.06-4500 pg/mL) and IL-13 (0.07-3200 pg/mL) as per the

manufacturer's instruction. Samples were analyzed in duplicates with CV < 10 % and absorbance was read on a spectrophotometer, and concentrations were calculated from the standard curve.

5.2.5. Immune cell phenotype analysis in splenocytes

Immune cell populations were identified by direct immunofluorescence assay as previously described (Field et al., 2000; C J Field et al., 1990). Briefly, immune cells (200,000) were incubated for 30 min at 4 °C with pre-labelled monoclonal antibodies applied in combination to quantify various immune cell phenotypes. The monoclonal antibodies were selected to minimize spectral overlap and compensation controls, and isotype controls and unstained controls were used to guide our gating strategy, published in Patel et al. (2021) (Chapter 5, Figure 4.2). Furthermore, the cells stained with CD25/CD3/CD4 combination were fixed and permeabilized using the Fixation and Permeabilization kit (eBiosciences, San Diego, California, USA). Subsequently, cells were stained with Anti-FoxP3 antibody for 20 minutes and washed according to the manufacturer's protocol. Cells were then fixed and acquired within 72 h by flow cytometry (FACSCalibur; Becton Dickinson, San Jose, California, USA).

5.2.6. Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM) unless stated otherwise. The study followed a factorial design with four independent variables (main effect); SPD, WD, OT treatment and sex. The study was powered to assess significant changes in immune function (cytokine production) as a primary outcome. Data analysis was conducted using PROC MIXED procedure in SAS (v9.4; Cary, North Carolina, USA). This allowed us to determine the main effect of SPD (ARA+DHA SPD compared to control SPD), WD (ARA+DHA WD compared to control WD), OT treatment effect (Ova compared to sucrose), sex effect (male compared to

female) and the interactions between 4 variables (SPD × WD, SPD × OT, WD × OT, SPD × sex, WD × sex, sex × OT, SPD × WD × OT, SPD × WD × sex, SPD × sex × OT, WD × sex × OT). Post hoc analysis was conducted when significance was found for the main effect or the interaction effect using LSMEANS with Bonferroni adjustment for multiple comparisons. Note, Bonferroni adjustment is more conservative and hence less likely to find a significant difference between groups (lowers type I error or false positives). Therefore, some significant interaction effects do not show a significant post hoc difference between groups (Cabral, 2008). In cases where a main effect and its interaction were simultaneously found significant, post hoc analysis and group comparison were only reported on groups as per the interaction. The current study was not sufficiently powered to perform 16 group comparisons when a significant 4-way interaction was observed. A main effect of OT treatment indicates, irrespective of the SPD, WD or sex, the Ova OT treatment group differs from the sucrose OT treatment group for the outcome of interest. Furthermore, a significant interaction effect (for example, SPD × OT interaction effect) indicates the direction of change for an outcome of interest associated with SPD that would differ between Ova and Sucrose OT treatment groups. Note, the assumptions for ANOVA were fulfilled prior to conducting the analysis and Akaike Information Criteria (AIC) was used to select the best-fitting model. Additionally, all data were analyzed for outliers (i.e., ≥ 3 standard deviations from the overall mean) and the outlier values were removed prior to analysis. In the case of two group comparisons, (such as diet fatty acid composition) data was analyzed using an unpaired student *t*-test. At $P \leq 0.05$ (two-sided) differences were considered significant.

5.3. Results

5.3.1. Anthropometric measurement

The SPD, WD, OT treatment nor sex had no effect on the body weight, body length, or liver weight of the offspring. We observed a three-way interaction between SPD, WD and OT treatment for spleen weight. The post hoc analysis showed that the spleen weight of offspring that received ARA+DHA SPD was significantly higher than control SPD, however, only for offspring that received control WD and sucrose OT treatment (Figure 5.2). However, the splenocyte count (330±19 x 10^6 cells per gram of spleen) did not differ between diets, OT treatment or sex groups. Additionally, the average daily food intake between 4 to 8 weeks did not differ amongst groups (Table 5.2).

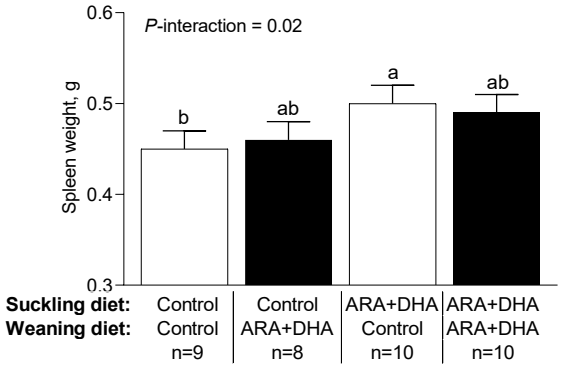

Figure 5.2. Interaction effect of suckling diet, weaning diet and OT treatment on the spleen weight of offspring at 8 weeks. Values are presented in mean ± SEM. The SD x WD x OT treatment was analyzed using 3-way ANOVA. Only data for sucrose OT groups are shown as Ova OT groups were not different. Bars that do not share the same letter indicate a difference at $P < 0.05$. Abbreviation: SD, suckling diet; WD, weaning diet.

Table 5.2. The average daily food intake in offspring from weeks 4 to 8 is based on their diet group.

Suckling diet: **Weaning Diet:**	**Control** **Control**	**Control** **ARA+DHA**	**ARA+DHA** **Control**	**ARA+DHA** **ARA+DHA**
gram of food/day	Mean ± SEM (*n* = 16)	Mean ± SEM (*n* = 17)	Mean ± SEM (*n* = 20)	Mean ± SEM (*n* = 22)
Week 4	17.7±0.8	16.3±0.4	17.6±1.4	13.6±0.5
Week 5	18.4±0.6	17.8±0.9	17.8±0.5	19.1±1
Week 6	20.9±0.7	20.9±0.6	21.3±0.8	21.5±1.2
Week 7	22±2.6	21±2.4	19.1±1.6	20.7±1.5
Week 8	27.5±2.2	25±1.6	25.7±1.9	25.2±1.4

Note: The food intake was measured as the difference between the weight of the food cup before and after refilling the food cup.

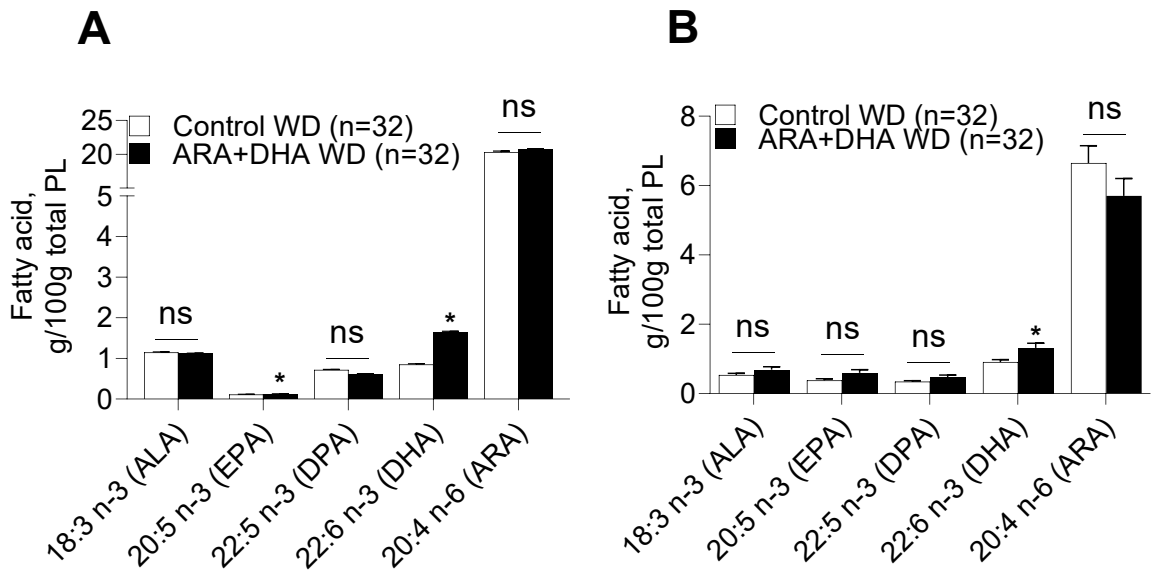

Figure 5.3. The effect of weaning diet (*n* = 32/ WD group) on 8-week Brown Norway offspring (A) splenocyte phospholipids and (B) plasma phospholipids fatty acid composition. Values are presented in mean ± SEM. There was no significant effect of the OT treatment group and maternal diet or interaction, so the OT treatment groups and maternal diet groups were combined. The weaning diet effect was calculated by unpaired Student *t*-test. * Indicate significant difference at $P < 0.05$. Abbreviation: ARA, arachidonic acid; DHA, docosahexaenoic acid; ALA, α-linolenic aid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

Table 5.3. The effect of suckling diet and weaning diet on the fatty acid composition of splenocyte phospholipids. [1]

Suckling diet:	**Control**	**Control**	**ARA+DHA**	**ARA+DHA**
Weaning diet:	**Control**	**ARA+DHA**	**Control**	**ARA+DHA**
	Mean ± SEM (*n* = 14)	**Mean ± SEM (*n* = 12)**	**Mean ± SEM (*n* = 18)**	**Mean ± SEM (*n* = 20)**
	g / 100 g fatty acid in total phospholipid			
14:0	0.28±0.05	0.32±0.04	0.45±0.1	0.36±0.06
15:0	0.16±0	0.16±0	0.16±0	0.15±0
16:0	24.39±0.26	24.37±0.22	24.48±0.18	24.4±0.18
18:0	20.79±0.23	20.72±0.22	21.12±0.19	21.32±0.31
20:0	0.31±0.02	0.31±0.01	0.34±0.01	0.37±0.01
24:0	0.58±0.03	0.6±0.02	0.65±0.02	0.7±0.03
16:1	0.93±0.02	0.96±0.02	0.9±0.02	0.82±0.02
18:1n-9	10.81±0.07	10.67±0.05	10.7±0.09	10.47±0.1
24:1n-9	3.56±0.09	3.4±0.07	3.58±0.04	3.41±0.07
18:2n-6 (LA)[2]	8.31±0.14 [ab]	8.13±0.12 [ab]	8.46±0.15 [b]	7.73±0.18 [a]
20:3n-6	1.67±0.02	1.68±0.02	1.73±0.02	1.66±0.03
20:4n-6 (ARA)	20.67±0.25	20.67±0.08	20.01±0.2	20.69±0.22
22:4n-6	0.13±0.02	0.09±0.01	0.09±0.01	0.13±0.02
22:5n-6[3]	0.23±0.01 §	0.13±0.01	0.19±0.01 §	0.13±0.01
18:3n-3 (ALA)	1.14±0.02 [ab]	1.15±0.02 [ab]	1.15±0.02 [a]	1.09±0.03 [b]
20:5n-3 (EPA)	0.11±0.01 §	0.1±0.01	0.11±0.01 §	0.13±0.01
22:5n-3	0.73±0.02	0.57±0.03	0.71±0.01	0.63±0.03
22:6n-3 (DHA)	0.86±0.03 §	1.7±0.03	0.84±0.02 §	1.62±0.06
Total SFA[4]	46.78±0.34	46.72±0.16	47.47±0.34	47.59±0.39
Total MUFA	18.28±0.12	17.96±0.07	18.12±0.11	17.57±0.11
Total PUFA	34.93±0.34	35.32±0.16	34.41±0.28	34.84±0.34
Ratio PUFA/SFA	1.91±0.02 §	1.97±0.01	1.9±0.02 §	1.98±0.02
Total n-6	31.98±0.33	31.69±0.15	31.47±0.28	31.24±0.3
Total n-3	2.95±0.04 §	3.63±0.05	2.95±0.03 §	3.6±0.06
Ratio n-6/n-3	10.87±0.18	8.75±0.15	10.69±0.12	8.72±0.15

[1] Abbreviation: LA, linoleic acid; ALA, α-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

[2] Differences between groups due to an interaction effect are shown with a different letter

[3] Differences between groups due to the WD effect are indicated by §

[4] Some trace fatty acids are not reported here. Therefore, the sum from the table may not match the total SFA

Table 5.4. The effect of suckling diet and weaning diet on the fatty acid composition of plasma phospholipids.

Suckling diet: **Weaning diet:**	**Control Control Mean ± SEM (*n* = 8)**	**Control ARA+DHA Mean ± SEM (*n* = 8)**	**ARA+DHA Control Mean ± SEM (*n* = 12)**	**ARA+DHA ARA+DHA Mean ± SEM (*n* = 10)**
	g / 100 g fatty acid in total phospholipid			
14:0	3.60±0.36 §	4.82±0.48	3.65±0.27 §	5.7±0.65
15:0	0.32±0.03	0.51±0.07	0.42±0.09	0.7±0.2
16:0	27.17±0.67	27.64±0.84	27.05±0.65	27.28±1.16
18:01[1]	35.69±1.04 *	34.45±1.34 *	32.12±0.96	31.77±0.71
20:0	0.73±0.13	1.1±0.24	0.56±0.06	1.13±0.16
24:0	0.63±0.03	0.69±0.03	0.68±0.1	0.97±0.13
16:1	0.42±0.09	0.42±0.07	0.28±0.03	0.71±0.21
18:1n-9	5.97±0.28 §	5.26±0.41	6.72±0.26 §	5.28±0.24
24:1n-9	0.44±0.01	0.41±0.02	0.54±0.05	0.94±0.2
18:2n-6 (LA)[2]	12.72±1 §	11.88±0.78	15.22±0.57 §	10.75±0.73
20:2n-6	0.59±0.06	0.76±0.18	0.48±0.08	0.71±0.18
20:3n-6	0.5±0.07 §	0.98±0.29	0.68±0.17 §	1.84±0.48
20:4n-6 (ARA)	6.08±0.51	5.82±0.37	7.19±0.36	5.61±0.46
22:5n-6	0.88±0.19	0.8±0.11	0.6±0.09	1.03±0.15
18:3n-3 (ALA)	0.68±0.11	0.71±0.12	0.44±0.03	0.68±0.13
20:5n-3 (EPA)	0.54±0.1 *	0.51±0.1 *	0.28±0.03	0.67±0.15
22:5n-3	0.36±0.05	0.4±0.07	0.33±0.03	0.52±0.09
22:6n-3 (DHA)	0.77±0.09 §	1.08±0.11	0.99±0.09 §	1.51±0.22
Total SFA[3]	68.67±1.28	69.85±1.44	65±0.89	68.39±1.31
Total MUFA	7.93±0.32	7.12±0.46	8.74±0.25	8.23±0.37
Total PUFA	23.17±1.23	22.98±1.07	26.24±0.73	23.36±1.04
Ratio PUFA/SFA	0.34±0.02	0.33±0.02	0.4±0.01	0.34±0.02
Total n-6	20.8±1.27 §	20.26±1.12	24.18±0.65 §	19.96±0.8
Total n-3	2.36±0.13 §	2.71±0.27	2.05±0.11 §	3.4±0.52
Ratio n-6/n-3	9.08±0.94 §	8.27±1.22	12.05±0.53 §	7.05±1.02

[1] Differences between groups due to the suckling diet effect are shown by *

[2] Differences between groups due to the weaning diet effect are indicated by §

[3] Some trace fatty acids are not reported here. Therefore, the sum from the table may not match the total SFA

5.3.2. Fatty acid composition of total phospholipid in 8-week offspring

Spleen: ARA+DHA supplementation in the suckling period (0-3 weeks) had no lasting effect on ARA and DHA in offspring at 8-week. However, ARA+DHA supplementation during the weaning period resulted in significantly higher DHA, EPA, total n-3 and PUFA/SFA ratio in splenocytes compared to control WD ($P < 0.01$, Figure 5.3 A). ARA+DHA WD did not affect the phospholipid composition of ARA, total SFA, total PUFA, total MUFA and total n-6. A significant SPD × WD interaction effect was observed for linoleic acid composition in splenocyte phospholipid, and the post hoc analysis showed that the ARA+DHA WD group was significantly lower than the control WD group (7.8±0.2 vs 8.5±0.1 g/100g total PL, $P = 0.003$), but only when they received ARA+DHA SPD (Table 5.3). Similarly, a significant SPD × WD interaction effect for splenocyte phospholipid of α-linolenic acid composition was also observed, which found that ARA+DHA WD was lower when compared to control WD (1.01±0.03 1.15±0.02 g/100g total PL, $P = 0.01$), however, only when they received ARA+DHA SPD (Table 5.3).

Plasma: ARA+DHA SPD had no lasting effect on ARA or DHA plasma phospholipids composition when observed in offspring at 8 weeks. However, offspring from the ARA+DHA WD group had significantly higher DHA (Figure 5.3 B) and total n-3 composition of plasma phospholipids, with no difference in ARA (Table 5.4). Further, linoleic acid was significantly lower in the ARA+DHA WD group than control WD group offspring. The WD had no effect on the plasma phospholipid composition of total SFA, total MUFA and total PUFA.

5.3.3. Plasma concentration of Ova specific immunoglobulins

Plasma Ova-IgG1: ARA+DHA SPD group offspring had significantly lower plasma Ova-IgG1 concentration compared to control (1297±147 versus 2040±374, $P = 0.02$, Figure 5.4 A). As

expected, plasma Ova-IgG1 was significantly lower in the Ova OT treatment group than the sucrose OT treatment group indicating successful induction of OT towards Ova (1266±134 versus 1965±337; $P = 0.01$). However, we did not observe an interaction effect between SPD and OT treatment (Figure 5.4. A show the SPD and OT group comparison). Moreover, WD or sex had no significant effect on plasma Ova-IgG1.

Plasma Ova-IgE: In 8-week offspring, SPD, WD and sex had no significant effect on the plasma concentration of Ova-IgE. Nevertheless, offspring from the Ova OT treatment group had plasma Ova-IgE concentrations that were significantly lower compared to sucrose OT treatment group offspring (14.8±1.2 versus 17.7±1.3; $P = 0.04$, Figure 5.4. B), further indicating OT towards Ova was induced in Ova OT group offspring.

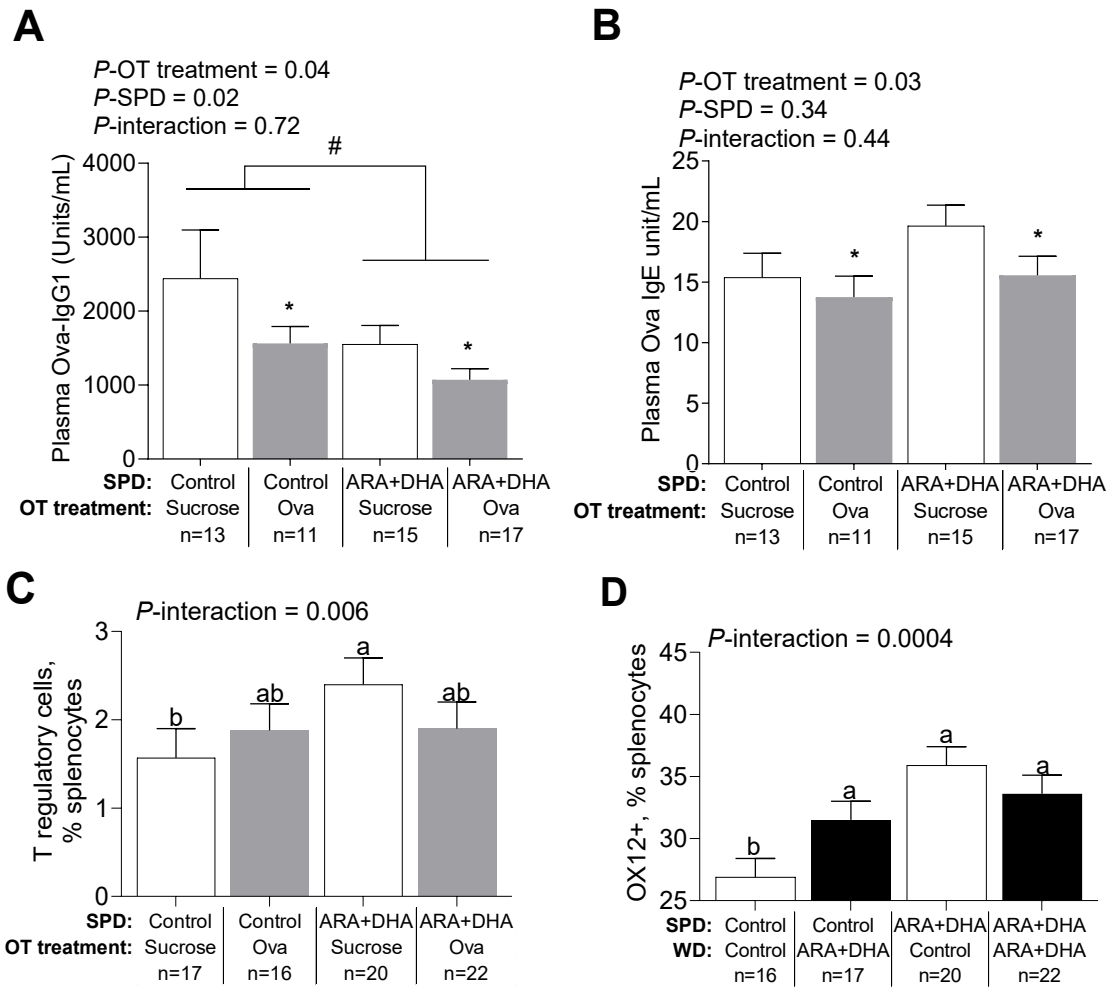

Figure 5.4. The effect of ARA+DHA SPD on OT development. Plasma Ova-specific IgG1 (A) and plasma Ova-IgE (B) concentration in 8-week Brown Norway offspring according to the SPD and OT treatment. The SPD x OT interaction was analyzed using 2-way ANOVA for T regulatory cells in the spleen (C). The SPD x WD interaction was analyzed using 2-way ANOVA on OX12+ (B cells subtype) splenocytes (D). * Indicates the difference between the OT treatment group and # indicates a difference between SPD groups at $P < 0.05$ based on unpaired student *t*-test or 2-way ANOVA. Two-way ANOVA was used to calculate the *P*-value for the main effect and interaction for SPD × OT treatment or SPD × WD. Bars that do not share the same letter indicate a difference at $P < 0.05$. Values are presented in mean ± SEM. Abbreviation: OT, oral tolerance; Ova, ovalbumin; SPD, suckling period diet; WD, weaning diet; IgG1, immunoglobulin G1; ARA, arachidonic acid; DHA, docosahexaenoic acid; IL-2, interleukin-2.

Table 5.5. Suckling diet effect on *ex-vivo* cytokine production by splenocytes with different stimulation conditions.[1]

Suckling diet	**Control**	**ARA+DHA**
	Mean ± SEM (n = 16)	Mean ± SEM (n = 17)
LPS (pg/ml)		
IL-1β	32.3±1.81	27.8±1.40
TGF-β	320±8.38	327±8.87
TNF-α	90±7.5	109±9.82
IL-6	443±20.4	393±21.9*
IL-10	258±10.9	231±9.71
IFN-γ	444±66.9	458±54.7
PMA+Ionomycin (pg/ml)		
IL-2	3307±220.3	3239±148.2
IL-6	97.86±9.84	113.31±13.39
TGF-β	221.42±8.35	235.24±9.88
TNF-α	81.51±11.86H	84.22±9.48
IL-10	80.67±11.69	85.26±6.7
IL-4	7.9±1.07	9.07±1.05
IL-13	12.27±1.14	10.67±1.03
IFN-γ	523±143	300±61.2
Ovalbumin (pg/ml)		
TGF-β	295±5.46	294±5.48
TNF-α	38.8±2.98	48.8±3.24
IL-10	55.2±5.23	47.6±5.69
IFN-γ	9.09±1.13	9.01±1.38
IL-6	192.8±21.6	226.7±28.3
Unstimulated (pg/ml)		
TNF-α	18.1±0.80	23.1±1.79
IL-10	3.8±0.1	3.4±0.0*

[1] Note: mean ± SEM values in a row that do not share a common symbol (*) are significantly different.

Table 5.6. The effect of SPD, WD and OT treatment on *ex-vivo* cytokine production to mitogens/antigens and phenotype by immune cells of spleen.

SPD:	Control				ARA+DHA										
WD:	Control		ARA+DHA		Control		ARA+DHA								
OT:	Sucrose	OVA	Sucrose	OVA	Sucrose	OVA	Sucrose	OVA	SPD	WD	SPD × WD	OT	SPD × OT	WD × OT	SPD× WD × OT
	n = 9	*n* = 7	*n* = 8	*n* = 9	*n* = 10	*n* = 10	*n* = 10	*n* = 12	*P* values						
LPS (pg/mL)															
IL-1β	38.4±4.30	34.2±4.50	27.3±1.90	29.1±2.50	27.6±2.60	28.3±4.20	27.2±2.40	27.9±2.30	0.30	0.13	0.13	0.66	0.67	0.47	0.46
IFN-γ	343±78.0	295±46.3	757±227	417±106	604±158	506±115	319±62.3	400±75.2	0.71	0.94	0.02	0.71	0.40	0.96	0.23
TNF-α	89.7±17.6	90.3±15.0	80.0±18.1	98.9±10.1	98.6±19.7	98.2±24.1	113±23.7	123±13.4	0.46	0.31	0.69	0.86	0.98	0.44	0.95
IL-6[1]	419±41.1	434±42.2	499±47.9	429±35.2	449±56.0*	387±40.9*	414±50.8*	339±27.2*	0.04	0.42	0.24	0.10	0.55	0.21	0.82
IL-10	249±18.8	287±15.6	259±35.1	245±13.7	239±30.3	231±19.0	240±13.9	217±13.8	0.23	0.33	0.98	0.86	0.42	0.30	0.38
TGF-β	314±19.5	320±21.5	321±17.2	324±12.0	322±13.2	33712.8	311±25.1	336±18.2	0.76	0.81	0.47	0.48	0.34	0.81	0.73
PMA+Ionomycin (pg/mL)															
IL-2[2]	3063±397	3897±550†	3031±418	3337±439†	3282±263	3604±342†	3023±284	3078±297†	0.50	0.20	0.83	0.05	0.51	0.30	0.80
IFN-γ[3]	270±215	662±373	612±300§	577±292§	321±119	266±129	346±122§	281±131§	0.26	0.03	0.27	0.66	0.17	0.34	0.41
TNF-α	49.5±19.7	89.5±33.0	102±22.3§	89.1±21.6§	78.4±21.1	78.1±20.6	92.2±22.2§	87.5±15.2§	0.45	0.01	0.10	0.33	0.64	0.15	0.21
IL-6[4]	73.3±9.2bc	92.5±21bc	138±26ab	95.5±19ab	183±42.4a	121±13.8a	85.5±17.5c	73.2±10.9c	0.59	0.69	<0.01	0.29	0.98	0.78	0.04
IL-4	5.30±1.10	9.00±2.50	8.5±2.40	9.20±2.50	8.80±2.20	7.00±1.00	11.30±3.10	9.20±1.80	0.14	0.36	0.78	0.89	0.11	0.29	0.15
IL-10	56.7±12.5	83.5±30.2	96.1±32.1§	88.8±20.2§	68.7±12.6	89.5±20.8	91.0±10.3§	89.5±9.00§	0.67	0.04	0.47	0.44	0.57	0.06	0.98
IL-13	9.40±1.90	14.10±2.80	14.00±2.60	12.20±2.00	12.90±3.30	10.50±1.90	9.80±1.30	9.60±1.60	0.76	0.85	0.29	0.95	0.18	0.34	0.05
TGF-β	250±17.9	220±5.70	199±15.4	213±18.2	224±30.9	264±19.3	232±13.7	224±12.4	0.23	0.11	0.76	0.82	0.62	0.95	0.05
Ovalbumin (pg/mL)															
TGF-β	297±15.7	293±12.9	302±4.20	289±8.30	284±7.70	293±12.6	303±11.5	296±11.8	0.73	0.67	0.52	0.55	0.51	0.50	0.75
TNF-α	37.5±5.60	37.0±7.30	36.1±4.50	43.9±6.90	53.2±7.00	51.8±7.00	44.7±6.40	46.2±6.20	0.27	0.49	0.09	0.66	0.68	0.44	0.78
IL-10	56.6±9.80	64.6±14.5	59.9±12.3§	42.3±5.80 §	49.0±12.2	66.5±16.4	47.8±9.40 §	30.5±4.8 §	0.56	0.01	0.70	0.34	0.62	0.01	0.29
IFN-γ	8.20±2.10	7.60±1.70	10.9±2.30	9.50±2.80	15.1±4.50	8.00±1.70	6.10±2.10	7.20±1.60	0.87	0.80	0.16	0.41	0.61	0.39	0.55
IL-6[5]	167±25.7	144±22.8	266±73.6§	192±27.4§	226±47.5	206±69.5	285±74.7§	196±37.5§	0.31	0.02	0.21	0.02	0.63	0.75	0.44
Unstimulated (pg/mL)															
TNF-α	17.4±1.10	18.3±1.50	18.2±2.40	18.5±1.50	24.7±4.80	27.8±5.20	20.6±1.60	19.7±1.60	0.25	0.81	0.25	0.40	0.93	0.39	0.39
IL-10	42.0±5.7a	45.8±5.6a	59.4±11a	49.5±9.30 a	52.1±9.8 a	48.2±9.3a	23.6±3.00 b	25.9±3.5b	0.02	0.11	0.00	0.66	0.67	0.74	0.31
% of gated splenocytes[6]															
CD25+ in CD3+CD8+	3.7±0.3 b	4.6±0.5 a	3.6±0.3 b	3.50±0.24 b	4.4±0.6 ab	4±0.42 ab	3.8±0.26 ab	4.±0.30 ab	0.70	0.41	0.69	0.25	0.11	0.72	0.01
CD4+ in CD28+	67.5±3.50	64.3±3.50	68.1±3.50	67.4±3.50	72.1±3.10	74.3±3.10	73.0±3.10	71.6±3.00	0.24	0.75	0.06	0.27	0.14	0.69	0.03
Total IgA+	1.8±0.1 a	1.90±0.2 a	1.8±0.1 ab	1.6±0.1 ab	1.5±0.2 b	1.4±0.1 b	1.80±0.10 ab	1.8±0.1 ab	0.28	0.92	0.00	0.76	0.14	0.69	0.08
Total IgG+	59.5±2.8 b	58.6±3.2 b	65±1.6 ab	66±2. ab	67.1±1. a	66.7±1.1 a	66.6±1.5 ab	64±1.9 ab	0.05	0.38	0.01	0.96	0.14	0.75	0.60
Total OX6+	24.3±1.5 b	24.7±2.2 b	29.0±1.9 a	30.6±1.9 a	31.8±1.8 ab	30.7±2. ab	28.2±1.3 ab	28±1.6 ab	0.56	0.32	0.00	0.71	0.14	0.63	0.73
Total OX12+	27.0±1 b	26.8±1.4 b	30.5±1.5 a	32.6±1.6 a	36.7±1.5 a	35.6±1.9 a	33.1±1.4 a	33.3±1.1a	0.07	0.23	<0.01	0.70	0.14	0.40	0.76
Total CD45RA+	61.1±1.1 b	61.6±1.8 b	63.9±1.7 a	64.1±1.8a	62±0.7 ab	61.6±1 ab	60.8±0.7 ab	61±0.9 ab	0.98	0.08	<0.01	0.45	0.14	0.91	1.00
Total CD11b/c+	10.1±0.6	9.7±0.9 †	9.4±0.4	10.3±0.5 †	8.60±0.6	9.50±0.6†	8.40±0.5	9.70±0.7†	0.84	0.90	0.73	0.03	0.14	0.19	0.60

[1] * Different from corresponding control suckling period diet groups, $P < 0.05$
[2] † Different from corresponding sucrose OT treatment groups, $P < 0.05$
[3] § Different from corresponding control weaning diet groups, $P < 0.05$
[4] Labeled means in a row without a common letter differ, $P < 0.05$.
[5] Group comparisons due to significant OT treatment effect on IL-6 are shown in Figure 5.5.
[6] Some immune cell markers were not significantly affected by SPD, WD, OT treatment or their interactions; therefore, they are not included here. These are total CD3+, total CD4+, total CD8+, CD3+CD4+, CD3+CD8+, CD27+, CD28+, CD68+, CD86+, CD3-CD161+, CD3+CD161+ and total foxP3+ cells.
Abbreviation; SPD, suckling period diet; WD, weaning diet; OT, oral tolerance; IL, interleukin; IFN-γ, interferon-γ; TGF-β, Transforming growth factor-β; TNF-α, Tumor necrosis factor-α; LPS, lipopolysaccharide, PMA, phorbol-myristate-acetate; CD, cluster of differentiation; Ig immunoglobulins.

5.3.4. Splenocyte *ex-vivo* cytokine production to mitogens or Ova stimulation

LPS: Compared to control SPD, ARA+DHA SPD group offspring produced significantly less IL-6 *ex-vivo* with LPS stimulation (Table 5.5.), however no differences in IL-1β, TGF-β, TNF-α, IL-10 and IFN-γ production was seen due to SPD. Further, no significant difference due to WD, OT treatment or sex was observed for IL-1β, TGF-β, TNF-α, IL-10 and IFN-γ by LPS-stimulated splenocyte.

Ova: The Ova stimulated splenocytes production of IL-6 showed two main effects, the WD effect and the OT treatment effect (*P's = 0.02*). First, offspring that received the ARA+DHA WD group had IL-6 levels significantly higher than the control (Table 5.6). Second, Ova OT treatment was significantly lower compared to the sucrose OT treatment group. WD × OT treatment interaction effect was observed for IL-10 production with Ova stimulation, which showed that offspring from ARA+DHA WD had significantly lower production than control WD, however only within the Ova OT treatment group (Figure 5.5 A). Lastly, IL-10 with Ova stimulation showed a significant SPD × OT treatment × sex interaction effect. However, the post hoc analysis (with Bonferroni multiple comparison adjustment) found no difference between groups. TGF-β and TNF-α production by splenocytes stimulated with Ova were not affected by SPD, WD, OT treatment or sex.

PMAi: IL-6 production by splenocytes stimulated with PMAi showed a significant SPD × WD interaction effect. The postdoc analysis found the ARA+DHA WD group had lower IL-6 than the control WD group but only when offspring were from ARA+DHA SPD (and not control SPD, Figure 5.5 C). IL-2 production to PMAi was significantly higher in Ova compared to the sucrose OT group (3428±192 vs. 3104±162, $P = 0.05$). SPD, WD and sex had no significant effect on IL-2 production by PMAi-stimulated splenocytes from 8-week offspring. Further, a

significant WD effect showed that the production of IL-10, IFN-γ and TNF-α with PMAi was significantly higher in the ARA+DHA WD group compared to control WD group offspring (Figure 5.5 B). A significant OT treatment × sex interaction effect showed that IL-10 production with PMAi stimulation was significantly higher in female offspring (but not male) that received Ova OT treatment group compared to sucrose OT group (108.2±18.1 vs 74.8±18.9 pg/mL, P = 0.02). We observed a significant SPD × sex interaction effect in TNF-α production by splenocytes with PMAi stimulation. In this, offspring (only female) showed significantly higher TNF-α when they received ARA+DHA SPD compared to control SPD (110±31 vs 76±31 pg/mL, P = 0.008), whereas male offspring showed no differences due to SPD supplementation. Next, we observed a significant SPD × WD × sex interaction effect for IL-4 production to PMAi, however, the post hoc analysis showed no differences between groups. TGF-β production by splenocytes from 8-week offspring showed three-way interaction between SPD × WD × OT treatment, however, post hoc analysis showed no differences between groups.

Unstimulated: Unstimulated splenocytes from the 8-week offspring showed no differences in TNF-α levels between groups. A significant SPD × WD interaction for IL-10 showed that offspring from ARA+DHA SPD followed by ARA+DHA WD produced lower IL-10 than offspring from other diet groups (control SPD and ARA+DHA WD, ARA+DHA SPD and control WD, and control SPD and control WD (Table 5.6). Furthermore, sex and OT treatment had no effect on IL-10 levels.

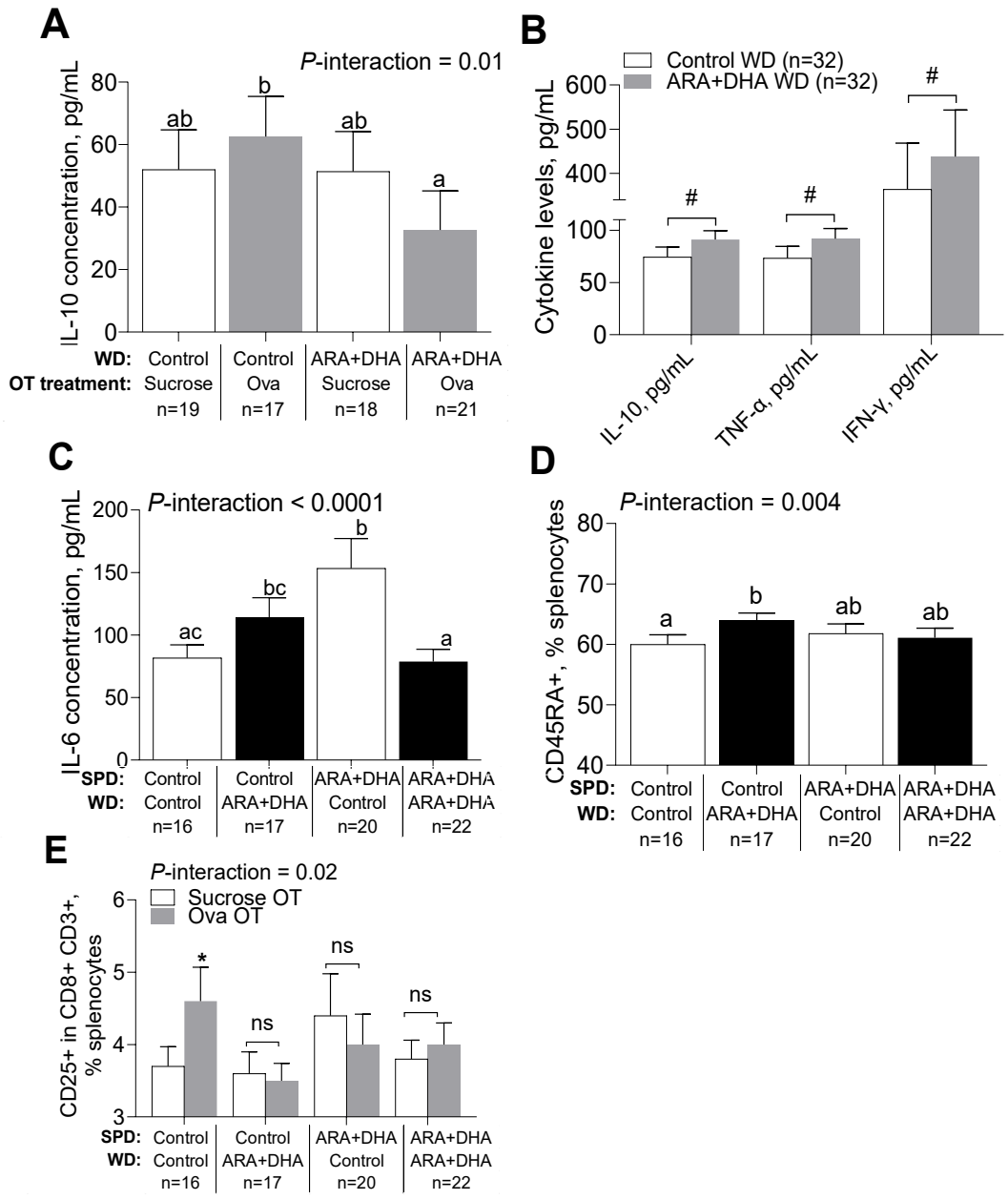

Figure 5.5. The effect of ARA+DHA WD on OT development. *Ex-vivo* IL-10 production with Ova stimulation in splenocytes (A). WD effect on cytokine production to PMAi stimulation (B) and SPD x WD interaction on IL-6 production to (C) in splenocytes. The SPD x WD interaction effect on splenocyte proportion of CD45RA+ (D) and the SPD x WD x OT treatment interaction effect on splenocyte proportion CD25+ in CD8+CD3+ (E). * Indicates a difference between the OT treatment group and # indicates a difference between WD groups at $P < 0.05$. Abbreviation: OT, oral tolerance; Ova, ovalbumin; SPD, suckling period diet; WD, weaning diet; IgG1, immunoglobulin G1; ARA, arachidonic acid; DHA, docosahexaenoic acid; IL-, interleukin-; IFN-γ, interferon-γ; TNF-α, Tumor necrosis factor-α.

5.3.5. Immune cell population phenotype in the spleen of offspring at 8-week

There was no effect of diet, sex, or OT treatment on the splenocyte population of major subtypes such as the relative proportion of total CD3+ cells, total CD4+ cells, total CD8+ cells, total CD161+ cells, total CD28+ cells, total OX62+, total CD68+, total CD284+, total IgE+ cells, total CD86+ cells, total CD27+, total FoxP3+ cells and total TCRαβ+ cells. The proportion of CD25+ in CD3+CD8+ (activated cytotoxic T cells, CTL) splenocytes showed a significant SPD × WD × OT treatment interaction effect. The post hoc analysis revealed Ova OT treatment group had higher CD25+ in CD3+CD8+ splenocytes than sucrose OT treatment when the offspring received control SPD and control WD (4.6±0.5 vs 3.7±0.3 % of gated splenocytes, $P = 0.01$) (Figure 5.5 E). The difference due to OT treatment was not present when offspring received the ARA+DHA diet during the suckling and/or weaning period (Figure 5.5 E). Splenocyte proportion of CD3+CD4+CD25+FoxP3+ (T regulatory cells) showed an SPD × OT treatment interaction effect in which ARA+DHA SPD was higher compared to control SPD, however, only when offspring were from the sucrose OT group (Figure 5.4 C).

The ARA+DHA SPD group had a significantly lower proportion of CD8+ CD152+ (1.67±0.72 vs 2.54±0.73; $P = 0.01$) and CD68+ CD284+ (38.9±4.0 vs 43.6±4.1; $P = 0.04$) splenocytes when compared to control SPD. The significant interaction effects on the proportion of CD4+CD28+ splenocytes and CD8+CD28+ splenocytes are not described here as there was no significant difference between the groups in the post hoc analysis.

A significant WD × sex interaction effect showed that the splenocyte proportion of total CD25+ and OX6+CD25+ was significantly higher in male offspring compared to female offspring only within ARA+DHA WD groups. We observed a significant SPD × OT treatment × sex interaction effect on OX6+OX62+ splenocytes, however, the post-hoc analysis showed no differences. A

significant SPD × WD interaction showed that the proportion of total IgG+ in splenocytes was significantly higher in offspring that received ARA+DHA SPD and control WD compared to offspring that received control SPD and control WD (Table 5.6). However, offspring that received ARA+DHA for SPD and WD showed no differences in total IgG+ splenocytes from offspring that received control for SPD and WD (Table 5.6). Next, the IgA+ splenocytes also showed a significant SPD × WD interaction, in that offspring that received ARA+DHA SPD and control WD had higher IgA+ splenocytes compared to offspring from the control SPD and control WD group (Table 5.6).

As shown by the post hoc analysis of the significant SPD × WD interaction effect, the splenocyte proportion of OX6+ cells was significantly higher in offspring from the ARA+DHA WD group compared to the control WD group, but only within control SPD groups (Table 5.6). When ARA+DHA SPD groups were compared based on WD there was no significant difference in OX6+ splenocytes. Next, in the proportion of total OX12+ splenocytes, there was a significant SPD × WD interaction effect and the post hoc analysis showed that the ARA+DHA SPD and ARA+DHA WD group is higher compared to the control SPD and control WD (Figure 5.4 D). The total CD45RA+ splenocytes had a significant SPD × WD interaction effect and the post hoc analysis showed offspring from the control SPD and ARA+DHA WD group were higher than control SPD and control WD (Figure 5.5 D). Additionally, the sex effect on CD45RA+ splenocytes showed that female offspring had a higher proportion of total CD45RA+ splenocytes than male offspring (62.7±0.7 vs 61.3±0.6, $P = 0.02$). The CD11b/c+ splenocytes showed a significant OT treatment effect where Ova OT group offspring had a higher proportion of CD11b/c+ splenocytes compared to sucrose OT group offspring (10.1±0.5 vs 9.4±0.6, $P = 0.03$).

5.4. Discussion

The current study investigated the effect of feeding ARA+DHA during two important phases of early life: the suckling period (0-3 weeks) and weaning period (3-8 weeks), on immune system development, using an allergy-prone Th2 dominant Brown Norway rat. A summary of fatty acid changes due to maternal SPD on dams' breastmilk and plasma has been previously reported (Patel et al., 2021). Feeding ARA+DHA WD resulted in higher DHA composition of plasma and splenocyte total phospholipids in 8-week offspring (Figure 5.3), without affecting ARA. Similar findings are also reported in previous experiments using healthy Sprague Dawley offspring at 6-week where an increase in DHA due to ARA+DHA supplementation did not result in changes in ARA in splenocytes (Caroline Richard et al., 2016e). This is consistent with the observation that when DHA is supplemented alongside ARA at the same ratio, does not result in significant changes in the ARA composition of tissues (Hahn et al., 2020; Caroline Richard et al., 2016e). It can be hypothesized that at a low dose (0.5% of ARA in total dietary fat) ARA may not be sufficient to cause significant changes in plasma phospholipids, although it can help maintain the ARA levels.

Changes in LCPUFA are thought to be responsible for the modulation of immune cell function and phenotype (Calder, 2008; Gutiérrez et al., 2019). We reported that feeding ARA+DHA during the suckling period resulted in a beneficial effect on OT, shown through suppression of plasma Ova-IgG1, and alteration in cytokine response (lower IL-6 production to *ex-vivo* LPS stimulation). This could counteract the Th2-biased immune system of Brown Norway offspring. Additionally, a higher B cell maturation was also associated with suckling period supplementation, where higher proportions of the B cell subtype (OX12+) and IgG+ B cells were observed in splenocytes of 8-wk old offspring. DHA-derived specialized pro-resolving mediators

have been shown to play an important role in B cell humoral response (Duffney et al., 2018; Ramon et al., 2014; Sesquile Ramon et al., 2012) and this could explain the higher proportion of B cells despite significantly lower food antigen-specific immunoglobulin response. ARA+DHA during the weaning period promoted the Th1 cytokine response with high TNF-α and IFN-γ, irrespective of SPD. This is further supported by higher proportions of total B cells (CD45RA+) and MHC-II+ cells (OX6+) in splenocytes with ARA+DHA WD. In terms of OT treatment, we have previously shown that a low dose exposure of Ova can induce OT in healthy Sprague Dawley offspring (Table 5.7.) (C. Richard et al., 2016). However, in the current study, we have demonstrated the effect of combined ARA+DHA supplementation during suckling and weaning periods on OT development in Th2-dominant Brown Norway offspring. A low IL-6 response towards Ova stimulation and a high CD11b/c+ proportion of splenocytes may be beneficial in the suppression of the Th2 dominant response in the Ova tolerized offspring compared to the placebo group. ARA+DHA supplementation of SPD and WD can respectively modulate *ex-vivo* IL-10 response to Ova and the IL-6 response to lymphocyte stimulant (PMAi), without affecting any major cell populations in splenocytes. This may support a better IL-2 response by lymphocytes in Ova tolerized offspring. Overall, ARA+DHA supplementation has an important role in suckling as well as weaning period diet on OT and immune system development in the Th2 dominant Brown Norway offspring (summarised in Table 5.7). Future studies focusing on the mechanisms behind the beneficial effects of ARA+DHA on oral tolerance development are required and are currently being explored by our group.

5.4.1. Suckling period ARA+DHA has a programming effect on the Th2 dominant immune system of 8-week Brown Norway offspring.

B cell antibody response is important in the prevention of food allergy, especially in Th2 dominant allergy prone condition during early infancy. The suckling period supplementation was associated with higher B cell subtype (OX12+) and IgG+ (only when followed by control WD), and lower co-inhibitory (CD152+CD8+) splenocytes irrespective of the WD supplementation. Although we did not observe changes in the *ex-vivo* cytokine production (to PMAi or Ova) by splenocytes, the plasma level of Ova-IgG1 was significantly lower in the supplemented group despite higher B cells as well as IgG+ cells in the spleen. A previous experiment with suckling period DHA supplementation had shown slightly lower OX12+ B cell proportion of splenocytes, however, weaning period DHA supplementation showed no changes in the B cell proportion in this Sprague Dawley model (Table 5.7.) (C. Richard et al., 2016). The B cells maturation process involves the differentiation of naïve B lymphocytes and the class-switching of their primary immunoglobulins such as IgM and IgA into secondary immunoglobulins such as IgG, IgE and IgD (Harwood & Batista, 2010). This indicates that ARA+DHA when provided during the suckling period is not only associated with higher mature B cell phenotype (Duffney et al., 2018) but also improved the OT response towards a common food antigen.

Table 5.7. Summary of main effects of SPD, WD and OT treatment observed in 8-week Brown Norway offspring and the comparison of SPD effects on OT between Brown Norway and Sprague Dawley rat model.

Summary of main effects	
Oral tolerance effect (Ova vs Sucrose)	**Suckling period diet effect (ARA+DHA vs control)**
↓ Ova-IgG1 and ↓ Ova-IgE levels in plasma ↓ IL-6 response to Ova stimulation ↑ IL-2 response to PMAi stimulation ↑ CD25+ in CD3+CD8+ splenocyte (only control SPD → control WD) ↑ CD11b/c+ splenocyte	↓ Ova-IgG1 levels in plasma ↓ IL-10 levels to unstimulated splenocytes ↓ IL-6 response to LPS stimulation ↑ Treg (only in the sucrose OT group) ↓ CD8+CD152+ & CD68+CD284+ in splenocytes ↑ OX12+, IgG+ & ↓ IgA+ (only in control WD)
Weaning diet effect (ARA+DHA vs control)	**Sex effect (females vs male offspring)**
↓ IL-6 response to PMAi (only in ARA+DHA SPD) ↑ TNF-α & IFN-γ response to PMAi ↑ IL-10 response to PMAi ↑ IL-6 response to Ova stimulation ↓ IL-10 response to Ova stimulation (only Ova OT group) ↓ IL-10 levels to unstimulated splenocytes (only in ARA+DHA SPD) ↑ OX6+ splenocytes (only in control SPD) ↑ CD45RA+ splenocytes (control SPD)	↑ TNF-α response to PMAi ↑ IL-10 response to PMAi ↓ CD25+ and OX6+CD25+ splenocytes ↑ CD45RA+ splenocytes
Comparisons of main outcomes between Brown Norway rats and Sprague Dawley rats	
Suckling period diet effects in Brown Norway rat (current findings)	**Suckling period diet effects in Sprague Dawley rat (Caroline Richard et al., 2016e, 2016f)**

↓ Ova-IgG1 but not Ova-IgE with ARA+DHA SPD in 8-week offspring compared to control SPD ↓ IL-10 with unstimulated splenocytes No change in IL-10 to PMAi/Ova stimulation with ARA+DHA SPD No change in inflammatory (LPS) response to adaptive (PMAi) response with suckling DHA ↑ Treg (only in the sucrose OT group)	↓ Ova-IgG1 & Ova-IgE with ARA+DHA SPD in 3-week and 6-week offspring than control SPD No change in IL-10 with unstimulated splenocytes ↑ IL-10 to ConA/Ova stimulation with ARA+DHA SPD than control SPD in 6-week offspring ↑ IL-10 & IFN-γ to LPS with ARA+DHA SPD ↑ IL-10/IFN to ConA with ARA+DHA SPD but only within control weaning group 6-week offspring
Oral Tolerance treatment effects in Brown Norway rat (current findings)	**Oral Tolerance treatment effects in Sprague Dawley rat (Caroline Richard et al., 2016e, 2016f)**
↓ Ova-IgG1 and ↓ Ova-IgE with Ova OT treatment in 8-week offspring ↑ IL-2 to PMAi with Ova OT group No change in IL-10 to PMAi with the Ova OT group No change in IL-10 to LPS with the Ova OT group ↓ IL-6 to Ova with Ova OT group IL-13 to Ova (not measured) No change in IL-10 & TGF-β to Ova at 8-week ↑ CD11b/c+ splenocytes with Ova OT group	No change in Ova-IgE or Ova-IgG with Ova OT treatment in 6-week offspring No change in IL-2 to ova with OT groups Less IL-10 to ConA with Ova OT group Less IL-10 to LPS with Ova OT group No change in IL-6 to ova with OT groups ↓ IL-13 to Ova with Ova OT group at 6-week No change in TGF-β to Ova with OT group No change in splenocyte phenotype

[1]Abbreviations: SPD, suckling period diet; WD, weaning diet; OT, oral tolerance; ARA, arachidonic acid; DHA, docosahexaenoic acid; Ova, Ovalbumin; Ova-Ig, Ova specific immunoglobulin; LPS, lipopolysaccharide, PMA, phorbol-myristate-acetate; ConA, Concanavalin A; CD, cluster of differentiation; Ig, immunoglobulin; IL, interleukin, IFN-γ, interferon-γ; TGF-β, Transforming growth factor-β; TNF-α, Tumor necrosis factor-α; Treg, T regulatory cell. Note: '↓' indicates a significantly lower change in comparison to control ($P < 0.05$); '↑' indicates a significantly higher change in comparison to control ($P < 0.05$).

IL-6 can act as a polarizing factor in promoting Th2 cell differentiation through IL-4-related pathways (Diehl & Rincón, 2002). It can also act as a suppressor of Th1 differentiation by inhibiting IFN-γ related activity (Detournay et al., 2005; Dodge et al., 2003). A lower IL-6 production with ARA+DHA SPD can act to suppress Th2 differentiation in Th2 dominant rodent model. The Brown Norway rat is known to have a dominant Th2 immune response, which is associated with higher Th2 cytokine response (IL-4, IL-5, IL-10 and IL-13) leading to heightened antibody production and suppression of Th1 cytokine (IFN-γ, TNF-α) production when exposed to a bacterial challenge such as LPS (Mills et al., 2000). Feeding ARA+DHA during the suckling period was associated with lower IL-6 response with LPS stimulation in the ARA+DHA SPD group compared to the control SPD group. This may be due to the observed lower proportion of activated macrophages (CD68+CD284+) in splenocytes of the ARA+DHA SPD group. LPS predominantly stimulate macrophages and other APCs to produce inflammatory cytokines, as well as to alter their ability to activate T cells. Having a lower IL-6 with no change in anti-inflammatory cytokines (IL-10 and TGF-β) may help to counteract the dominant Th2 profile of Brown Norway offspring as a lower IL-6 may be able to reduce the promotion of Th2 differentiation. Cell culture studies have reported anti-inflammatory effect of DHA on inflammatory cytokine to *ex-vivo* LPS stimulation by selectively lowering the proinflammatory cytokine IL-6 (Honda et al., 2015a; Titos et al., 2011). In previous experiments using Sprague Dawley rats(that have a lower Th2 dominant response(Renzi et al., 1996; Sirois & Bissonnette, 2001)), we reported an increased *ex-vivo* IL-6 production to LPS in high DHA fed 3-week-old offspring (Caroline Richard et al., 2016f). Therefore, DHA in a healthy rodent model may have been beneficial considering a higher inflammatory response to LPS is expected. However, in the context of Th2 dominant Brown Norway offspring, the immunoregulatory properties of DHA

through lower IL-6 can be beneficial for attaining Th1 skewed response in early infancy. Especially, when other cytokines (IL-1β, TGF-β, TNF-α, IL-10 and IFN-γ) were not affected by the SPD supplementation.

5.4.2. Weaning period ARA+DHA supplementation promotes lymphocyte function through TNF-α, IFN-γ and IL-10 in the Th2 dominant Brown Norway offspring.

A low level of Th1 cytokines such as TNF-α and IFN-γ and elevated Th2 cytokines have been reported in those with food allergies (Osterlund et al., 1999; Österlund & Suomalainen, 2002; Turcanu et al., 2003). Therefore, a dominant response to food allergens with Th2 cytokines is associated with food allergy whereas a Th1 skewed response is present when there is OT towards food antigen (Sampath & Nadeau, 2019; Turcanu et al., 2003). Clinical studies with fish oil supplementation have been shown to improve IFN-γ response (Lauritzen et al., 2005), which might be beneficial in preventing food allergies. In the current study, we reported that feeding ARA+DHA WD resulted in higher TNF-α and IFN-γ response (Figure 5.5 B) to *ex-vivo* lymphocyte stimulation (PMAi) compared to the control WD group, irrespective of SPD. This can be vital in countering the Th2 dominant immunity of Brown Norway offspring and could be beneficial in preventing food allergies. In previous experiments with Sprague Dawley rats (C. Richard et al., 2016), we have reported a similar increase in Th1 cytokines, TNF-α and IFN-γ. Particularly, feeding a high DHA diet during the suckling period resulted in high TNF-α and IFN-γ to Concanavalin A stimulation (non-specific polyclonal lymphocyte stimulant) (C. Richard et al., 2016). In addition, irrespective of SPD, ARA+DHA WD also resulted in a higher IL-10 response to a lymphocyte stimulant (Figure 5.5 C). A higher IL-10 may have resulted in the suppression of humoral response by B cells towards food antigen which is shown by significantly lower concentrations of plasma Ova-IgG1 and Ova-IgE in Ova tolerized group

compared to placebo exposed group. In the context of higher Th1 cytokines (TNF-α and IFN-γ) by lymphocytes, a higher IL-10 can indicate a better immune response in countering the Th2 dominant immunity of Brown Norway offspring. DHA has been shown to beneficially affect the B cell function through changes in lipid microdomain organization in the B cells plasma membrane (Gurzell et al., 2013). A study analyzing the effect of feeding n3 LCPUFA on B cell function from the spleen has shown upregulation of CD69 (early activation marker), MHC-II and CD11c after stimulation with LPS (Rockett et al., 2010). Similarly, the current study reported ARA+DHA WD resulted in a higher proportion of MHC-II (OX6+, Table 5.6.) and B cell (CD45RA+) in splenocytes of 8-week offspring when compared to the control WD group, however, only when they received ARA+DHA SPD. However, the IgG+ B cells, IgA+ B cells or B cell subtype (marked by OX12+) were not different between these diet groups. The role of DHA and n3 LCPUFA in B cell development and maturation needs to be further studied in the context of OT development in Th2 dominant model.

5.4.3. ARA+DHA in early infancy is beneficial for OT development by supporting a better IL-2 response and suppression of Th2 dominant response in Brown Norway offspring.

A higher proportion of CD11b/c+ splenocytes in Ova tolerized offspring through their role in the presentation of orally fed antigen to lymphocytes and suppressing Th17 differentiation can benefit the development of OT (Ehirchiou et al., 2007). Briefly, IL-6 together with TGF-β can promote Th17 cells. These cells produce IL-17 which is known to promote allergic inflammation by enhancing the Th2 response (Chen et al., 2019). However, Th17 can be suppressed by CD11b+ cells (Ehirchiou et al., 2007). Therefore, a lower IL-6 along with higher CD11b/c+ splenocytes may have played a mechanistic role in the suppression of *in-vivo* plasma

concentration of Ova-IgG1 and Ova-IgE that promoted the OT development (Chantal Kuhn et al., 2017).

The cell surface maker CD25, which is a receptor for IL-2 produced by lymphocytes, is highly expressed on Tregs. Therefore, IL-2 can act as a tolerogenic cytokine that controls the generation and survival of Tregs through CD25 (Abbas, 2020; Chinen et al., 2016). We reported a lower IL-10 response to Ova with ARA+DHA WD, which can potentially explain the better proliferation response (through higher IL-2) in tolerized offspring. Since IL-10 is an inhibitory cytokine, a lower IL-10 towards Ova may have promoted IL-2-related Tregs suppression in tolerized offspring. Previous studies using Sprague Dawley offspring showed a lower *ex-vivo* IL-10 response to Concanavalin A and LPS stimulation, but not to Ova stimulation, in Ova tolerized group compared to placebo-exposed controls (Table 5.7.) (Caroline Richard et al., 2016e). Although the development of OT towards Ova was reported, the *ex-vivo* cytokine (IL-2, IL-6, or TGF-β) response towards Ova stimulation showed no difference between tolerized and placebo control groups (Caroline Richard et al., 2016e). Additionally, a lower IL-6 to lymphocyte stimulant (PMAi) was reported when ARA+DHA was provided in both SPD and WD compared to when either SPD or WD was supplemented. IL-6 can be produced by lymphocytes and APCs with dual anti- and pro-inflammatory properties (Diehl & Rincón, 2002). A lower IL-6 by lymphocytes can also support the suppression of the Th2 dominant response through IL-4, as well as a lower inflammatory response in Brown Norway offspring. Although we reported a higher Treg proportion of splenocytes with ARA+DHA SPD, the tolerized offspring showed no significant difference in the proportion of Tregs due to SPD. Therefore, with the same proportion of Treg cells in ARA+DHA SPD and a lower IL-10 to Ova with ARA+DHA WD, the Ova tolerized offspring generated a better suppression response (Ova-IgG1 and Ova-IgE, Figure 5.4

A), this indicates an efficient response due to the ARA+DHA supplementation during early infancy. However, the multifunctional role of IL-10 needs to be further studied in the context of OT as it can suppress Th1 and Th2 response and promote regulatory response towards food antigen and our findings only partly explain its role in OT development (Ng et al., 2013).

There are limitations pertaining to the current study. As the study had several main effects (SPD, WD, OT and sex) and their interactions, we utilized multiple comparison Bonferroni adjustments to reduce false positives. As such adjustments are more conservative in estimating differences, some parameters that showed statistically significant main effects did not result in post-hoc differences between groups (for instance, SPD × OT treatment × sex interaction effect for IL-10 levels with Ova stimulation). Caution must be taken in interpreting these findings. Although we have reported the differences pertaining to the sex of the offspring, our study was not sufficiently powered to study the sex effect. Therefore, future studies focusing on the sex differences in early life supplementation are required. We have reported statistically significant differences in *ex-vivo* cytokine production and *in-vivo* antigen-specific Ig's using allergy-prone Brown Norway offspring, nonetheless, the biological relevance needs to be tested in infants prone to developing food allergy.

5.5. Conclusion

In summary, the suckling period ARA+DHA showed no lasting effect on the fatty acids composition of splenocytes. Yet, it showed a programming effect on OT development through IL-6 response to bacterial challenge and B cell function (lower plasma Ova-IgG1 levels) in 8-week offspring. Next, our findings also supported the importance of the weaning period (3 - 8 weeks) ARA+DHA supplementation in promoting a Th1 response (through TNF-α and IFN-γ) which can counter the Th2 dominant immune response of Brown Norway offspring. In the Ova

tolerized offspring, suppression of Th2 response is supported by low production of IL-6 and IL-10 to food antigen and a higher proportion of CD11b/c+ splenocytes associated with ARA+DHA supplementation. Overall, the importance of DHA supplementation alongside ARA is shown to be beneficially affecting the OT development by suppressing the Th2 dominant immune response of Brown Norway offspring and promoting Th1-related cytokines.

5.6. References

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Chapter 6. Maternal diet supplementation with high docosahexaenoic acid canola oil, along with arachidonic acid, promotes immune system development in allergy-prone BALB/c mouse offspring at 3-week of age[1]

6.1. Introduction

Immune system at birth is immature and early life diet plays an important role in its maturation (Calder et al., 2006a; Saavedra & Dattilo, 2017). Infants heavily rely on the innate immune cells (macrophages, dendritic cells (DC), natural killer (NK) cells and granulocytes) as well as humoral immunity during the breastfeeding period when the adaptive immune cells are still naïve (Basha et al., 2014; Kelly & Coutts, 2000). Suckling period in infants not only corresponds to the rapid expansion of adaptive immune cells, but also involves exposure to foreign antigens resulting in the development of antigen-specific acquired immunity that is more effective, and long-lasting (antigenic memory) (Drutman et al., 2012; Pérez-Cano et al., 2005; Pérez-Cano et al., 2007; Pérez-Cano et al., 2012). Infant immune system is also characterized by a dominant T helper type-2 (Th2) cytokines (interleukin (IL)-4, IL-5 and IL-13) response derived through passive transfer during pregnancy and lactation (Prescott, 2003; Wilson & Kollmann, 2008; Zaghouani et al., 2009). This is replaced by Th1 immune response (IL-2, interferon (IFN)-γ and tumour necrosis factor (TNF)-α) as infant's immune system becomes more adult-like (Raphael et al., 2015). However, infants susceptible to developing allergies or atopic disease fail to generate a robust Th1 immune response (Van Der Velden et al., 2001) which also makes them prone to bacterial and viral infections (Basha et al., 2014).

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Diet plays a vital role in the development of immune system. The immunomodulatory properties of the dietary long chain polyunsaturated fatty acid (LCPUFA) such as omega-3 (n-3) docosahexaenoic acid (DHA) and n-6 arachidonic acid (ARA) are well established (Calder, 2008; Kim et al., 2010). Anti-inflammatory and pro-inflammatory mediators synthesised from DHA and ARA, respectively, are known to influence the polarization and differentiation of adaptive immune cells (Field et al., 2001; L D Peterson et al., 1998). It is established that n-3 LCPUFA supplementation is beneficial for the prevention of allergies in neonates that are susceptible to developing allergies (Alm et al., 2009; Clausen et al., 2018; Furuhjelm et al., 2011a; Kull et al., 2006; Nagakura et al., 2000). Therefore, the supplementation of both DHA and ARA during early infancy is important for the optimal development of the immune system (Caroline Richard et al., 2016a). Maternal diet supplementation with ARA and DHA, increases the breastmilk concentration of ARA and DHA, and provides a rich source of LCPUFA for breastfeeding infants (van Goor et al., 2009). Previous studies have investigated the effect of DHA at high dosage, and in the absence of ARA, resulting in a dominant anti-inflammatory effect on immune cells (Kim et al., 2010; Stables & Gilroy, 2011). This maybe due to the reduction in the proportion of ARA, that is replaced by DHA, in cell membranes (Philip C. Calder, 2013a; Philip C. Calder et al., 2010). Therefore, dietary supplementation of DHA along with ARA that allow for an increase in DHA concentration of immune cells while maintaining the ARA concentration is considered desirable for the development of immune system (Caroline Richard et al., 2016c, 2016e; C. Richard et al., 2016). Few recent studies have shown the importance of combined ARA+DHA supplementation in suckling and weaning diets on the early development of immune system using Th2 dominant rodent model (Dhruvesh Patel et al., 2022; Patel et al., 2021; Weise et al., 2011a).

Therefore, the main objective of the current study is to determine the effect of dams' maternal diet supplementation with DHA+ARA (1% of total fat), and the same amount of ARA, on the immune system development of Th2 skewed BALB/c mouse offspring. The primary outcome was to study the *ex-vivo* cytokine production by splenocytes to different stimulation conditions. Additionally, immune cell phenotype and fatty acid concentration in spleen was investigated in the offspring after 3-week suckling period to support the observed differences in the primary outcome. We hypothesized that feeding dams with DHA and ARA will increase the breastmilk concentration of DHA resulting in higher DHA concentration of pups splenocytes. The high DHA levels in splenocytes will promote immune system development through increasing Th1 cytokine response in the 3-week offspring.

6.2. Material and method

6.2.1. Animal experimental protocol and diet

All the animal care and experimental protocols were conducted in accordance with the Canadian Council of Animal Care and approved by the University of Alberta Animal Ethics Committee (AUP00000125). Timed pregnant BALB/c mouse were obtained from Charles River Laboratories (Montreal, Quebec, Canada) on day 14 of gestation and were individually housed in temperature- and humidity-controlled environment with a 12/12 reverse light cycle. For acclimatization (48 hours), all the dams were fed standard chow purified diet (Lab diet 5001, PMI international, Brentwood, Montana, USA). Five days prior to parturition, dams were randomized to one of the two nutritionally complete experimental diets: DHA+ARA diet (1 % DHA and 1 % ARA w/w of total fat, n=10) or control diet (0 % DHA and 0 % ARA of total fat, n=10). Note, DHA concentration was selected to attain upper end of breastmilk DHA levels found in the human population (Brenna et al., 2007) and the concentration ARA was selected to

match DHA (1:1 ratio) based on the recommendation for infant formulas by the World Health Organization ("Standard for infant formula and formulas for special medical purposes intended for infants," 2007). Both experimental diets were isocaloric, isonitrogenous and nutritionally adequate. The fat mixture added (20 % w/w diet) to the semi-purified basal diet (described elsewhere (Lewis et al., 2016b)) was obtained by mixing lard, Mazola canola oil, Mazola corn oil, ARAsco (provided by DSM, Nutritional Products, Columbia, Maryland, USA) and Nuseed canola oil (provided by Nuseed, Calgary, Alberta, Canada). The experimental diets were matched to have similar n-6/n-3 fatty acid ratio and PUFA/SFA ratio (Table 1) to avoid confounding with the immunomodulatory effects of other fatty acids groups, besides DHA and ARA. Nonetheless, there were small difference in the dietary fat acid concentrations that may influence the outcome. Diets were prepared biweekly (stored at 4 °C) and food cups were changed every 2-3 days to limit oxidation.

The litters were cross fostered within the same diet group to attain similar number of pups and sex ratio. Dams were fed the experimental diets ad libitum throughout the 3-week suckling period, during which pups consumed their mother's breastmilk. Dietary intake and body weight were monitored regularly throughout the intervention. At the end of 3-week suckling period, all the dams and at least 2 pups (1 male and 1 female) from the same dams were euthanized using CO_2 asphyxiation. Dam's breastmilk (collected by scraping the mammary gland) and pup's stomach content were collected and snap-frozen in liquid nitrogen and stored at -80 °C until analysis. Spleen was collected aseptically, and immune cells were isolated (see below) for further analysis.

6.2.2. Splenocyte stimulation with mitogen and *ex-vivo* cytokine production measurement

Immune cells isolation protocol has been previously described (C. J. Field et al., 1990). Briefly, small cut spleen tissues were disrupted through a nylon mesh screen in sterile Kreb-Ringer HEPES buffer with bovine serum albumin (5 g/l) (Sigma-Aldrich Canada Ltd, Oakville, Ontario, Canada). Erythrocytes were lysed with ammonium chloride lysis buffer (155mM NH_4Cl, 0.1mM EDTA, and 10mM KHCO3; Fisher Scientific, Alberta, Canada) and cells were washed and resuspended in complete cell culture media (RPMI 1640 media with 5% v/v fetal calf serum, 2.5mM 2-mercaptoethanol, and 1% antibiotic / antimycotic, pH 7.4; Thermo Fisher Scientific, Mississauga, Ontario, Canada). Cells were counted on a hemocytometer using trypan blue dye (Sigma-Aldrich Canada Ltd.) exclusion and diluted to 1.25×10^6 cells/ml.

Ex-vivo cytokine production by stimulated immune cells was measured as described earlier (Blewett et al., 2009). Briefly, immune cells from the spleen were incubated in the presence or absence of phorbol-myristate-acetate and ionomycin (PMAi, 2 µl/ml, cell stimulation cocktail, Thermo Fisher Scientific) and lipopolysaccharide (LPS, 2 µl/ml, Thermo Fisher Scientific) for 48 h at 37 °C and 5 % CO_2. Note, although PMAi is known to predominantly stimulate lymphocytes but due to its non-specific nature, it can also stimulate NK cells and some macrophages. Cells were then centrifuged, and supernatant collected and stored at -80 °C. Commercial electrochemiluminescence kits (Proinflammatory Panel - 1, Meso Scale Discovery (MSD), Rockville, Maryland, USA) were used to quantify cytokines as per the manufacturer's instructions. Electrochemiluminescence was measured using MSD instrument (MESO QuickPlex SQ 120, MSD) and concentrations were calculated using a standard curve (Mesoscale Discovery 4.0 software, MSD). The range of detections are as follows: IFNγ, 0.0226 – 764 pg/ml; IL-10, 0.292 – 3540 pg/ml; IL-12, 4.95 - 32500 pg/ml; IL-1β, 0.0689 - 1530 pg/ml; IL-2, 0.204 - 2530 pg/ml; IL-4, 0.156 - 1790 pg/ml; IL-5, 0.0675 - 994 pg/ml; IL-6, 0.439 - 5240

pg/ml; CXCL-1 (keratinocyte chemoattractant/ growth-regulated oncogene, KC/GRO), 0.265 – 2020 pg/ml and TNFα, 0.04 - 639 pg/ml. All measurements were conducted in duplicate with a CV < 15%.`

Table 6.1. Fatty acid composition in total lipids of the experimental diets fed to BALB/c dams during the 3-week suckling period[1]

Fatty acid[2] (g/100 g of total fatty acid)	Control diet	DHA diet
C14:0	0.85±0.05	1.13±0.04
C16:0	15.96±0.13	21.2±1.01
C18:0	10.16±0.91	11.28±1.42
C20:0	0±0	0.53±0.03
C16:1	1.1±0.1	1.32±0.18
C18:1n-9 (oleic acid)	53.75±0.44	39.68±0.73
C18:2n-6 (LA)	14.46±0.34	17.63±0.9
C20:4n-6 (ARA)[3]	**0±0**	**1.02±0.02**
C18:3n-3 (ALA)	3.72±0.12	4.83±0.48
C20:4n-3	0±0	0.17±0.03
C20:5n-3 (EPA)	0±0	0.06±0.01
C22:5n-3 (DPA)	0±0	0.11±0.02
C22:6n-3 (DHA)	**0±0**	**1.04±0.02**
Total MUFA	54.85±0.54	41.00±0.91
Total PUFA	18.18±0.45	24.86±1.45
Total SFA	26.97±0.99	34.15±2.36
Ratio PUFA/SFA	0.68±0.04	0.73±0.09
Total n-6	14.46±0.34	18.65±0.88
Total n-3	3.72±0.12	6.20±0.57
Ratio n-6/n-3	3.89±0.03	3.02±0.13

[1] Fatty acid analysis of the diet was conducted using gas-liquid chromatography.
[2] Trace fatty acids are not presented as they were not relevant, this may result in a mismatch between the total fatty acids such as total SFA, MUFA, PUFA, n-6 and n-3.
[3] Differences in DHA and ARA are indicated in bold.
Data are presented as the mean ± SEM of 2 batches of diet mix.

6.2.3. Splenocyte phenotyping using flow cytometry.

The phenotype of immune cells from the spleen was assessed by direct immunofluorescence assay as previously described (Field et al., 2000). In brief, specific immune cells were identified using a combination of fluorophore-conjugated monoclonal antibodies (mAb) from the following panels: TGF-β/FoxP3/CD4/CD25/CD3, CD28/CD8α/CD86/CD3/CD25/CD4/ cytotoxic T-lymphocyte-associated protein 4 (CTLA-4 or CD152), CD8α /CD3 /CD4/C-X-C motif chemokine receptor 3 (CXCR3 or CD183)/C-C motif chemokine receptor-4 (CCR4 or CD194) /CCR6 (CD196), CD45R/CD138/CD19/CD23/IgG/IgM, CD4/CD11c/CD19/CD103/MHC-II (I-A^d/I-E^d specific to d haplotype)/CD86/CD8α, CD86/TLR-4(CD284)/CD68/MHC-II (I-A^d/I-E^d)/CD163/CD11b and CD27/CD45RB/CD4/CD49b/NKp46 (CD335)/CD3/CD8α. All the mAb were purchased from BD Biosciences (San Diego, California, USA) or BD Biosciences (Mississauga, Ontario, Canada). Briefly, immune cells ($0.2 – 0.5 \times 10^6$ per panel) were incubated for 30 min at 4 °C with cell surface mAb. For intracellular staining, after cell surface staining was completed, cells were fixed, and permeabilized using the fixation and permeabilization kit (eBiosciences, Thermo Fisher Scientific). Subsequently, cells were stained with FoxP3 mAb for 20 min and prepared for further analysis according to the manufacturer's protocol. Cells were then fixed and acquired within 72 h by flow cytometry (FACSCalibur; Becton Dickinson, San Diego, California, USA) according to the relative fluorescence intensity and analysed using FlowJo v10.8.1 software (Ashland, Oregon, USA).

6.2.4. Fatty acid concentration analysis in stomach content, breastmilk and splenocytes

The total lipids were extracted from the diet, stomach content, breastmilk (mammary gland tissue) and splenocytes using modified Folch protocol as described elsewhere (C. J. Field et al., 1988; Caroline Richard et al., 2016c). For isolating phospholipid, lipid extract from

splenocytes and breastmilk were scraped from the phospholipid band on the silica G plate and then the fatty acid methyl esters were formed. Followed by their separation using automated gas-liquid chromatography (Agilent Technologies, Mississauga, Ontario, Canada) on 100m CP-Sil 88 fused capillary column (Varian Instruments, Mississauga, Ontario, Canada) using flame-ionization detector method. The methylated fatty acids were quantified as relative percent of total fatty acids content (Cruz-Hernandez et al., 2004).

6.2.5. Statistical analysis

Data are reported as mean ± standard error of the mean (SEM). The study was powered to assess significant changes in the immune cell function as the primary outcome and lipid analysis (change in phospholipids concentration of breastmilk and spleen) as secondary outcomes. For dams, statistical analyses were conducted using unpaired Student *t* test. For the 3-week pups, data was analysed using 2-way analysis of variance by PROC MIXED procedure in SAS (v9.4, Cary, North Carolina, USA). The suckling diet and sex of the pups were considered the main effects. When interaction effect between suckling diet and sex was significant, LSMEANS procedure was used to conduct *post-hoc* comparison. Additionally, post-hoc analysis were adjusted using Bonferroni adjustment for multiple comparison. In the absence of significant interaction effect, an unpaired Student *t* test was used to compare mean ± SEM of diet groups. Dams were considered the experimental unit so the data from pups from the same dams were pooled together. Differences at $p \leq 0.05$ (2-sided) were considered significant.

6.3. Results

6.3.1. Growth parameters and food intake

There was no difference in dams' average food intake for week 1 and week 2, body weight and splenocyte count due to suckling diet (supplementary table 1). Only the average food intake of

dams in week-3 was higher in DHA+ARA than control group (14.96 ± 1.15 vs. 11.38 ± 0.75, p = 0.029), yet the bodyweight of dams at the end of suckling period was comparable. In 3-week pups, suckling diet had no significant effect on pups' bodyweight (13.5 ± 0.80 g), spleen weight (0.19 ± 0.01 g) and splenocyte count (20.31 ± 1.79 10^6 cells/mL).

Table 6.2. The effect of suckling diet on anthropometric measurements of BALB/c dams and 3-wk pups at the end of the suckling period

	Control (n = 12)	DHA+ARA (n = 10)	P value
Dams			
Bodyweight (g)	23.52 ± 0.47	23.17 ± 0.45	0.608
Splenocyte count (10^6 cells/mL)	15.58 ± 1.6	17.15 ± 1.65	0.297
Food intake (g/day):			
Week-1	6.44 ± 0.38	5.23 ± 0.79	0.163
Week-2	11.21 ± 0.98	11.26 ± 1.02	0.248
Week-3	11.38 ± 0.75	14.96 ± 1.15	**0.029**
3-wk pups			
Bodyweight (g)	13.95 ± 0.86	13.02 ± 0.77	0.270
Spleen weight (g)	0.18 ± 0.02	0.2 ± 0.01	0.580
Splenocyte count (10^6 cells/mL)	19.91 ± 1.27	20.72 ± 2.31	0.060

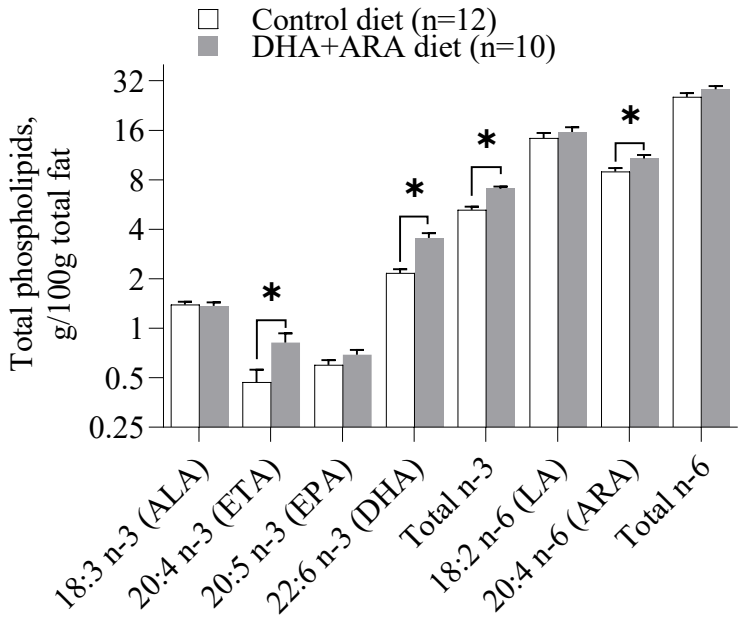

Figure 6.1. The effect of suckling diet on fatty acid concentration in the phospholipids of the breastmilk from BALB/c dams at the end of 3-week suckling period. Values are reported in mean ± SEM. * indicate significant difference ($p < 0.05$) between the control and DHA+ARA diet groups dams. ALA, a-linolenic acid; LA, linoleic acid; ARA, arachidonic acid; ETA,

eicosatetraenoic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; n-3, omega-3

6.3.2. Fatty acid composition of total lipids in stomach content, and phospholipids in milk from mammary gland and splenocytes

In the DHA+ARA group, the fatty acid concentration of the phospholipids from dams' milk (mammary gland) resulted in significantly higher levels of DHA, eicosatetraenoic acid and total n-3, whereas the n-3 precursor ALA was not different when compared to control group (Figure 1). The DHA+ARA group showed significantly higher level of ARA compared to control, however, LA and total n-6 were not different (Figure 1). Furthermore, a significantly higher total SFA in dams' milk phospholipids was balanced with lower total MUFA, whereas the total PUFA showed no significant difference when DHA+ARA group was compared with control (supplementary table 2). The n-6/n-3 ratio was found to be significantly lower in DHA+ARA group than control (4.03±0.16 vs. 4.79±0.11; $p < 0.001$). Additionally, dams' splenocyte phospholipid concentration of fatty acids is described in supplementary table 3 for reader's benefit but not described here.

In the DHA+ARA group, the fatty acid concentration of the stomach content from 3-week pups (presented in supplementary table 4) is representative of breastmilk from their respective dams. In the total lipid concentration of stomach content, DHA+ARA group pups showed significantly higher levels of DHA, ETA, and total n-3, whereas the n-3 precursor ALA was not different, compared to control. DHA+ARA group also resulted in higher proportion of ARA levels and total n-6 fatty acids, with no differences in n-6 precursor LA. The higher proportion of total PUFA and SFA in DHA+ARA group was balanced with significantly lower proportion of total

MUFA. However, the suckling diet resulted in no changes in the ratio of total PUFA/SFA or the ratio of total n-6/n-3 in total lipids of stomach content (supplementary table 4).

In 3-week pups, the splenocyte phospholipids concentration showed 60% higher DHA levels (5.76 ± 0.21 vs 3.54 ± 0.19, $p < 0.001$) as well as significantly higher total n-3 in DHA+ARA group compared to the control, although ALA proportion was significantly lower in DHA+ARA group (Figure 2). The proportion of LA (6.26 ± 0.19 vs. 5.72 ± 0.1), ARA (12.31 ± 0.49 vs. 13.86 ± 0.28) and total n-6 (21.52 ± 0.33 vs. 22.6 ± 0.29) showed no differences due to suckling diet. However, the ratio of n-6/n-3 was significantly lower in DHA+ARA group pups than control. The total MUFA (16.07 ± 0.14 vs 19.85 ± 0.29, $p < 0.001$), which includes C16:1, C18:1 and C24:1n-9, was significantly lower in DHA+ARA group than control group pups' splenocyte phospholipid concentration. The suckling diet showed no effect on the ratio of PUFA/SFA. The sex of pups showed no significant effect on the fatty acid concentration in splenocyte phospholipids.

Table 6.3. The effect of the suckling diet on fatty acid composition in the phospholipids of the mammary gland milk from BALB/c dams at the end of the 3-week suckling period[1]

Fatty acid (g/100 g of total fatty acid)[1]	Control (n=12)	DHA+ARA (n=10)	*p* value
C14:0	0.75 ± 0.09	0.75 ± 0.07	0.943
C16:0	19.22 ± 0.67	19.5 ± 1.07	0.356
C18:0	21.36 ± 0.6	23.61 ± 1.13	0.096
C20:0	0.1 ± 0.01	0.07 ± 0.01	0.244
C24:0	0.78 ± 0.07	0.61 ± 0.04	0.135
C16:1	1.46 ± 0.17	1.16 ± 0.16	0.296
C18:1	25.2±1.2	18.44±1.37	**0.016**
C24:1 n-9	0.58 ± 0.04	0.41 ± 0.04	**0.015**
C18:2 n-6 (LA)	14.37 ± 1.03	15.61 ± 1.06	0.680
C18:3 n-6	0.22 ± 0.03	0.19 ± 0.02	0.840
C20:2 n-6	0.41 ± 0.03	0.45 ± 0.03	0.740
C20:3 n-6	1.22 ± 0.06	1.25 ± 0.06	0.786
C20:4 n-6 (ARA)	8.98 ± 0.45	10.79 ± 0.51	**0.024**
C22:5 n-6	0.04 ± 0	0.05 ± 0	0.155
C18:3 n-3 (ALA)	1.39 ± 0.06	1.37 ± 0.07	0.290
C20:4 n-3	0.47 ± 0.09	0.82 ± 0.13	**0.025**
C20:5 n-3 (EPA)	0.6 ± 0.04	0.69 ± 0.05	0.193
C22:5 n-3 (DPA)	0.61 ± 0.03	0.6 ± 0.06	0.886
C22:6 n-3 (DHA)	2.17 ± 0.12	3.54 ± 0.25	**<0.001**
Total SFA[2]	42.23 ± 0.55	44.58 ± 0.46	**<0.001**
Total MUFA	27.24 ± 1.36	19.93 ± 1.56	**0.001**
Total PUFA	30.52 ± 1.53	33.98 ± 2.12	0.425
Total n-6	25.26 ± 1.32	28.36 ± 1.36	0.160
Total n-3	5.26 ± 0.23	7.05 ± 0.24	**<0.001**
Ratio PUFA/SFA	0.72 ± 0.02	0.76 ± 0.02	**0.020**
Ratio n-6/n-3	4.79 ± 0.11	4.03 ± 0.16	**<0.001**

[1] Fatty acid analysis was conducted using gas-liquid chromatography.

[2] Trace fatty acids are not presented as they were not relevant, this may result in a mismatch between the total fatty acids such as total SFA, MUFA, PUFA, n-6 and n-3.

Values are presented as the mean ± SEM.

Abbreviation, ALA, a-linolenic acid; LA, linoleic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; n-3, omega-3, PUFA, polyunsaturated fatty acid; MUFA, monounsaturated fatty acid; SFA, saturated fatty acid.

Table 6.4. The effect of the suckling diet on fatty acid composition in the phospholipids of the splenocytes from BALB/c dams at the end of 3-week suckling period[1]

Fatty acid (g/100g of total fatty acid)[1]	Control (n=12)	DHA+ARA (n=10)	*p* value
C14:0	0.32 ± 0.01	0.38 ± 0.04	0.100
C16:0	27.36 ± 0.2	28.59 ± 0.35	**0.008**
C18:0	25.21 ± 0.46	27.27 ± 0.77	**<0.001**
C20:0	0.18 ± 0	0.2 ± 0.02	0.344
C24:0	1.27 ± 0.08	1.01 ± 0.07	**0.039**
C16:1	1.11 ± 0.02	1.06 ± 0.05	0.188
C18:1	12.72±0.27	9.76±0.18	**<0.001**
C24:1n-9	1.75 ± 0.06	1.31 ± 0.08	**<0.001**
C18:2n-6 (LA)	6.4 ± 0.11	5.91 ± 0.24	0.123
C18:3n-6	0.43 ± 0.02	0.47 ± 0.03	0.335
C20:2n-6	0.66 ± 0	0.62 ± 0.02	**0.034**
C20:3n-6	1.44 ± 0.06	1.55 ± 0.09	0.321
C20:4n-6 (ARA)	15.09 ± 0.22	14.89 ± 0.41	0.275
C22:5n-6	0.18 ± 0.01	0.2 ± 0.02	0.518
C18:3n-3 (ALA)	0.78 ± 0.01	0.57 ± 0.04	**<0.001**
C20:4n-3	0.23 ± 0.02	0.35 ± 0.06	0.073
C20:5n-3 (EPA)	0.51 ± 0.03	0.59 ± 0.05	0.372
C22:5n-3 (DPA)	1.19 ± 0.06	1.12 ± 0.1	**0.037**
C22:6n-3 (DHA)	3.08 ± 0.08	4.06 ± 0.24	**<0.001**
Total SFA[2]	54.36 ± 0.42	57.47 ± 0.78	**<0.001**
Total MUFA	15.6 ± 0.32	12.14 ± 0.23	**<0.001**
Total PUFA	30.03 ± 0.3	30.38 ± 0.61	0.777
Total n-6	24.22 ± 0.2	23.66 ± 0.38	0.171
Total n-3	5.8 ± 0.12	6.71 ± 0.35	**0.018**
Ratio PUFA/SFA	0.55 ± 0.01	0.53 ± 0.02	**0.054**
Ratio n-6/n-3	4.19 ± 0.07	3.57 ± 0.15	**0.001**

[1] Fatty acid analysis was conducted using gas-liquid chromatography.

[2] Trace fatty acids are not presented as they were not relevant, this may result in a mismatch between the total fatty acids such as total SFA, MUFA, PUFA, n-6 and n-3.

Values are presented as the mean ± SEM.

Abbreviation, ALA, a-linolenic acid; LA, linoleic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; n-3, omega-3, PUFA, polyunsaturated fatty acid; MUFA, monounsaturated fatty acid; SFA, saturated fatty acid.

Table 6.5. The effect of the suckling diet on fatty acid composition in the total lipids of the stomach content from BALB/c pups at the end of 3-week suckling period[1]

Fatty acid (g/100 g of total fatty acid)	Control (n=8)	DHA+ARA (n=8)	*p* value[2]
C14:0	1.61 ± 0.47	3.45 ± 0.87	**0.001**
C16:0	16.04 ± 0.86	22.45 ± 1.32	**<0.001**
C16:1	1.21 ± 0.03	1.55 ± 0.06	**<0.001**
C18:0	9.76 ± 0.54	10.5 ± 0.41	0.085
C20:0	0.04 ± 0.02	0.07 ± 0.02	0.555
C24:0	0.15 ± 0.01	0.12 ± 0.01	0.149
C18:1	47.01±0.89	33.95±0.80	**0.010**
C24:1n-9	0.05 ± 0.01	0.16 ± 0.02	**0.003**
C18:2n-6 (LA)	14.33 ± 0.45	14.91 ± 0.57	0.073
C18:3n-6	1 ± 0.15	0.9 ± 0.11	0.864
C20:2n-6	0.37 ± 0.03	0.56 ± 0.03	**0.013**
C20:3n-6	0.57 ± 0.07	0.66 ± 0.05	0.298
C20:4n-6 (ARA)	0.31 ± 0.05	1.64 ± 0.24	**<0.001**
C22:5n-6	0.02 ± 0	0.02 ± 0	0.055
C18:3n-3 (ALA)	6.83 ± 0.48	6.64 ± 0.54	0.617
C20:4n-3 (ETA)	0.05 ± 0.01	0.19 ± 0.02	**<0.001**
C20:5n-3 (EPA)	0.33 ± 0.05	0.35 ± 0.05	0.951
C22:5n-3 (DPA)	0.13 ± 0.02	0.33 ± 0.03	**<0.001**
C22:6n-3 (DHA)	0.1 ± 0.01	1.46 ± 0.07	**<0.001**
Total MUFA[3]	48.27 ± 0.86	35.66 ± 0.81	**<0.001**
Total PUFA	24.1 ± 0.86	27.71 ± 1.19	**0.002**
Total SFA	27.61 ± 1.17	36.62 ± 1.9	**<0.001**
Ratio PUFA/SFA	1.19 ± 0.11	1.45 ± 0.18	0.586
Total n-6	16.63 ± 0.43	18.72 ± 0.72	**0.003**
Total n-3	7.46 ± 0.55	8.98 ± 0.55	**0.011**
Ratio n-6/n-3	2.33 ± 0.11	2.15 ± 0.08	0.109

[1] Fatty acid analysis of stomach content was conducted using gas-liquid chromatography.

[2] *P* values for the suckling diet effect on 3-week pups' outcome

[3] Trace fatty acids are not presented as they were not relevant, this may result in a mismatch between the total fatty acids such as total SFA, MUFA, PUFA, n-6 and n-3.

Data are presented as the mean ± SEM.

Abbreviation, ALA, a-linolenic acid; LA, linoleic acid; ARA, arachidonic acid; ETA, eicosatetraenoic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; n-3, omega-3, PUFA, polyunsaturated fatty acid; MUFA, monounsaturated fatty acid; SFA, saturated fatty acid.

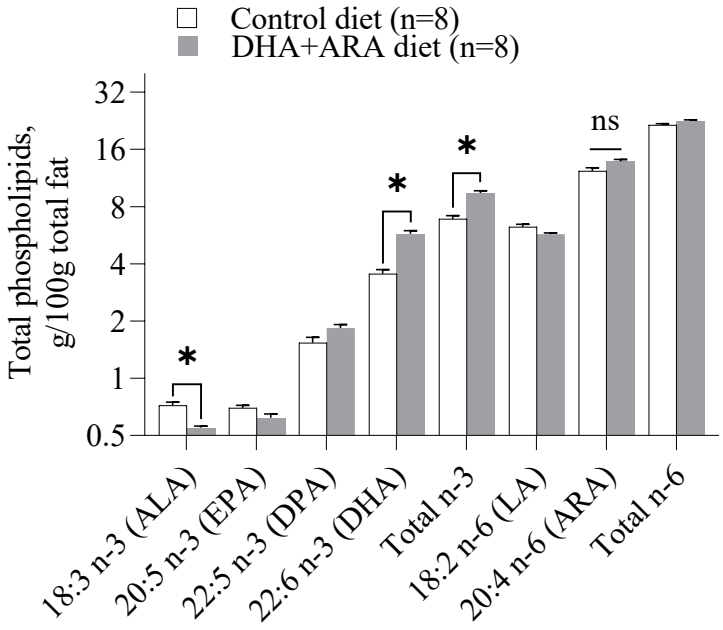

Figure 6.2. The effect of suckling diet and sex on fatty acid composition in the phospholipids of the splenocytes from BALB/c pups at the end of the 3-week suckling period. Values are reported in mean ± SEM. * indicate a significant difference ($P < 0.05$) between the control and DHA diet group. ALA, a-linolenic acid; LA, linoleic acid; ARA, arachidonic acid; ETA, eicosatetraenoic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; n-3, omega-3

6.3.3. *Ex vivo* cytokine responses to mitogens by splenocytes

In dams' splenocytes, *ex-vivo* cytokines levels (IFN-γ, IL-10, IL-12, IL-1β, IL-2, IL-4, IL-5, CXCL1 and TNF-α) at baseline (with no stimulation) did not differ significantly between suckling diet groups (supplementary table 5). The DHA+ARA group dams showed lower IL-6 levels in unstimulated splenocytes than control. The splenocytes stimulated with PMAi (predominantly stimulating lymphocytes in non-specific manner and some NK cells) showed no differences between diet groups. However, splenocytes stimulated with LPS (a component of gram-negative bacterial cell wall that triggers TLR4 stimulating innate immune cells) elicited 30% lower IL-10 in DHA+ARA-fed dams versus control, with no differences in other anti-

inflammatory cytokine (IL-4) or inflammatory cytokines (IL-1β, IFN- γ, TNF-α, CXCL1, IL-2, IL-12 and IL-5) (supplementary table 5).

In the 3-week pups, baseline levels of IL-2 and CXCL1 from unstimulated splenocyte was significantly higher in DHA+ARA group than control, however, there was no difference in IFN-γ, IL-10, IL-1β, IL-4, IL-5, IL-6 and TNF-α levels (Table 2). In the PMAi stimulated splenocytes, *ex-vivo* CXCL1 production was higher in DHA+ARA group pups compared to controls (Table 2). Additionally, a significant interaction suckling diet × sex effect showed that IL-1β production by PMAi stimulated splenocytes was higher in DHA+ARA group than control (0.80 ± 0.09 vs 0.58 ± 0.08; $p < 0.001$) but only in female pups. Next, pups from DHA+ARA group showed significantly higher production of inflammatory cytokines (IFN-γ, IL-1β and TNF-α) by LPS stimulated splenocytes than control group pups (Table 2). However, the production of IL-10, IL-2, IL-4, IL-5, IL-6 and CXCL1 by LPS stimulated splenocytes showed no difference between the suckling diet group pups. Although, LPS stimulated IL-5 production showed a significant suckling diet and sex interaction, no differences were observed in the post-hoc analysis.

Table 6.6. The Effect of suckling period diet on *ex-vivo* cytokine production by mitogen-stimulated splenocytes from BALB/c dams at the end of a 3-week suckling period.

Cytokine (pg/ml)	Control (n = 12)	DHA+ARA (n = 10)	P value[1]
Unstimulated			
IFN-γ	2.73 ± 0.37	3.54 ± 0.37	0.250
IL-10	17.58 ± 1.39	14.87 ± 1	0.139
IL-12	ND	ND	-
IL-1β	1.77 ± 0.16	1.47 ± 0.18	0.214
IL-2	39.43 ± 4.24	47.78 ± 5.31	0.272
IL-4	9.55 ± 1.73	8.75 ± 0.91	0.467
IL-5	1.31 ± 0.15	1.44 ± 0.29	0.840
IL-6	296.54 ± 29.95	249.19 ± 29.2	**0.045**
CXCL1	89.79 ± 18.21	63.91 ± 17.19	0.181
TNF-α	156.44 ± 57.27	83.7 ± 21.37	0.332
PMAi (lymphocyte stimulant)			
IFN-γ	34.24 ± 16.78	25.7 ± 10.09	0.681
IL-10	90.14 ± 31.9	56.89 ± 22.04	0.626
IL-12	ND	ND	-
IL-1β	1.99 ± 0.2	1.74 ± 0.11	0.425
IL-2	852.26 ± 343.71	1021.08 ± 484.61	0.792
IL-4	75.25 ± 29.23	56.38 ± 26.67	0.659
IL-5	23.67 ± 8.61	24.12 ± 11.66	0.905
IL-6	365.39 ± 77.9	245.24 ± 40.93	0.485
CXCL1	97.92 ± 24	73.63 ± 13.19	0.773
TNF-α	27.98 ± 4.18	26.57 ± 3.37	0.054
LPS (bacterial challenge)			
IFN-γ	518.31 ± 126.32	460.26 ± 109.35	0.914
IL-10	626.31 ± 49.04	434.71 ± 20.86	**0.001**
IL-12	16.08 ± 1.85	15.14 ± 2.47	0.791
IL-1β	62.36 ± 7.57	45.47 ± 8.36	0.152
IL-2	14.3 ± 1.75	14.63 ± 1.46	0.748
IL-4	6.93 ± 2.2	4.22 ± 2.24	0.218
IL-5	2.34 ± 0.26	2.34 ± 0.16	0.952
IL-6	2066.47 ± 190.52	2077.7 ± 177.5	0.401
CXCL1	873.57 ± 67.19	968.4 ± 79.37	0.383
TNF-α	702.08 ± 54.83	737.78 ± 94.13	0.264

[1] P values for the suckling diet effect on dams' outcome. Values are presented as the mean ± SEM. Significant differences are in bold. PMAi, phorbol myristate acetate + ionomycin; LPS,

lipopolysaccharide; IFN interferon, IL interleukin, TNF tumour necrosis factor, CXCL1, C-X-C motif chemokine ligand 1, LPS lipopolysaccharide

Table 6.7. The Effect of suckling period diet and sex on *ex-vivo* cytokine production by mitogen-stimulated splenocytes from BALB/c pups at the end of 3-week suckling period.

Cytokine (pg/ml)	**Control (*n* = 8)**	**DHA+ARA (*n* = 8)**	**SD *P*** value[1]	**Sex *P*** value[2]	**SD*sex *P*** value[3]
Unstimulated					
IFN-γ	0.42 ± 0.09	0.54 ± 0.15	0.080	**0.045**	0.235
IL-10	11.3 ± 1.11	13.13 ± 0.83	0.203	0.743	0.149
IL-1β	0.67 ± 0.06	0.58 ± 0.04	0.137	0.757	0.359
IL-2	9.79 ± 1.03	13.13 ± 1.07	**0.004**	0.339	0.953
IL-4	1.65 ± 0.27	1.42 ± 0.12	0.840	0.832	0.496
IL-5	0.25 ± 0.11	0.42 ± 0.12	0.340	0.785	0.105
IL-6	139.18 ± 18.88	153.19 ± 22.16	0.272	0.350	0.182
CXCL1	36.07 ± 6.62	53.51 ± 8.92	**0.007**	0.511	0.122
TNF-α	40.63 ± 8.46	30.97 ± 7.76	0.429	0.715	0.102
PMAi					
IFN-γ	3.88 ± 1.32	2.42 ± 0.8	0.076	0.622	0.621
IL-10	28.49 ± 3.97	28.04 ± 5.76	0.155	0.443	0.506
IL-1β	0.69 ± 0.06	0.63 ± 0.05	0.239	0.975	**<0.001**
IL-2	282.52 ± 83.36	298.93 ± 148.55	0.141	0.724	0.702
IL-4	11.83 ± 3	4.94 ± 1.64	0.911	0.303	0.251
IL-5	2.26 ± 0.67	1.59 ± 0.44	0.174	0.501	0.368
IL-6	216.64 ± 30.3	209.23 ± 39.09	0.362	0.785	0.333
CXCL1	47.83 ± 7.64	71.17 ± 15.29	**0.005**	0.967	0.325
TNF-α	17.46 ± 4.78	15.98 ± 2.84	0.888	0.891	0.304
LPS					
IFN-γ	135.83 ± 22.81	153.05 ± 42.54	**<0.001**	0.094	0.438
IL-10	727.31 ± 58.47	735.26 ± 46.15	0.829	0.507	0.625
IL-1β	51.68 ± 6.46	60.81 ± 8.93	**0.019**	0.936	0.443
IL-2	10.09 ± 0.74	11.81 ± 1.44	0.433	0.406	0.824
IL-4	6.79 ± 1.28	5.35 ± 1.38	0.401	0.812	0.224
IL-5	1.3 ± 0.12	1.48 ± 0.1	0.267	0.565	**0.037**
IL-6	3221.44 ± 489.88	3167.73 ± 420.03	0.215	0.260	0.872
CXCL1	1135.56 ± 92.24	1079.41 ± 72.76	0.769	0.285	0.365
TNF-α	1002.34 ± 77.74	1058.13 ± 107.64	**0.027**	0.982	0.230

[1] *P* values for the suckling diet's main effect in the mixed model on 3-week pups' outcome

[2] *P* values for the sex main effect in the mixed model on 3-week pups' outcome

[3] *P* values for the suckling diet and sex main effect interaction in the mixed model on 3-week pups' outcome. Values are reported in mean ± SEM. Significant differences are in bold. PMAi, phorbol myristate acetate + ionomycin; LPS, lipopolysaccharide; IFN interferon, IL interleukin, TNF tumour necrosis factor, CXCL1, C-X-C motif chemokine ligand 1, LPS lipopolysaccharide

6.3.4. Immune cell phenotype of dams' splenocytes

All the immune cell phenotypes analysed for the dams splenocytes are described in supplementary table 6; however, only relevant immune cell phenotype differences are described here. Splenocytes from dams in the DHA+ARA group showed significantly lower proportion of CD25 marker (involved in proliferation and activation of T cells and Treg) and CD86 marker on T cells (including Th (CD3+ CD4+) and cytotoxic T cells (CTL, CD3+ CD8+)) than control group. Note, CD86 is classically expressed on APC and its role in T cells is not fully established but implicated in survival of activated cells (Mukherjee et al., 2002)). However, CD27 marker (involved in the memory function and survival of activated T cells (Borst et al., 2005)) on T cells (Th and CTL) were significantly higher in DHA+ARA group dams than control. Additionally, DHA+ARA diet resulted in higher proportion of Th cells with CXCR3+CCR6- phenotype (enriched for Th1) than in control. Although, the DHA+ARA group showed a lower proportion of B cells (CD19+) in splenocytes, the IgG+ proportion of B cells (CD19+) was higher than control group. Additionally, the proportion of innate immune cells, such as CD3-CD49b+ (NK cells), CD11c+ (DCs and macrophages), CD11b+ CD68+(macrophages), in splenocytes was significantly lower in DHA+ARA group than control.

Table 6.8. Effect of suckling diet on the phenotype of splenocytes from BALB/c dams at the end suckling period

% of gated splenocytes[1]	Control (n = 12)	DHA+ARA (n = 8)	P value[2]
Total CD3+	29.39 ± 0.65	27.35 ± 1.73	0.390
CD4+	54.6 ± 1.55	56.88 ± 1.45	**0.028**
CD8+	36 ± 0.69	37.33 ± 1.68	0.986
CD25+	14.93 ± 1.15	11.88 ± 0.89	**0.024**
CD27+	69.62 ± 1.87	76.51 ± 1.01	**<0.001**
CD28+	86.56 ± 0.77	87 ± 1.39	0.735
CD86+	65.1 ± 0.91	55.36 ± 3.46	**0.002**
CD152+	15.21 ± 1.7	23.57 ± 4.91	0.375
CD45RB+	72.43 ± 1.97	79.65 ± 1.43	**0.006**
% of cytotoxic T cells (CD3+ CD8+):			
CD25+	9.73 ± 0.76	7.45 ± 0.43	**0.011**
CD27+	88.6 ± 1.6	94.02 ± 0.76	**<0.001**
CD28+	88.5 ± 0.77	91.01 ± 0.62	**0.033**
CD86+	72.53 ± 1.69	59.28 ± 6.59	0.054
CD152+	12.33 ± 1.74	25.52 ± 7.44	0.092
% of Th cells (CD3+ CD4+):			
CD25+	17.94 ± 0.65	13.07 ± 0.83	**<0.001**
CD27+	82.69 ± 1.02	88.22 ± 0.66	**<0.001**
CD28+	95.55 ± 0.38	96.73 ± 0.23	0.060
CD86+	57.02 ± 0.69	44.48 ± 3.18	**<0.001**
CD152+	14.1 ± 1.14	22.68 ± 6.44	0.129
CD183+ CD196- (enriched for Th1)	10.39 ± 0.74	16.91 ± 2.24	**0.005**
CD194+ CD196+ (enriched for Th17)	9.26 ± 0.67	5.69 ± 0.63	**<0.001**
CD194+ CD196- (enriched for Th2)	8.34 ± 1.52	7.19 ± 1.11	0.585
CD25+ FoxP3+ (enriched for Treg)	10.04 ± 0.34	8.76 ± 0.45	**0.006**
CD19+ (Total B cells)	49.54 ± 0.96	44.88 ± 1.51	**0.019**
% B cells (CD19+):			
CD45R- (enriched for B1 cells)	32.43 ± 7.79	29.06 ± 6.24	0.760
CD45R+ (enriched for B2 cells)	77.45 ± 2.02	75.25 ± 2.44	0.830
CD45R+ CD23- (marginal B2 cells)	12.25 ± 0.74	8.95 ± 0.52	**0.004**
CD45R+ CD23+ (follicular B2 cells)	58.49 ± 6.61	61.75 ± 6.39	**0.023**
IgM+	59.21 ± 2.64	56.35 ± 3.27	0.827
IgG+	69.7 ± 3.05	78.31 ± 2.44	**0.020**
Total CD45R+ (peripheral B cells)	40.52 ± 4.62	40.12 ± 3.42	0.082
% Peripheral B cells (CD45R+)			
CD19- CD138+ (plasma cells)	3.20 ± 0.83	3.17 ± 0.79	0.895
CD19- CD138+ IgM+ (plasma IgM cells)	1.12 ± 0.27	1.19 ± 0.3	0.265
CD19- CD138+ IgG+ (plasma IgG cells)	1.63 ± 0.43	1.31 ± 0.35	**0.010**
CD3- CD49b+ (NK cells)	14.76 ± 1.18	10.28 ± 0.72	**<0.001**

% of gated splenocytes[1]	Control (n = 12)	DHA+ARA (n = 8)	P value[2]
% NK cells (CD3- CD49b+):			
CD27+	23.85 ± 0.87	22.78 ± 1.57	0.935
CD335+	39.1 ± 3.27	36.22 ± 2.72	0.277
CD3+ CD49b+ (NKT cells)	4.28 ± 0.49	3.24 ± 0.32	0.147
% NKT cells (CD3+ CD49b+):			
CD27+	67.47 ± 1.01	70.32 ± 0.94	0.052
CD335+	15.05 ± 3.15	9.01 ± 1.45	**<0.001**
Total CD11c+ (DC)	13.18 ± 0.59	10.74 ± 0.53	**<0.001**
CD11c+ CD4- CD8+ (enriched for cDC1)	12.4 ± 0.96	11.51 ± 1.24	0.251
CD11c+ CD8- (enriched for cDC2)	8.34 ± 0.81	7.63 ± 0.74	**0.016**
CD11b+ CD68+ (Total macrophages)	4.37 ± 0.46	3.32 ± 0.32	**<0.001**
CD11b+ CD68+ CD86+ (enriched for M1 phenotype)	4.02 ± 0.43	2.94 ± 0.30	**<0.001**
CD11b+ CD68+ CD163+ (M2 phenotype)	1.24 ± 0.2	0.64 ± 0.08	**0.001**

[1] Values are in percent of total gated splenocytes, except when reported otherwise (using italics).

[2] P values for the suckling diet effect on dams' outcome

Values are reported in mean ± SEM.

Significant differences are in bold.

Abbreviations, SD, suckling diet; CD, cluster of differentiation; DHA, docosahexaenoic acid; Th, T helper cells; NK, natural killer; NKT, natural killer T cells; DC, dendritic cells, cDC, conventional dendritic cells.

6.3.5. Immune cell phenotype of 3-week pups' splenocytes

Adaptive immune cells: In 3-week pups, the percent of total CD3+ T cells in splenocytes was not different between DHA+ARA and control group. However, the percent of CD3+CD4+ Th cells was significantly lower in DHA+ARA group pups than control. The proportion of CD25 marker on Th cells (CD3+ CD4+) as well as CTL (CD3+ CD8+) with was significantly lower in DHA+ARA than control group pups. The proportion of CD28+ on Th cells was significantly lower in DHA+ARA groups than control. However, CD27+ CTL was significantly higher in DHA+ARA group than control. The proportion of CD86+ T cells (Th and CTL) showed significant suckling diet × sex interaction, where female was lower than male pups but only from DHA+ARA group (Figure 3A). The proportion of CTLA-4+ T cells also showed a significant interaction effect and the post-hoc analysis showed that the female were higher than male but only from DHA+ARA groups (Figure 3B). Similarly, a significant interaction effect on the proportion of Th cells with CCR4+ CCR6+ (enriched for Th17, gating described in Figure 3C) indicated female from DHA+ARA had higher proportion compared to male from DHA+ARA and females from control group. A significant sex effect showed that the fraction of CD45RB+ CD3+ splenocytes (naïve T cells) were lower in female than male pups irrespective of their suckling diet. In terms of B cells, the DHA+ARA group pups had significantly higher proportion of CD19+ (total B cells) and IgG+ B cells in splenocytes than control. Additionally, sex had no significant effect on the proportion of B cell phenotype in splenocytes.

Innate immune cells: A significant sex effect was found in the proportion of total CD11c+ cells, which were higher in females vs. males (8.5±1.27 vs. 6.14 ± 0.82; $p = 0.019$, Figure 3D). The splenocyte proportion of CD11b+ CD68+ (macrophages) and CD86+ macrophages (enriched for M1 phenotype) were significantly higher in DHA+ARA group pups than in control (Figure 4).

The suckling diet did not affect the proportion of CD3- CD49b+ (NK cells) and CD11c+ (present mostly on dendritic cells and some macrophages) in 3-week pups' splenocytes. Table 3 presents suckling diet effects on specific subsets of immune cell phenotypes of splenocytes.

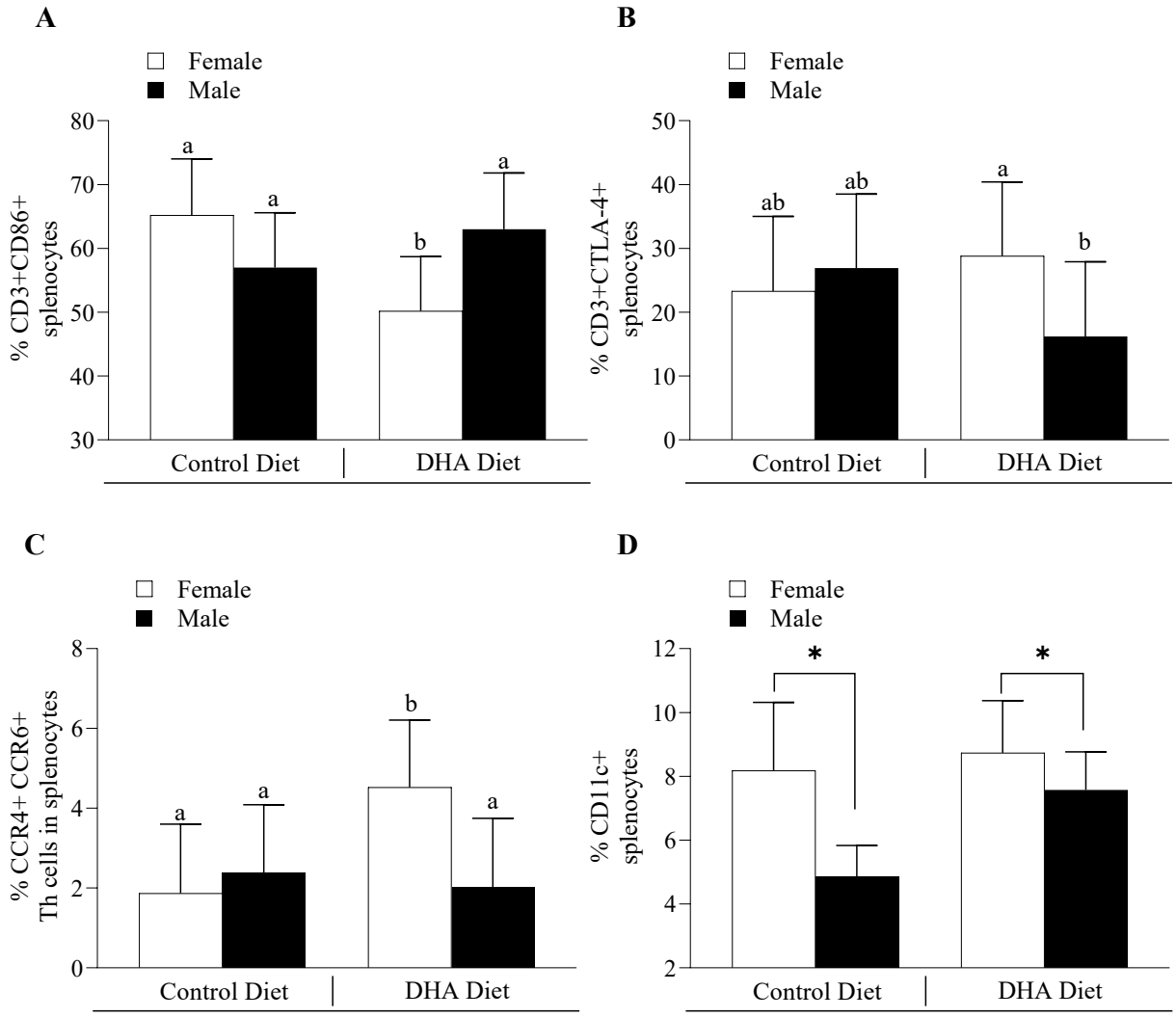

Figure 6.3. The interaction effect of suckling diet and sex in T cells splenocytes from 3-week pups. Panel A shows CD3+ CD86+ cells, panel B shows CD3+ CTLA4+, panel C shows CCR4+ CCR6+ in Th cells and panel D shows CD11c+ splenocytes. Note, Th cells were gated using CD3 followed by CD4 marker. This was followed by gating a double positive population using CCR4+ and CCR6+ to identify the subpopulation of Th cells enriched for Th17 cells. Values are

presented in means ± SEM. Note, CD86 is a marker of effector cells, CD152 is a marker of co-inhibitory CTLA4 molecule on T cells, Th17 is characterized by CCR4+ CCR6+ in Th cells and CD11c mostly identifies dendritic cells and some macrophages.

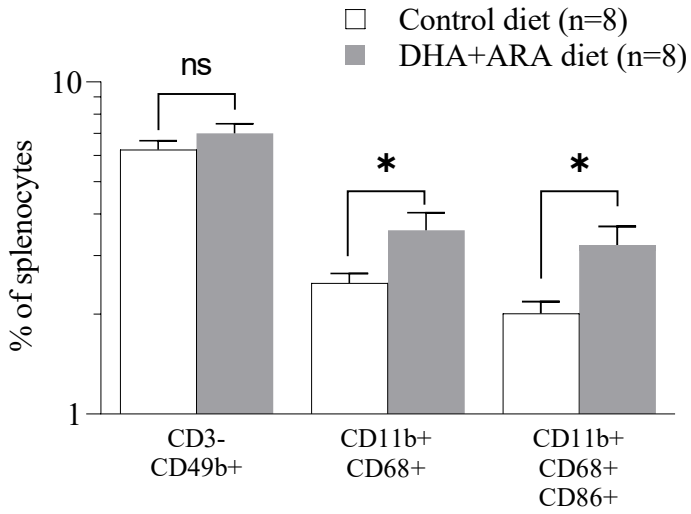

Figure 6.4. The effect of suckling diet on innate immune cells in splenocytes. Values are presented in means ± SEM. Abbreviation; CD, cluster of differentiation; Th, T helper cells; DHA, docosahexaenoic acid; ns, not significant. Note, CD3- CD49b+ markers of NKs, CD11b+ CD68+ is a marker of macrophages and the expression of CD86+ is high on macrophages with M1 phenotype although it does not fully capture M1 macrophage.

Table 6.9. The effect of the suckling diet on the immune cell phenotype of the splenocytes from BALB/c pups at the end of 3-week

% of gated splenocytes[1]	Control (n = 8)	DHA+ARA (n = 8)	SD P value[2]	Sex P value[3]	SD*sex P value[4]
Total CD3+	13.79 ± 0.73	13.34 ± 0.7	0.318	0.918	0.069
% of T cells (CD3+):					
CD4+	49.13 ± 2.78	42.74 ± 2.99	**0.020**	0.333	0.503
CD8+	30.52 ± 1	32.63 ± 1.2	0.530	0.818	0.100
CD25+	12.21 ± 0.74	11.22 ± 0.63	0.668	0.259	0.779
CD27+	7.53 ± 0.89	6.42 ± 0.86	0.106	0.801	0.594
CD28+	84.88 ± 2.09	77.65 ± 2.27	0.412	0.877	0.205
CD86+	64.13 ± 2.5	54.17 ± 4.1	0.349	0.598	**0.025**
CD152+	19.87 ± 3.1	26.1 ± 3.64	0.488	0.076	**0.045**
CD45RB+	77.23 ± 2.94	62.57 ± 4.53	0.302	**0.049**	0.288
% of cytotoxic T cells (CD3+ CD8+):					
CD25+	8.27 ± 0.62	8.19 ± 0.9	**0.042**	0.096	0.230
CD27+	74.85 ± 3.21	86.02 ± 1.73	**0.005**	0.325	0.785
CD28+	61.38 ± 2.42	65.71 ± 3.2	0.296	0.428	0.113
CD86+	72.49 ± 2.85	56.25 ± 5.09	0.220	0.264	**0.049**
CD152+	15.45 ± 2.76	24.23 ± 4.53	0.907	0.268	0.085
% of Th cells (CD3+ CD4+):					
CD25+	15.02 ± 0.53	10.82 ± 0.76	**0.003**	0.328	0.096
CD27+	78.71 ± 2.87	80.95 ± 2.66	0.427	0.994	0.736
CD28+	44.77 ± 2.32	43.48 ± 2.68	**0.008**	0.648	0.560
CD86+	68.21 ± 2.08	56.26 ± 4.01	0.112	0.575	**0.011**
CD152+	17.98 ± 2.01	26.08 ± 3.6	0.586	0.113	**0.038**
CD183+ CD196- (enriched for Th1)	8.48 ± 1.99	14.16 ± 2.8	0.835	0.586	0.154
CD194+ CD196+ (enriched for Th17)	1.41 ± 0.21	3.79 ± 0.76	0.131	0.156	**0.036**
CD194+ CD196- (enriched for Th2)	7.46 ± 0.55	6.78 ± 0.82	0.924	0.101	0.143
Total CD19+ (Total B cells)	29.76 ± 2.6	37.87 ± 2.13	**0.025**	0.750	0.720
% B cells (CD19+)					
CD45R- (enriched for B1 cells)	21.35 ± 2.52	28.26 ± 4.36	0.424	0.072	0.208
CD45R+ (enriched for B2 cells)	67.81 ± 3.07	52.22 ± 4.03	0.074	0.662	0.682

% of gated splenocytes[1]	Control (n = 8)	DHA+ARA (n = 8)	SD P value[2]	Sex P value[3]	SD*sex P value[4]
CD45R+ CD23- (marginal B2 cells)	28.82 ± 0.93	30.66 ± 2.33	0.509	0.421	0.172
CD45+ CD23+ (follicular B2 cells)	50.64 ± 2.56	42.14 ± 2.86	0.857	0.105	0.763
IgM+	17.12 ± 1.51	21.34 ± 1.81	0.089	0.650	0.904
IgG+	18.01 ± 1.9	25.22 ± 1.79	**0.022**	0.832	0.821
Total CD45R+ (peripheral B cells)	32.18 ± 2.33	37.75 ± 3.26	0.102	0.583	0.829
% peripheral B cells (CD45R+)					
CD19- CD138+ (plasma cells)	2.88 ± 0.28	3.67 ± 0.65	0.637	0.103	0.158
CD19- CD138+ IgM+ (plasma IgM cells)	1.1 ± 0.17	1.63 ± 0.33	0.634	0.120	0.153
CD19- CD138+ IgG+ (plasma IgG cells)	0.28 ± 0.06	0.87 ± 0.19	0.196	0.312	0.256
CD3- CD49b+ (NK cells)	6.26 ± 0.39	7.01 ± 0.47	0.215	0.064	0.063
% NK cells (CD3- CD49b+):					
CD27+	23.46 ± 1.01	27.31 ± 1.73	0.073	0.404	0.192
CD335+	24.19 ± 0.99	29.65 ± 2.74	0.291	0.192	0.266
CD3+ CD49b+ (NKT cells)	16.49 ± 0.83	14.26 ± 0.66	0.566	0.828	0.321
% NKT cells (CD3+ CD49b+):					
CD27+	59.33 ± 1.36	57.73 ± 3.19	0.242	0.754	0.794
CD335+	9.94 ± 1.61	18.24 ± 3.16	0.675	0.095	0.847
Total CD11c+ (DC)	6.31 ± 1.12	8.18 ± 1.01	0.766	**0.019**	0.896
CD11c+ CD4- CD8+ (enriched for cDC1)	0.65 ± 0.05	0.65 ± 0.04	0.967	0.799	0.107
CD11c+ CD8- (enriched for cDC2)	3.57 ± 0.98	5.58 ± 0.98	0.784	0.125	0.973
CD11b+ CD68+ (Total macrophages)	2.48 ± 0.17	3.58 ± 0.46	**0.028**	0.384	0.736
CD11b+ CD68+ CD86+ (enriched for M1 phenotype)	2.01 ± 0.17	3.23 ± 0.44	**0.033**	0.212	0.389
CD11b+ CD68+ CD163+ (M2 phenotype)	1.18 ± 0.21	0.73 ± 0.16	0.080	0.167	0.203

[1] Values are in percent of total gated splenocytes, except when reported otherwise (using italics).
[2] P values for the suckling diet main effect in the mixed model on 3-week pups' outcome
[3] P values for the sex main effect in the mixed model on 3-week pups' outcome
[4] P values for the suckling diet and sex main effect interaction in the mixed model on 3-week pups' outcome
Values are reported in mean ± SEM. Significant differences are in bold
Abbreviations, SD, suckling diet; CD, cluster of differentiation; DHA, docosahexaenoic acid; Th, T helper cells; Treg, T regulatory cells; NK, natural killer; NKT, natural killer T cells; DC, dendritic cells, cDC, conventional dendritic cells.

6.4. Discussion

The current study investigates the effect of feeding DHA in a diet, along with ARA, to breastfeeding dams on immune system development of offspring post-suckling period. Feeding dams diet supplemented with high DHA canola oil and ARA resulted in 60% higher DHA and 20% higher ARA concentration of total phospholipids in milk from mammary gland of the dams. In 3-week pups, these changes resulted in 60% higher DHA concentration in the splenocyte phospholipid concentration, although no difference in ARA concentration when compared to control. We found DHA+ARA supplemented offspring had a higher inflammatory cytokine (IL-1β, IFN-γ and TNF-α) response to a bacterial challenge, despite lower proportion of CD25+ (marker of activation and regulatory function) and CD28+ (co-stimulatory marker) T cells in splenocytes. Further, DHA+ARA supplementation was associated with higher proportion of adaptive immune cells, such as CD19+ B cells and IgG+ B cells (class-switched mature B cell), in 3-week pups' splenocytes. Lastly, sex differences were observed in immunological responses with higher CD11c+ (dendritic cells and some macrophages) in females compared to males, irrespective of diet. Overall, DHA+ARA supplementation of dams' diet during suckling period was associated with beneficial effects on the immune system of the 3-week pups (see below).

6.4.1. The maternal diet increases DHA in the mammary gland of dams and splenocytes from 3-week pups.

Feeding DHA and ARA (each 1% w/w of total fatty acids) to lactating dams during the 3-week suckling period resulted in 60% higher DHA and 20% higher ARA in dams' mammary gland milk. These changes are consistent with a population consuming diet high in DHA (Sheila M. Innis, 2014; van Goor et al., 2009). Literature suggests that the DHA level of breastmilk is dose-dependent and in the absence of ARA supplementation it reduces ARA levels in breastmilk (Del

Prado et al., 2001). The dose of DHA and ARA used in the current study allowed us to increase DHA and ARA levels in breast milk, which is consistent with previous experiments from our lab (Patel et al., 2021; Caroline Richard et al., 2016c). The supplementation of LCPUFA in pups, through dams' mammary gland milk, will provide essential immunomodulatory properties during the early phase of immune system development. Consistent with the increase in dams' breastmilk composition, pups' splenocytes phospholipid composition of DHA was also significantly higher. However, ARA levels in splenocytes showed no changes despite a small increase in breast milk composition. Findings from the current study are consistent with past studies in the healthy and allergy-prone rodent model (Hadley et al., 2017; Patel et al., 2021; Caroline Richard et al., 2016c). Although there was a small decrease in the ALA composition of splenocytes in the DHA group compared to the control, due to the low conversion rate from ALA to EPA and DHA (Igarashi et al., 2006), it is less likely to affect the n-3 LCPUFA levels in splenocytes.

6.4.2. DHA+ARA promotes immune system development through better inflammatory response in 3-week pups.

DHA+ARA suckling diet in 3-week pups resulted in higher inflammatory cytokines, such as IFN-γ, IL-1β and TNF-α to bacterial antigen (macrophage and B cell stimulant), CXCL1 to lymphocyte mitogen (T cell and B cell stimulant), and IL-2 and CXCL1 in unstimulated splenocytes (baseline levels). A past study using Sprague Dawley rat offspring has shown similar increase in inflammatory cytokine TNF-α and IL-6 to bacterial challenge with the supplementation of DHA (0.8% DHA of total fat) and ARA (0.4% of total fat) during suckling period (Caroline Richard et al., 2016f). The same study also reported a higher IFN- γ production to T cell mitogen by splenocytes from DHA and ARA supplemented suckled offspring than

controls (Caroline Richard et al., 2016f). Clinical trials with fish oil (containing EPA and DHA but not ARA) supplementation in infants have reported elevated TNF-α and IFN-γ production to lymphocytes mitogen (phytohemagglutinin) (D'Vaz et al., 2012) and IFN-γ to bacterial challenge (Lauritzen et al., 2005). At birth infant's immune system is highly dependent on innate immune responses as the repertoire of adaptive immune cells is not fully developed. During this time the Th2-mediated allergic response is dominant as the counteracting Th1 response is still developing (Mills et al., 2000; Pérez-Cano et al., 2007). We used BALB/c mouse to model the Th2 dominant characteristics of infant immune system. In BALB/c mouse, the Th2 skewed immunity is resultant of elevated prostaglandin E2 levels (an ARA metabolite), which can suppress the IFN-γ (Th1 cytokine) production and promote IL-12 (Th2 cytokine) by innate immune cells (Kuroda et al., 2000). Therefore, it is likely that the higher DHA levels in splenocytes may have suppressed ARA-derived immunomodulatory metabolite (prostaglandin E2) resulting in the promotion of IFN-γ in the BALB/c offspring. In the context of allergy sensitive Th2 dominant immunity, promoting Th1 cytokines can be beneficial for immune system maturation.

Suckling diet supplementation with DHA+ARA was associated with important phenotypic changes in the splenocytes of 3-week pups. We found that DHA+ARA supplementation resulted in significantly lower proportion of activated Th cells (CD3+CD4+CD25+) and activated CTL (CD3+CD8+CD25+) in splenocytes (Table 3). It is important to note CD3+ CD4+ CD25+ cells are enriched in Foxp3+ Tregs and future experiment with Foxp3 intracellular staining should be conducted to understand the relation between Treg and activated Th cells. A cell culture study has reported lower antigen-specific T cell activation markers (CD25 and CD69) when stimulated using DCs cultured with DHA or ARA (Carlsson et al., 2015). Similarly, clinical trial in participants with atopic dermatitis showed a reduction in CD25+ lymphocytes in peripheral

mononuclear cells after 4-months of EPA+DHA supplementation (6 g per day), which resulted in 5x increase in EPA and 3x increase in DHA (Søyland et al., 1994). *In-vitro* cell culture studies using fish oil (EPA and DHA) have suggested various mechanism involved in the activation of T cells (Gutiérrez et al., 2019; Zaloga, 2021) but the same remains to be confirmed in preclinical models using physiologically achievable n-3 LCPUFA levels (David et al., 2000). Further research is required to understand the effect of supplementation dose and ratio of DHA with ARA on T cell activation markers. Moreover, specific activation marker (CD69 and CD44) needs to be studied to confirm the hypothesis of 'DHA supplementation reducing T cell activation markers.' Nevertheless, the current study reports no change in IL-2 production by PMAi stimulated splenocytes in pups that received higher DHA+ARA than controls. This confirms the lack of any functional differences in T cell upon ex-vivo stimulation due to suckling period supplementation, despite a higher baseline IL-2 (unstimulated splenocytes) in DHA+ARA group than control.

6.4.3. DHA+ARA during the suckling period results in the maturation of immune cell phenotype in the spleen of 3-week pups.

Pups that receiving higher DHA+ARA during suckling period showed phenotypic changes in the adaptive and innate immune cells of spleen (representative of immune system). First, DHA+ARA supplemented pups had significantly higher proportion of total B cell (CD19+) and class-switched mature IgG+ B cells with no change in primary IgM+ B cells (Table 3). Past studies from our lab using allergy prone Brown Norway (Patel et al., 2021) and healthy Sprague Dawley (Caroline Richard et al., 2016c) rat offspring have also shown similar increases in the proportion of B cells (OX12+) in splenocytes with ARA+DHA supplementation. Next, splenocyte proportion of innate immune cells such as macrophage (CD11b+CD68+) and CD86+

macrophage (maybe involved in M1 related pro-inflammatory function) was significantly higher in DHA+ARA supplemented pups, with no differences in DCs or natural killer cells compared to controls. The higher proportion of antigen presenting B cells and macrophages in spleen can partly explain the higher inflammatory cytokine response (discussed earlier) towards bacterial component (LPS). The B cells form major component of adaptive immunity of neonates. Therefore, the higher proportion of mature B cells, and higher production of IFN-γ, IL-1β and TNF-α by LPS stimulated splenocytes observed in the current study may be beneficial for the development of infant immune system. Studies using fish oil supplementation (in the absence of ARA) have shown that the changes in membrane order and lipid raft through increasing EPA and DHA levels can affect the B cell function (cytokine production, antigen presentation and antibody production) (Rockett et al., 2012). Some studies have also shown higher production of IL-6 and TNF-α by B cells from mice fed DHA enriched fish oil (Gurzell et al., 2013; Teague et al., 2014).

The maturation of adaptive immunity is marked by the development of lymphocyte repertoire with memory phenotype, described using CD45RO, CD62L, CCR7, CD69 and CD27 (Martin & Badovinac, 2018). Findings from the current study showed an association between DHA+ARA supplementation and higher proportion of CD27+ CTL (representing a subset of memory marker) in splenocytes from 3-week pups. In mice, CD27 plays an important role in the expansion and survival of effector CTL that are involved in response to influenza viral infection (Hendriks et al., 2003). Similar findings have been reported in clinical trial where DHA+ARA supplementation during gestation and breastfeeding resulted in 2x higher memory CTL (CD45RO+CD8+ lymphocytes) in 4 months old infants that were exclusively breastfed (Granot et al., 2011). The current study also identified that the dams' splenocyte proportion of

CD3+CD27+, at the end of suckling period, was also higher in DHA+ARA diet group than control. Therefore, the passive transfer of cellular immunity (including effector memory (CD45RO+, $CD62L^{low}$) T cells (Myles & Datta, 2021)) through breastmilk along with other immune components can likely explain our findings (Field, 2005), but it needs to be confirmed with future studies. The breastmilk derived memory B cells are vital in maintaining antibody levels in infants (Duchamp et al., 2014; Tuaillon et al., 2009) making it important for the development of adaptive immune response. Therefore, DHA+ARA during suckling period can positively influence the immune system of 3-week pups through phenotypic changes in the B cells and T cells proportions of spleen.

6.4.4. Sex effect on immune system development in 3-week pups.

Sex related differences in the immune response are evident early in life and several factors play an important role, which includes hormones, genotype, environmental exposure, nutrition, and microbiome (Christoforidou et al., 2019; Ghosh et al., 2021; Hensel et al., 2019; Klein & Flanagan, 2016). We identified some significant sex differences in the immune system of 3-week pups prior to puberty. The female pups had higher IFN-γ levels in unstimulated splenocytes, as well as when splenocytes were stimulated with bacterial component, along with higher CD11c+ (DCs and some macrophages) splenocytes when compared to male pups. Differences in DC phenotype and function of inflammatory cytokine production due to sex have been reported (Klein & Flanagan, 2016). Specifically, DCs isolated from peripheral mononuclear cells of women showed higher levels of interferon-α when stimulated with toll-like receptor 7 on DCs in comparison to men (Berghöfer et al., 2006; Griesbeck et al., 2015). It should be noted that the clinical significance of small but significant changes between sexes must be further investigated. In terms of adaptive immune cells, females have higher Th cells and males have higher CTL in

peripheral blood, however, such differences were absent in the current study. Interestingly, we found that female pups from DHA+ARA group had significantly higher CCR4+ CCR6+ Th cells (enriched for Th17) and CTLA-4+ T cells proportion in spleen, such sex differences were absent in control group pups. In terms of *ex-vivo* cytokine production, female pups showed significantly higher IL-1β levels by splenocytes stimulated with PMAi (non-specific lymphocyte stimulant) than males, whereas IL-2, TNF-α or IFN-γ showed no sex effect and production of Th17 specific cytokines (IL-17 and IL-22) were not studied. Th17 mediate proinflammatory response are implicated in countering T regulatory cells, and Th2 specific responses (Schnyder-Candrian et al., 2006). Clinical trial have shown altered Th17 response in neonates (Razzaghian et al., 2021) and children (Dhuban et al., 2013) with food allergies suggesting its role in the prevention of allergic response to food antigens. The role of n-3 on the function of Th17 cells need to be further studied specially when there are sex-related differences in T cell subtypes and function (Monk et al., 2013). In the light of sex specific differences in immune response, future nutritional intervention studies should be sufficiently powered to evaluate sex effect on immune system development.

To our knowledge, this is the first study to evaluate the effects of a novel DHA enriched canola oil (provided by Nuseed) on immune system development during infancy. The use of a commercially viable source of DHA will help reduce the risk associated with the consumption of fish and seafood, which include heavy metal toxicity. In addition, for population with vegan or vegetarian dietary practices, the integration of high-DHA Canola in the can be useful. The current study also has few limitations. First, we use a combination of antibodies to identify specific markers associated with specific immune cells. However, since there is overlap between some of these cell surface markers, the specific immune cells need to be confirmed with genetic

transcription factors. For instance, CD11c is widely used marker for DCs in splenocytes of mouse, however it is also expressed by some macrophages (Drutman et al., 2012). Second, while the change in proportion of specific immune cells in spleen do not directly correlate with differences in immune cell function, nevertheless the higher proportion of specialized cells can improve specific functions, which can include antigen screening, antigen presentation, cytokines, and antibodies production. Next, the current study reported *ex-vivo* cytokine response after 48 hours after stimulation, which might be different from shorter period of stimulation (i.e., 6 hr). Cytokines released after short period of stimulation are predominantly by innate immune cells, but longer period of stimulation also involves cytokines produced by adaptive immune cells. Last, we used adjustment for multiple comparison to estimate the pos-hoc differences between groups resulting in a conservative estimate of difference. This may have resulted in some parameters being suppression of statistically significant differences after multiple comparison adjustment. Of note, the influence of dietary LCPUFA on microbiota and mucosal immune system during early life is important to consider and should be investigated in future research (Brown et al., 2012).

6.5. Conclusion

The current study uses a novel source bioactive canola oil enriched in DHA to demonstrate the importance of LCPUFA supplementation in the immune system development during early infancy in allergy prone BALB/c mouse offspring. Feeding 1% of DHA, along with the same amount of ARA, to breastfeeding dams resulted in a significant increase in DHA concentration of dams' mammary gland milk. Which was reflected in 60% higher DHA in splenocytes of the pups measured at the end of suckling period. Higher DHA was associated with better inflammatory cytokine response (IFN-γ, IL-1β and TNF-α) upon stimulation of macrophages,

DC and B cells (APCs) using bacterial component LPS, this may support T cell polarization to Th1 skewed since IFN- γ and TNF-α are known Th1 inducers. Further, DHA+ARA supplementation may have also promoted maturation of some adaptive immune cells (higher IgG+ B cells and CD27+ CTL splenocytes). Interestingly, immune system of pre-pubertal 3-week pups, also showed differences associated with the sex where female had higher inflammatory (IFN-γ to LPS and IL-1β) characteristic than males. Overall, increasing DHA in the diet of BALB/c mouse offspring, promoted better inflammatory response and the maturation of immune system at 3-weeks.

6.6. References

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Chapter 7. The programming effect of plant-based DHA, along with equivalent ARA, on the immune system and oral tolerance development in 6-week allergy-prone BALB/c pups[1]

7.1. Introduction

The recent rise in the prevalence of food hypersensitivity has been associated with a higher ratio of dietary n-6 to n-3 polyunsaturated fatty acid (PUFA) (Elghoudi & Narchi, 2022; Lack, 2012; Prescott et al., 2013). This is mostly due to the increased consumption of vegetable oils which are high in n-6 PUFAs and low in n-3 PUFAs (Lack, 2012). Further, the plant-based oils are considered poor sources of dietary long chain PUFA (LCPUFA), especially n-3 LCPUFA. The n-3 LCPUFAs are commonly obtained from oily fish and sea foods and more recently from genetically modified single-cell organisms and novel plant oils. Due to low intake of these foods, the n-3 LCPUFA status remains suboptimal and is considered to be a risk factor for food allergies (Jia et al., 2015; Papanikolaou et al., 2014; Caroline Richard et al., 2016a; Sartorio et al., 2021). Recently, a commercially available genetically modified canola has been shown to produce high DHA. The canola plant was genetically modified to introduce a set of desaturase and elongase enzymes involved in the n-3 and n-6 bioconversion pathways. The high level of DHA in these seeds is achieved by the conversion of oleic acid to n-3 LCPUFAs such as EPA, DPA and DHA (Petrie et al., 2020). Therefore, the intake of n-3 LCPUFA (docosahexaenoic acid, DHA) from novel high-DHA canola oil (developed by Nuseed) can provide an efficient alternative to improve DHA status.

The infant immune system after birth undergoes a significant transition to become mature and more adult-like. First, the non-specific responses by innate immunity transition to more specific and robust through the expansion of adaptive immunity (T cells and B cells) (Wilson &

[1] A version of this chapter has been submitted for publication in is currently under peer-reviewing process in Journal of Nutrition.

Kollmann, 2008). Second, the T helper cell (Th) type-2 (Th2)-biased immune responses (IL-4, IL-5, IL-13, Immunoglobulin (Ig)-G1 and IgE) transition to Th1 dominant responses (TNF-α, IFN-γ, IL-12 and IgG2a) (Raphael et al., 2015). The risk of developing food allergies during this period is also higher during this period (Campbell et al., 2015). However, the immune system during early life can generate a specific suppression, or ignorance, of cellular and humoral immune responses towards orally ingested food protein, defined as oral tolerance (OT) (Satitsuksanoa et al., 2018; Weiner et al., 2011; Yang et al., 2021). The failure to develop OT results in food allergies. Infants with prolonged Th2-biased immune responses promote the development of food hypersensitivity, mediated by antigen-specific-IgE (Johnston et al., 2014) and may hinder the development of OT during early life (Georas et al., 2005; Weiner et al., 2011).

The first year after birth is considered a critical window of opportunity for infant immune system development (Saavedra & Dattilo, 2017). The immune system adaptation obtained during this period can have a long-term programming effect that can be observed later in life (Philip C. Calder et al., 2010; Palmer, 2011). Therefore, providing optimal nutrition is paramount during this period (Saavedra & Dattilo, 2017).The supplementation of DHA (n-3 LCPUFA) and arachidonic acid (ARA, n-6 LCPUFA) may be useful due to their immunomodulatory properties (Dyall et al., 2022). Depending on the dose and the balance of these two lipids they can be either anti-inflammatory or inflammatory (Miles et al., 2021; Nettleton & Salem Jr., 2019). Therefore, studies that provide a balance of n-3 and n-6 LCPUFA may be crucial for immune system development. Past studies from our lab group using DHA and ARA in preterm and full-term infants have shown beneficial effects on immune system development (Field et al., 2000; Field et al., 2008b). Furthermore, similar findings have also been seen in studies evaluating a low-dose

supplementation of DHA alongside ARA in rodents with a healthy immune system (C. Richard et al., 2016) or allergy-prone immune system (Dhruvesh Patel et al., 2022). Therefore, it was hypothesised that the supplementation of relatively higher dose of DHA along side ARA during early infancy in allergy prone could promote the maturation of infant immune function and assist in the prevention of food hypersensitivity through OT development (Fussbroich et al., 2020; Gunaratne et al., 2015; Weise et al., 2011a).

The overall objective of the study was to determine the effects of DHA supplementation (1% of total fat), along with ARA, during the suckling and weaning period on the development tolerance towards ovalbumin (ova) and maturation of the immune system. We used allergy-prone BALB/c mice in the study to model infants born with a genetic predisposition to developing atopic conditions such as food hypersensitivity and allergies. The primary outcomes measured were *ex-vivo* cytokine and antibody response to different stimuli. Additionally, the main outcome evaluated for OT analysis was the *ex-vivo* production of cytokines and immunoglobulin by splenocytes stimulated with ova. We also analyzed plasma levels of ova-specific-Ig to evaluate *in-vivo* response to OT. The differences in immune cell phenotypic were used to explain the observed changes in the immune cell functions. The hypothesis was that the DHA would promote beneficial changes in the immune function of BALB/c mouse offspring, resulting in the suppression of antigen-specific response to ova *in-vivo* and *ex-vivo* to induce OT.

7.2. Methods

7.2.1. Diet and study design

All the animal experiment and procedures were completed in compliance with Canadian Council on Animal Care and were approved by the University of Alberta Animal Care and Use Committee. Timed pregnant BALB/c mouse were obtained from Charles River laboratories and housed in temperature- and humidity- controlled environment on a 12 h to 12 h reverse light

cycle. Dams were acclimatized until 14 d of gestation during which standard rodent chow diet (Lab diet 5001; PMI Nutrition International) was fed. Dams were randomized 5- to 7- days before parturition to consume one of the two suckling period diet (SPDs): DHA+ARA SPD (1% DHA wt/wt of total fat and 1% ARA wt/wt of total fat, *n*=10) and control SPD (0% DHA or ARA, *n*=12) for 3-week suckling period (Figure 7.1.). Dams were housed in individual cages with their pups during which pups consumed dam's mammary gland milk. Following suckling period, pups from each of the SPD groups were randomly assigned to one of two weaning diets (WD); DHA+ARA WD (1% DHA wt/wt of total fat and 1% ARA wt/wt of total fat, *n*=16 pups) and control WD (0% DHA and ARA, *n*=16 pups), until pups were 6-week-old. This resulted in 4 diet group combination for the pups (i.e., control SPD to control WD, control SPD to DHA+ARA WD, DHA+ARA SPD to control WD, and DHA+ARA SPD to DHA+ARA WD). Note, after OT group allocation (described below) there were 8 group combinations formed and each group consisted of *n*=8 pups of 6-week age. The 6-week-old pups were considered the experimental unit for the current experiment.

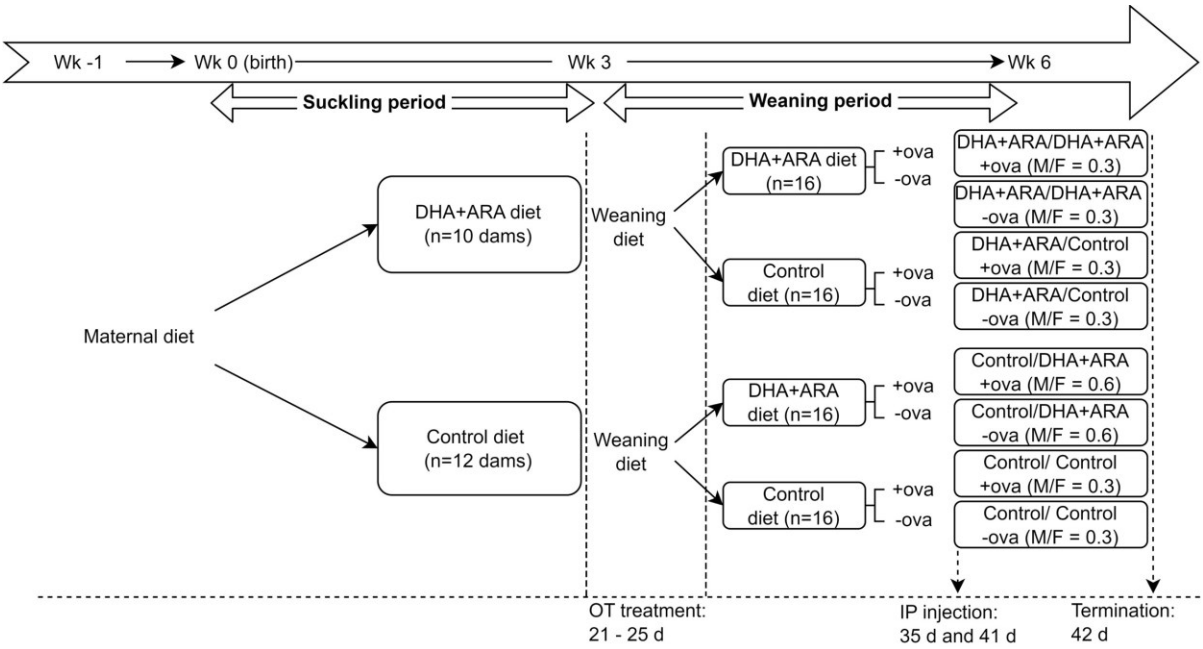

Figure 7.1. The study design shows BALB/c mice were randomized to consume either DHA+ARA maternal diet (1% DHA wt/wt of total fat and 1% ARA wt/wt of total fat, n=10 dams) or control maternal diet (no ARA or DHA, n=12 dams) beginning from 5 to 7 days pre-parturition to 3-week suckling period, when pups consumed their mother's breastmilk. At 3 wk, pups from each maternal diet group were randomly allocated to either DHA+ARA diet (1% DHA wt/wt of total fat and 1% ARA wt/wt of total fat, n=16 pups) or control diet (no ARA or DHA, n=16 pups) for addition 3-wk, this was referred to as weaning period. For inducing oral tolerance, one-half of the pups from each weaning diet groups was exposed to ovalbumin or sucrose placebo for 5 consecutive days starting from 21 d. The maternal diet, weaning diet and oral tolerance interventions resulted in 8 groups with n=8 pups/group. The 6-week-old pups were considered the experimental unit for the current experiment. Intraperitoneal injections of ovalbumin and adjuvant was administered to all the pups to induce systemic immune response on 35 d and 41 d. All the pups were killed at 6-wk of age and relevant tissues were collected. Abbreviation: ARA, arachidonic acid; DHA, docosahexaenoic acid; IP, intraperitoneal; M/F, male to female ratio; OT, oral tolerance; ova, ovalbumin

All the experimental diets were isocaloric, isonitrogenous and nutritionally complete. Food and water were provided *ad libitum*. The experimental diets were high fat diet prepared with mixing non-fat powder diet (described previously (Lewis et al., 2016b)) with 20 % wt/wt oil mix (described in table 7.1). We used a combination of oils to attain a similar PUFA/SFA ratio and n-6/n-3 ratio in both experimental diets such that the effects studied were primarily due to DHA and ARA supplementation. The fatty acid composition of the diet is now described in table 7.1 (D Patel et al., 2022). The DHA concentration of 1% in human milk is found in population with lifelong intake of fish, which also allows to maintain DHA status of mother while improving status of DHA in infants (Brenna et al., 2007; Jackson & Harris, 2016; Yuhas et al., 2006). Therefore, the concentration of DHA in diet was selected to model the upper end of levels found in human breastmilk and the concentration of ARA in maternal diet was selected to match DHA as per the current dietary recommendations for infant supplementation (Codex Alimentarius Commission, 2007). All the pups were euthanized at 6 weeks after birth using CO_2 asphyxiation and cervical dislocation. Blood was collected with cardiac puncture and spleen was aseptically collected for further analysis.

Table 7.1. Composition of experimental diets. The mixture of commercial oils was used to prepare a 20% wt/wt high-fat experimental diet. One kg diet was prepared by mixing 800 g of fat-free powder diet mix and 200 g of the oil mix.

Ingredients (g / 1kg diet)	Control diet	DHA+ARA diet
Casein (high protein)	270	270
Starch	241	241
Sucrose	126	126
Vitamin mix[1]	19	19
Salt mix[2]	50	50
Calcium phosphate dibasic	3.4	3.4
Inositol	6.3	6.3
Cellulose	80	80
L-cysteine	1.8	1.8
Choline bitartrate	2.5	2.5
Total powder (fat-free) diet	800	800
Lard	95	119
Mazola canola oil	105	0
Mazola corn oil	0	29.5
ARAsco[3]	0	5.5
Nuseed canola oil[4]	0	46
Total oil mix	200	200
Fatty acids (g/100g of total fatty acids)		
C16:0	16.0±0.1	21.2±1.0
C18:0	10.2±0.9	11.3±1.4
C18:1n-9 (oleic acid)	53.8±0.4	39.7±0.7
C18:2n-6 (LA)	14.5±0.4	17.6±0.9
C20:4n-6 (ARA)	0±0	1.0±0.0
C18:3n-3 (ALA)	3.7±0.1	4.8±0.5
C20:5n-3 (EPA)	0±0	0.1±0.0
C22:5n-3 (DPA)	0±0	0.1±0.0
C22:6n-3 (DHA)	0±0	1.1±0.0
Total MUFA	54.8±0.5	41.0±0.9
Total PUFA	18.2±0.5	24.9±1.5
Total SFA	27.0±1.0	34.2±2.4
Ratio PUFA/SFA	0.7±0.0	0.7±0.1
Total n-6	14.5±0.3	18.7±0.9
Total n-3	3.7±0.1	6.2±0.6
Ratio n-6/n-3	3.9±0.0	3.0±0.1

[1] AIN-93-VX vitamin mix
[2] Bernhart-Tomarelli salt mixture

[3] Arachidonic acid from single cell organism was provided by DSM Nutritional Products
[4] Nuseed high docosahexaenoic acid canola oil was provided by Nuseed
Data are presented as the mean ± SEM of 2 batches of diet mix. Abbreviation, ALA, a-linolenic acid; LA, linoleic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; n-3, omega-3, PUFA, polyunsaturated fatty acid; MUFA, monounsaturated fatty acid; SFA, saturated fatty acid.

7.2.2. Ova administration and immunization protocol.

To induce oral tolerance towards ova (food antigen derived from egg white protein, Sigma Aldrich), the 3-week pups were orally fed ova (0.01 mg/g body weight) dissolved in sucrose water (8% wt/wt) using a syringe once a day from 21 d to 25 d of age. For placebo control, half of the pups from all the 4 diet groups were fed sucrose water (8% wt/wt) using a syringe once a day from 21 d to 25 d. To induce systemic immune response towards ova all the pups received intraperitoneal injection of ova (10 µg Ova in 100 µl PBS) with an adjuvant (1:1, Imject^{TM} alum adjuvant; Thermo-Scientific) at 35 d (7 d before termination) and 41 d (24 h before termination, as a booster dose). At 6-week (42 d), pups were euthanized, and blood was collected in K2 EDTA-containing tubes using a 2-mL syringe. One-hour from collection, blood tubes were centrifuged (1734 x g; 10 min; 22 °C) and plasma was collected and frozen at – 80 °C. The *in-vivo* analysis of OT was based on plasma levels of ova specific IgE (ova-IgE) and ova specific IgG (ova-IgG). Commercially available kits for ova-IgE and ova-IgG from MyBioSource, Inc. was used with a detection of range of 0.312-20ug/mL and 0.16-10ng/mL respectively. The *ex-vivo* analysis of OT was based on antibody response and cytokine response towards ova stimulation of splenocytes.

7.2.3. Immune cell isolation and *ex-vivo* cytokine response to mitogens and ova.

The procedure to collect spleen and isolate immune cells has been previously described (C. J. Field et al., 1990). Briefly, spleen tissue was cut into small pieces and pushed through nylon mesh to obtain a single-cell suspension. The red blood cells in the cell suspension were lysed using ammonium chloride lysis buffer (155 mmol/L NH_4Cl, 0.1 mmol/L EDTA and 10 mmol/L $KHCO_3$; Fisher Scientific). Cells were washed with 0.5% bovine serum albumin in Krebs-Ringer bicarbonate buffer and resuspended in complete cell culture medium (RPMI 1640 medium

supplemented with 5% vol/vol fetal calf serum (FCS), 2.5 mmol/L 2-mercaptoethanol, and 1% antibiotic/antimycotic; Invitrogen). Cells were counted with the use of trypan blue dead cell exclusion and single cell suspension with a concentration of 1.25 x 10^6 cells/mL was prepared to be used for cell stimulation assay as described below.

The immune cell function was evaluated by cytokine production to *ex-vivo* stimulation with mitogens as described elsewhere (Blewett et al., 2009). We tested the splenocyte cytokine production function with no mitogen, lipopolysaccharides (LPS), phorbol–myristate–acetate and ionomycin (PMAi), anti-CD3/anti-CD28 or ova. In brief, splenocytes were incubated with LPS (2 µL/mL, Sigma-Aldrich Canada Ltd) in 5% CO_2 and 37 °C to evaluate innate immune cells (macrophage and dendritic cells (DC) and B cells) or PMAi (2 µL/mL, cell stimulation cocktail, Thermo Fisher Scientific) in 5% CO_2 and 37 °C to evaluate adaptive immune cells (lymphocyte). The immune cell response to the model food antigen was evaluated by stimulation with ova (200 ug/mL, Sigma) in 5% CO_2 and 37 °C. For T cell stimulation, 12-well plates were coated with anti-CD3 antibody (2ul/mL prepared in phosphate buffer saline (PBS)) overnight and washed with PBS prior to adding splenocytes (1.25 x 10^6 cells/mL) cell culture medium. Then anti-CD28 antibody (0.43 µL/mL, BD Biosciences) was added, and cell were cultured for 72 h in 5% CO_2 and 37 °C.

The *ex-vivo* cytokine production was quantified using commercially available electrochemiluminescence kits (Proinflammatory Panel - 1, Meso Scale Discovery (MSD)). The range of detection for measured cytokines are as follows; : IFN-γ, 0.0226 – 764 pg/mL; IL-10, 0.292 – 3540 pg/mL; IL-12, 4.95 - 32500 pg/mL; IL-1β, 0.0689 - 1530 pg/mL; IL-2, 0.204 - 2530 pg/mL; IL-4, 0.156 - 1790 pg/mL; IL-5, 0.0675 - 994 pg/mL; IL-6, 0.439 - 5240 pg/mL; chemokine C-X-C motif ligand 1 (CXCL-1, known as keratinocyte chemoattractant/ growth-

regulated oncogene, KC/GRO), 0.265 – 2020 pg/mL and TNF-α, 0.04 - 639 pg/mL. The *ex-vivo* levels of TGF-β1, 7.8 – 500 pg/mL, was quantified using commercial kits (LEGEND MAX, Bio Legend). The *ex-vivo* levels of total IgG, IgG1 (Th2 type response) and IgG2a (Th1 type response) were quantified using commercial kits (Condrex, Inc.) with 1.56 – 100 ng/mL detection range. Note, IgG2c was not measured. The dilution factor was calculated based on the estimated sample concentration for each cytokine/antibody *ex-vivo* response prior to analysing samples.

7.2.4. Splenocyte immunofluorescence phenotyping.

Splenocyte populations were identified by direct immunofluorescence (IF) assay as previously described (Field et al., 2000; Field et al., 2008b). Five to seven fluorescent labelled monoclonal antibodies (mAb) were used in combination to identify specific immune cell populations in splenocytes as follows CD28/CD25/CD4/CD152/CD8a/CD86/CD3, CD183/CD194/CD4/CD196/CD8a/CD3, CD45R/CD138/CD19/CD23/IgG/IgM, CD4/CD11c/CD19/CD103/MHC-II/CD86/CD8a, CD86/CD284/CD68/MHC-II/CD163/CD11b, CD27/CD45RB/CD4/CD49b/CD335/CD3/CD8a and latent-associated peptide (LAP)/FoxP3/CD4/CD25/CD3. All antibodies were purchased from Cedarlane and Thermo Fisher (developed by BD Biosciences or eBiosciences). Titration experiments were conducted to optimize the dilution factor for each mAb within a panel. The mAbs were selected to minimize spectral overlap. Further, compensation controls, isotype controls and unstained controls were used to guide our gating strategy. Splenocytes from three different stimulation conditions; no stimulation (freshly isolated cells), LPS stimulation (collected after 12 h incubation with mitogen) or PMAi stimulation conduction (collected after 12 h incubation in mitogen) were used for immunofluorescence assay. Briefly, splenocytes (200,000 to 500,000) were incubated for 30

min at 4 °C with either of the monoclonal antibody combinations described earlier. Additionally, to identify T regulatory cells, the cell stained with LAP/CD4/CD25/CD3 combination were fixed and permeabilized as per manufacturers guideline (eBiosciences) followed by staining with anti-FoxP3 fluorescent labelled antibody for 20 min and washed as per manufacturers protocol. Cells stained with surface mAb were then washed with IF buffer (4% FCS in PBS stored and used at 4°C) and cells were fixed with 1% paraformaldehyde buffer (10 g/L, stored and used at 4°C; Thermo Fisher Scientific). The fluorescently labelled cells were acquired within 48 hours by flow cytometry according to relative fluorescence intensity and analyzed using FlowJo software. Detailed gating strategy is provided in the Figure 7.2 [a] to Figure 7.2. [g].

7.2.5. Statistical analysis

All the parameters are presented as mean ± SEM unless stated otherwise. The sample size of n=8 per group was determined to be sufficient to assess a difference of 20% or more in the primary outcome variable, *ex-vivo* cytokine production. Data analysis was conduced with PROC MIXED procedure 3-factor ANOVA (SAS 9.4 software) and have been previously described (Dhruvesh Patel et al., 2022; Patel et al., 2021). The study followed a statistical model to evaluate three main independent variables (main effects); SPD, WD and OT as well as their interactions (SPD × WD, SPD × OT, WD × OT and SPD × WD × OT). The effect of pups' sex and related interaction effect are not included in the current study and will be appropriately discussed with findings from subsequent studies. When a main effect was found to be significant (in the absence of any statistically significant in associated interactions), the means of groups within the main effects were reported for easy of understanding. When the main effect was found to be statistically significant along with significant interaction effect then only the interaction effects are described and *post-hoc* analyses were conducted to compare group means associated with the

interaction effect using Bonferroni adjustment for multiple comparison. This adjustment is conservative in estimating P-values to minimize type I errors (false positives) due to which some parameters may appear to be different but may not show statistical significance. Furthermore, limiting the statistical power to observe the difference between groups. For instance, when *P*-value for SPD x WD interaction was statistically significant, means from 4 diet groups were compared (control SPD and control WD, control SPD and DHA+ARA WD, DHA+ARA SPD and control WD, and DHA+ARA SPD and DHA+ARA WD). Note, prior to conducting inferential statistics, the assumptions were checked, and data was appropriately transformed to meet the assumption. Differences were assumed to be significant at $P \leq 0.05$.

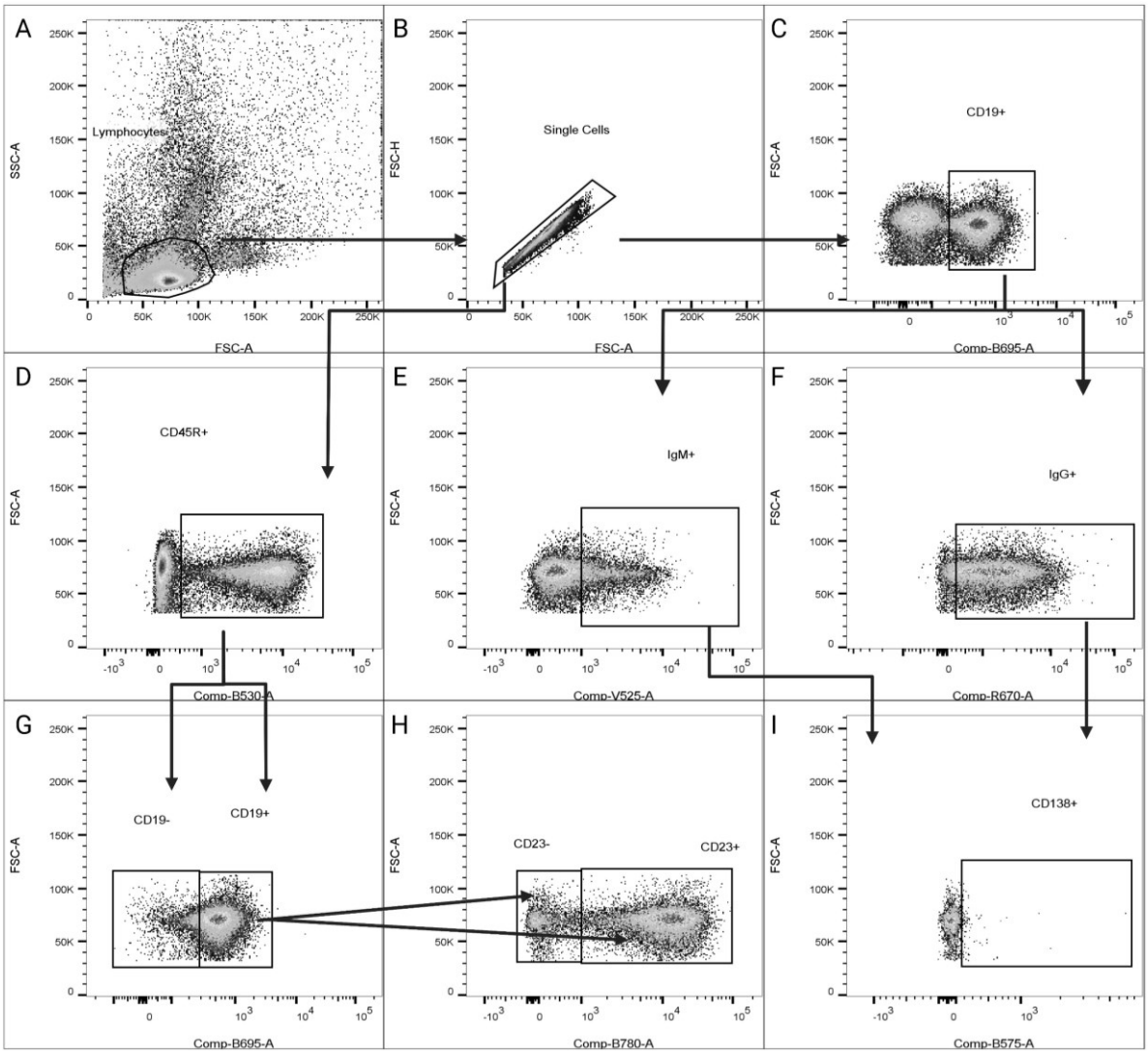

Figure 7.2. [a] Representative plots for **B cell gating strategy**. Splenocytes were first gated to include lymphocytes based on side-scatter area versus forward-scatter area dot-plot (A) followed by the exclusion of doublets and debris based on forward-scatter height versus forward-scatter area (B). Single cells were then analyzed for frequency (%) of CD19 (C) and CD45R (D) using specific antibodies, representing total B cell populations. Subsequently, total CD19+ cells were gated on IgM (E) or IgG (F) specific antibodies. The CD19+ cells were subsequently used for CD23+ (H) cells and CD23- cells. The CD138 markers were used to identify plasma cells (I) after gating on CD45R+ CD19- IgM+ or CD45R+ CD19- IgG+ or CD19+ IgM+ or CD19+ IgG+. Primary B cells were identified from CD19+ IgM+ cells. Secondary B cells were identified from CD19+ IgG+ cells. Follicular B cells were identified from CD19+ CD23+ cells. At the same time, marginal B cells were identified from CD19+ CD23- cells.

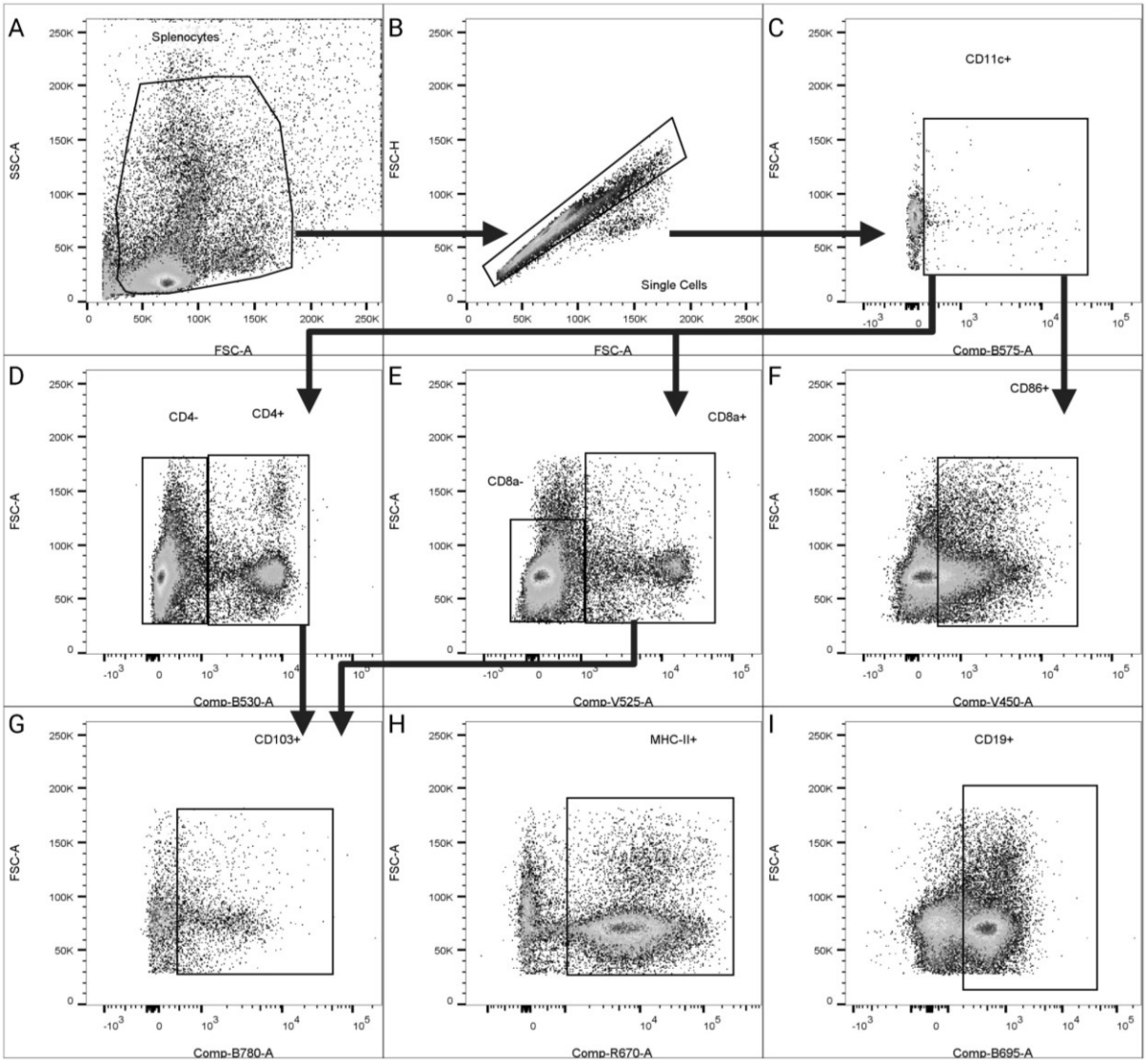

Figure 7.2. [b] Representative plots for **Dendritic cell gating strategy**. Splenocytes were first gated to include all the mononuclear cells based on side-scatter area versus forward-scatter area dot-plot (A) followed by the exclusion of doublets and debris based on forward-scatter height versus forward-scatter area (B). Single cells were then analyzed for frequency (%) of CD11c+ (C) DCs and some macrophages, MHC-II (H) using I-A^d/I-E^d antibody, CD19+ (I) B cells. The CD86+ cells (F) were gated on CD11c+ cells to identify co-stimulatory markers on DCs. The CD103+ cells (G) were gated on CD11c+CD4+ or CD11c+CD8a+ to identify tolerogenic dendritic cell subpopulation. Plasmacytoid DCs were identified from CD11c+ CD4+ as CD11c+ CD4+MHC-II+. Conventional dendritic type-1 cells were identified from CD11c+ CD4- as CD11c+ CD4- CD8a+. Conventional dendritic type-2 cells were identified from CD11c+ CD8-.

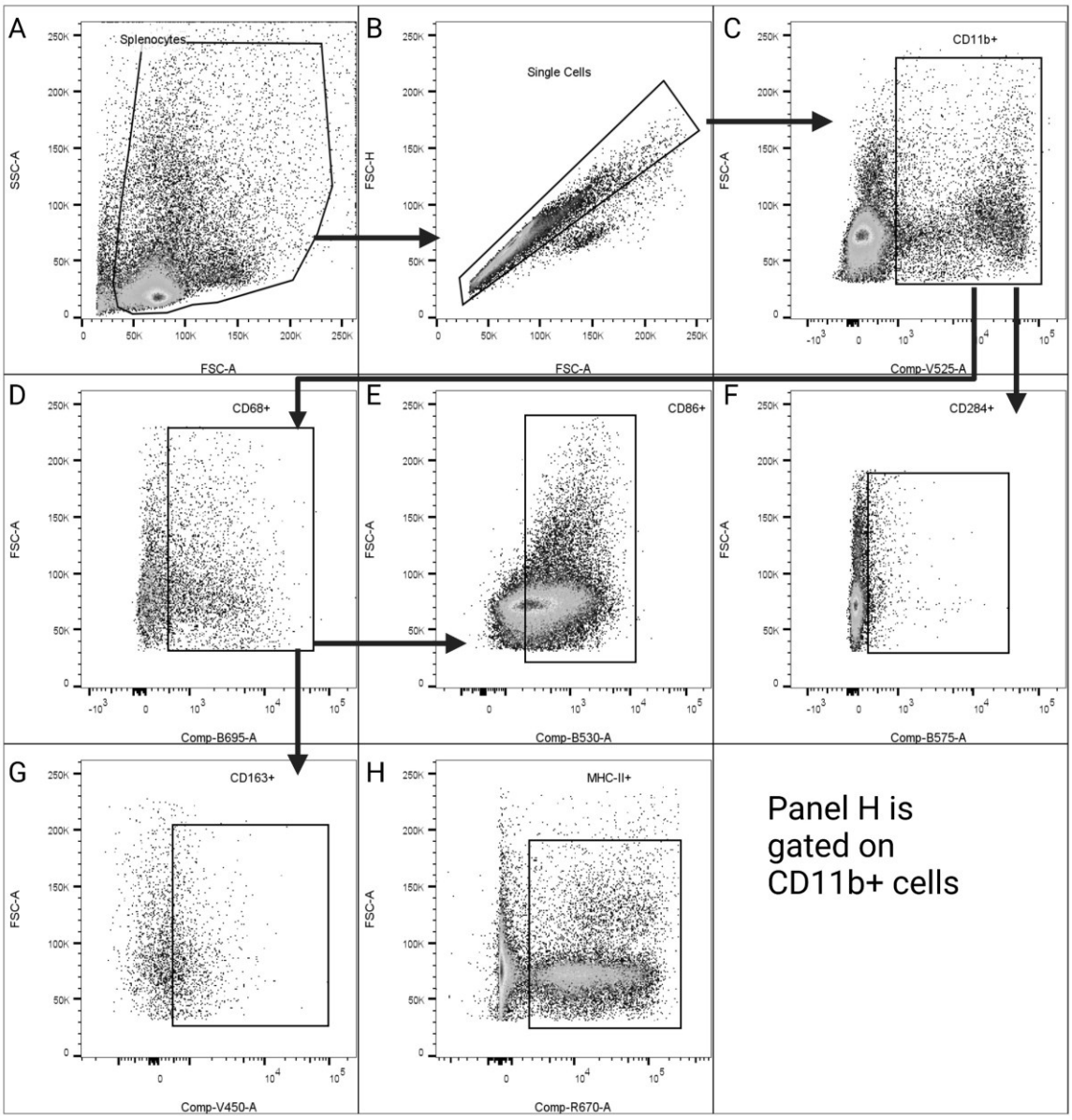

Figure 7.2. [c] Representative plots for **macrophage gating strategy**. Splenocytes were first gated to include all the mononuclear cells based on side-scatter area versus forward-scatter area dot-plot (A) followed by the exclusion of doublets and debris based on forward-scatter height versus forward-scatter area (B). Single cells were then analyzed for frequency (%) of CD11b+ (C), followed by gating on CD68+ (D) to identify total macrophages. The CD86 marker (E) and CD163 marker (G) were gated on CD11b+ CD68+. The CD11c+ was used to gate on CD284+ (F) and MHC-II (H). The M1 phenotype macrophages were identified from CD11b+ CD68+ CD86+ MHC-II+. The M2 phenotype macrophages were identified from CD11b+ CD68+ CD163+.

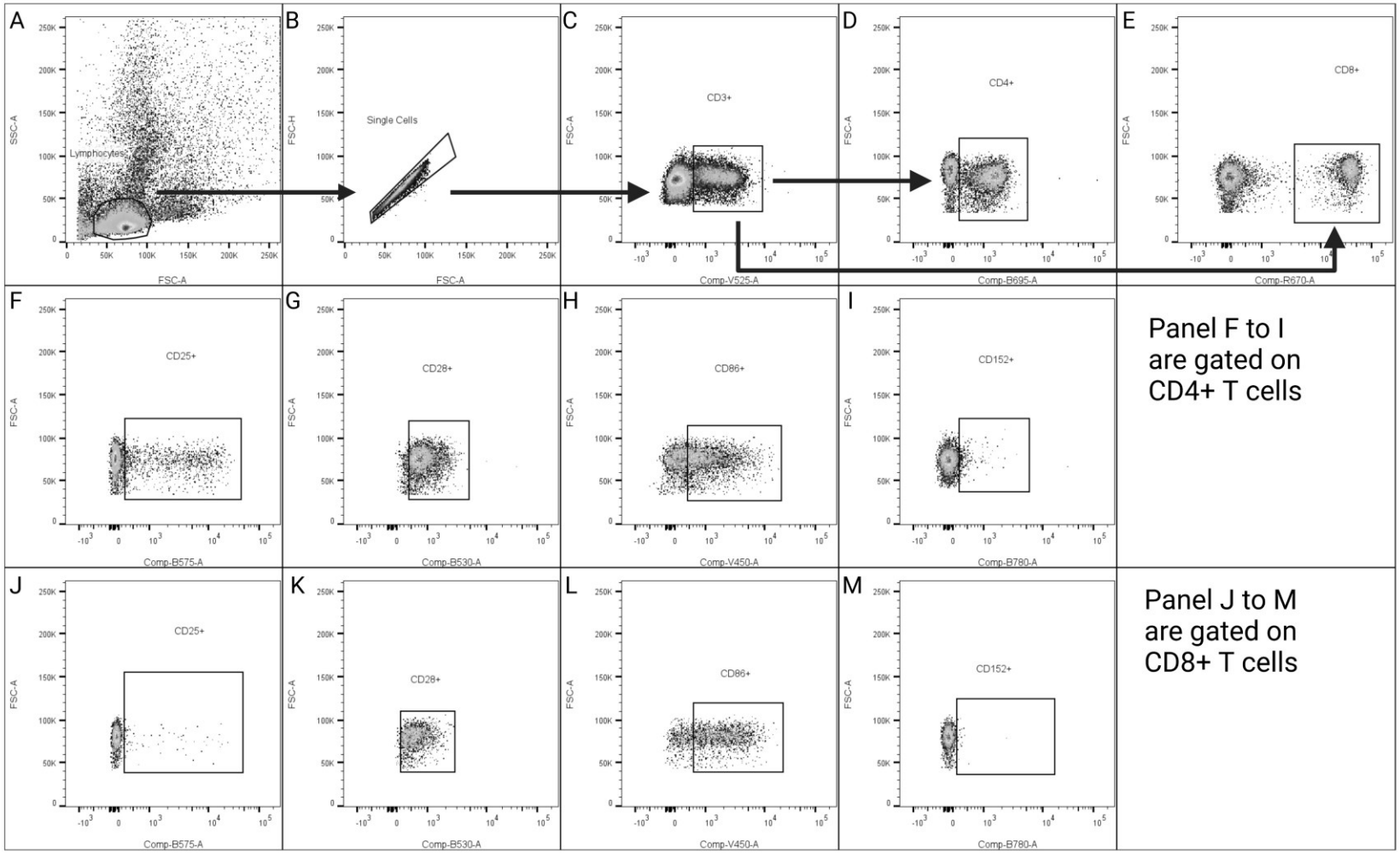

Figure 7.2. [d] Representative plots for **T cell gating strategy**. Splenocytes were first gated to include all the lymphocytes based on side-scatter area versus forward-scatter area dot-plot (A) followed by the exclusion of doublets and debris based on forward-scatter height versus forward-scatter area (B). Single cells were then analyzed for frequency (%) of CD3+ (C), followed by gating on CD4+ (D) T helper cells or CD8+ (E) T cytotoxic cells. The CD25+ (F), CD28+ (G), CD86+ (H) or CD152+ (I) were gated on CD4+ T cells. The CD25+ (J), CD28+ (K), CD86+ (L) or CD152+ (M) were gated on CD8+ T cells.

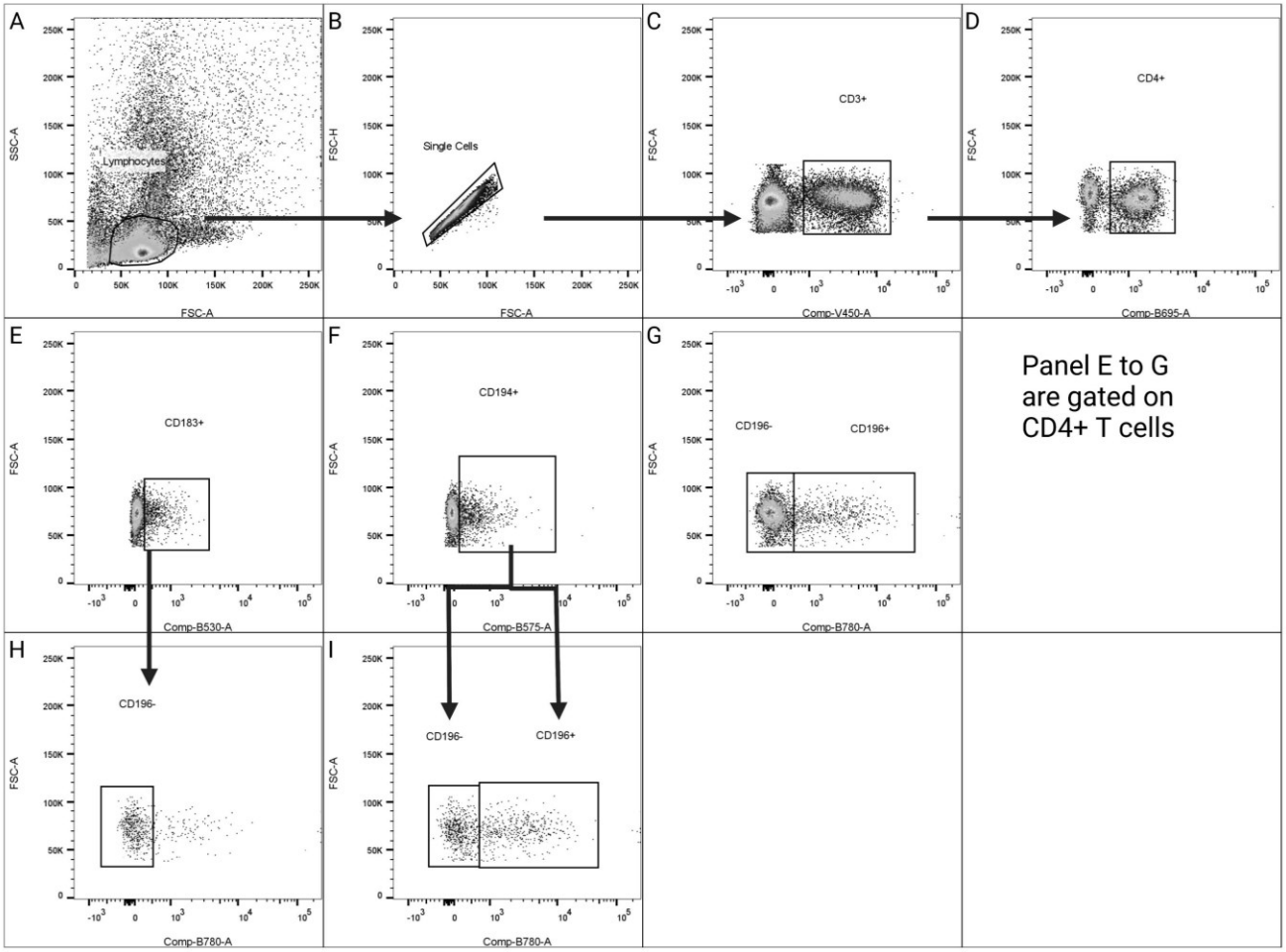

Figure 7.2. [e] Representative plots for **T helper cell subtype gating strategy**. Splenocytes were first gated to include all the lymphocytes based on side-scatter area versus forward-scatter area dot-plot (A) followed by the exclusion of doublets and debris based on forward-scatter height versus forward-scatter area (B). Single cells were then analyzed for frequency (%) of CD3+ (C) followed by CD4+ (D). The CD183+ (E), CD194+ (F) and CD196+ (G) were gated on CD4+ T cells. The T helper type 1 was identified from CD3+ CD4+ CD183+ CD196-. The T helper type 2 was identified from CD3+ CD4+ CD196- CD194+. The T helper type 17 was identified from CD3+ CD4+ CD196+ CD194+.

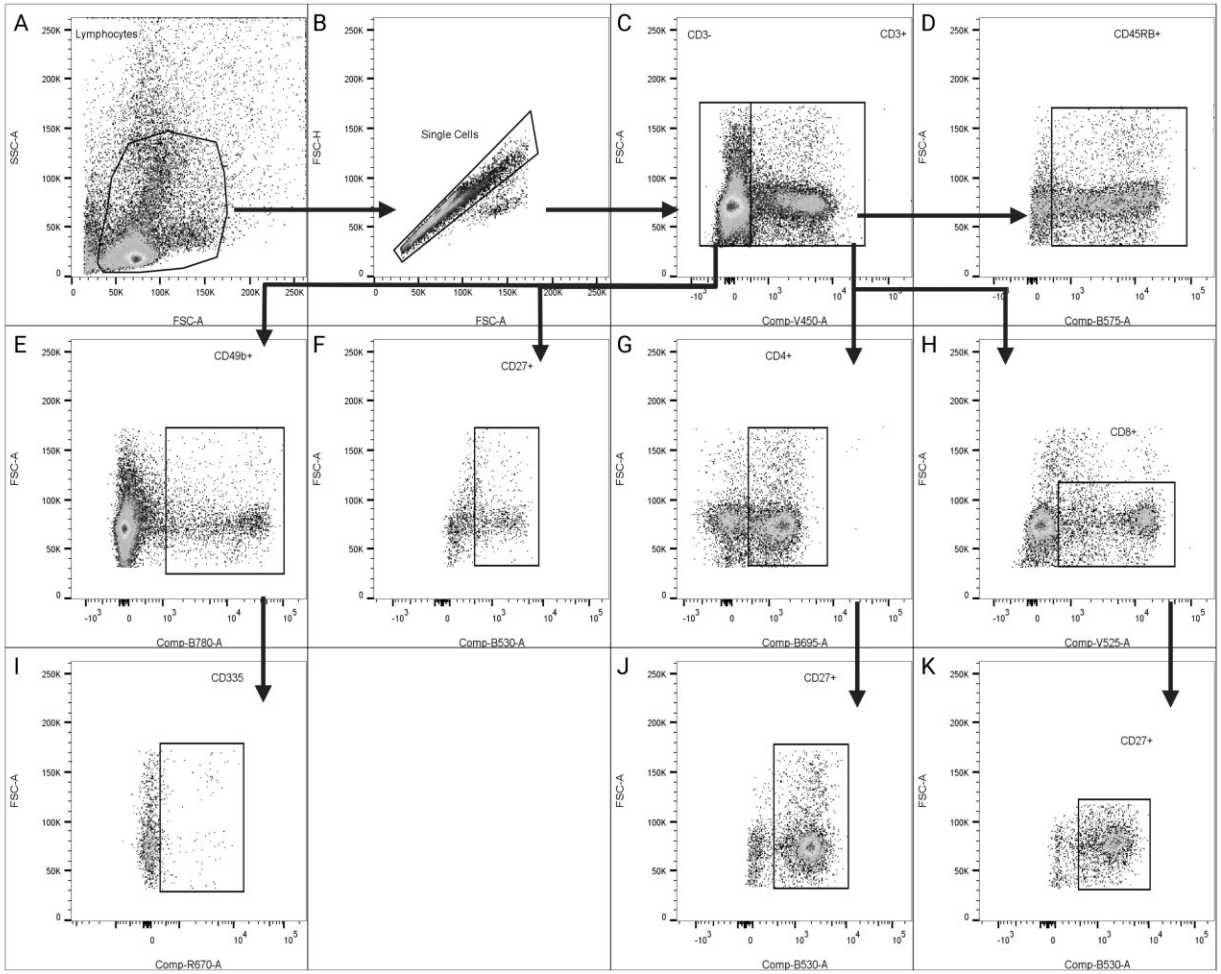

Figure 7.2. [f] Representative plots for **natural killer cells gating strategy**. Splenocytes were first gated to include all mononuclear cells based on side-scatter area vs. forward-scatter area dot-plot (A) followed by the exclusion of doublets and debris based on forward-scatter height versus forward-scatter area (B). Single cells were then analyzed for frequency (%) of CD3 (C) for CD3+ T cells and CD3– non-T cells. The CD49b+ (E) or CD27+ (F) were gated on CD3– cells. The CD4+ (G) or CD8+ (H) were gated on CD3+ cells. The CD335+ (I) or CD27+ (F) were gated on CD3– CD49b+ (NK cells). The NKs were identified from CD3- CD49b+. The natural killer T cells were identified from CD3+ CD49b+. The naïve T cells were identified from CD3+ CD45RB+.

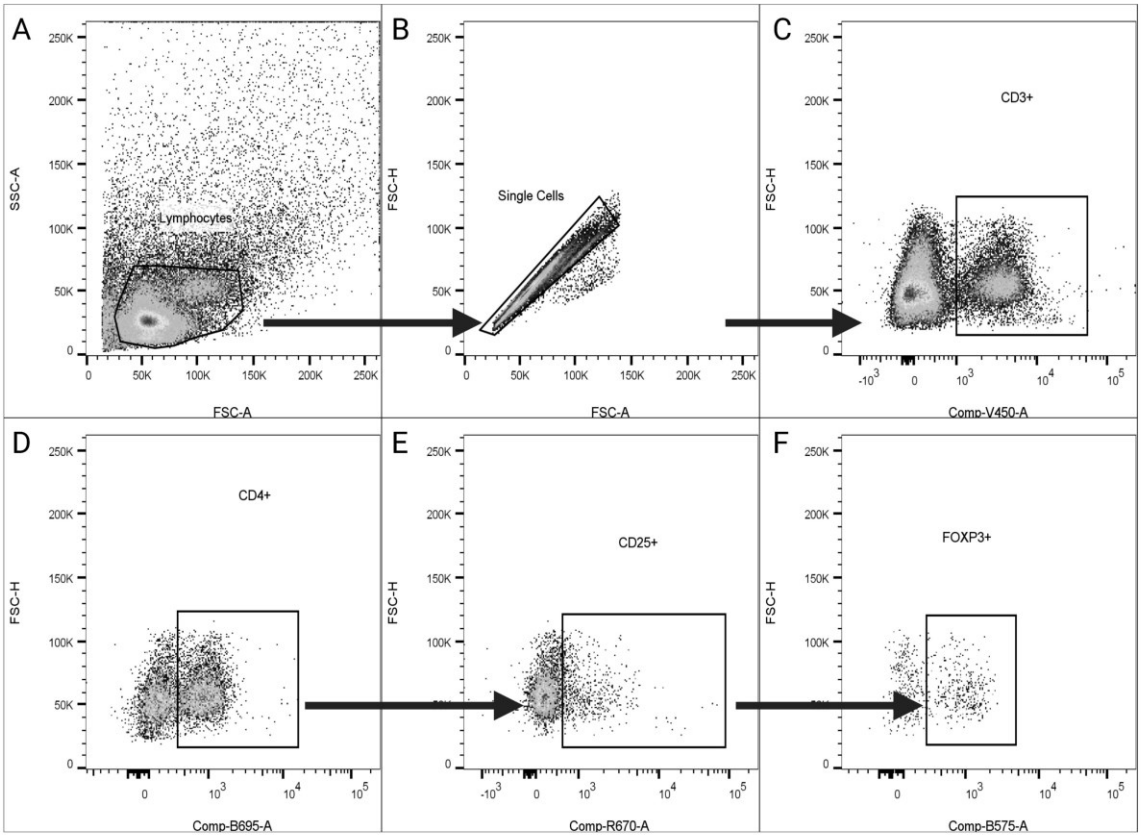

Figure 7.2. [g] Representative plots for **T regulatory cell subtype gating strategy**. Splenocytes were first gated to include all the lymphocytes based on side-scatter area versus forward-scatter area dot-plot (A) followed by the exclusion of doublets and debris based on forward-scatter height versus forward-scatter area (B). Single cells were then analyzed for frequency (%) of CD3+ (C) followed by CD4+ (D) and CD25+ (E). Subsequently, intracellular staining of FoxP3 was used to identify T regulatory cells. The T regulatory cells were identified from CD3+ CD4+ CD25+ FoxP3+ cells.

7.3. Results

7.3.1. Anthropometric measurements of 6-week-old pups.

Bodyweight: The SPD and OT intervention effect or their interaction effect did not have influence on the bodyweight of 6-wk pups. However, the pups' fed DHA+ARA WD had significantly high bodyweight than controls (19.1±0.4 vs. 18.0±0.3; $P = 0.02$).

Food intake: The SPD and OT intervention did not have influence on the average food intake between week 3 to week 6. A small but significant reduction in daily average of food intake was observed in DHA+ARA WD group pups compared to control WD (week-4: 2.8±0.1 vs. 3.5±0.2; $P < 0.001$, week-5: 5.0±0.3 vs. 5.7±0.4; $P = 0.01$, week-6: 7.3±0.5 vs. 7.5±0.6; $P = 0.03$).

Spleen weight and cell count: The weight of spleen and splenocyte count showed no significant effect of SPD, WD, or OT intervention.

7.3.2. Plasma concentration of ova-IgE and ova-IgG in 6-week pups.

A significant SPD × OT interaction effect was observed for ova-IgE (Figure 7.3 A). Post-hoc analysis showed feeding DHA+ARA SPD resulted in lower levels of plasma ova-IgE than control SPD (7.6±1.4 vs. 25.1±2.9, $P = 0.01$) but only when pups were exposed to sucrose (placebo exposed) OT. Similarly, in ova OT group pups, Ova-IgE was found to be lower but did not reach significant in DHA+ARA SPD fed pups compared to control SPD ($P = 0.30$). This indicated providing DHA+ARA SPD lowered plasma ova-IgE.

A significant OT effect was observed for ova-IgG (Figure 7.3 B). Contrary to our hypothesis, pups exposed to ova showed higher ova-IgG concentration than sucrose exposed groups (6.2±0.4 vs. 5.4±0.3; $P = 0.01$), irrespective of SPD or WD intervention.

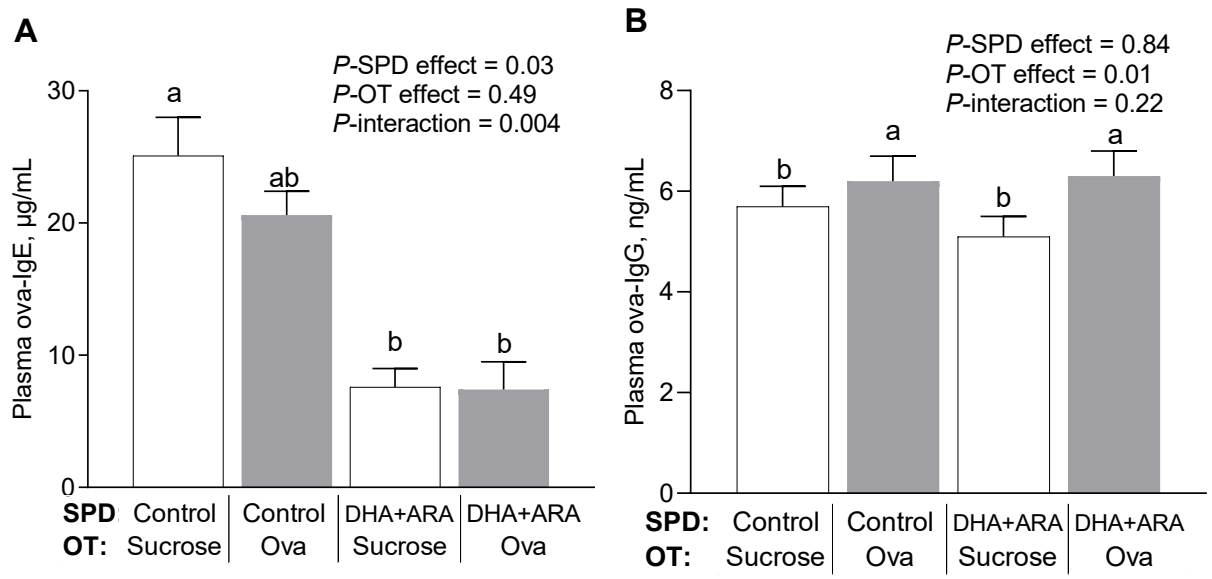

Figure 7.3. The effect of SPD and OT effects on in-vivo responses. The plasma concentration of and ova-IgE (A) and ova-IgG (B). Statistical analysis was conducted using 3-way ANOVA and results from SPD × OT interactions are described here. Bars without a common letter differ significantly at $P < 0.05$. Values are presented in mean ± SEM. Abbreviation: ARA, arachidonic acid; DHA, docosahexaenoic acid OT, oral tolerance; ova, ovalbumin; ova-IgG, ova-immunoglobulin G; SPD, suckling period diet.

7.3.3. *Ex-vivo* antibody response (IgG subtypes) by splenocytes with different stimuli.

Ova stimulation: The *ex-vivo* production of total IgG and IgG1 by splenocytes after stimulation with ova was significantly lower in Ova exposed pups compared to sucrose group (both $P <$ 0.001), irrespective of SPD or WD (shown in Figure 7.4 A and 7.4 B respectively). The difference in IgG1 levels between ova and sucrose OT, was greater in DHA+ARA WD group pups (1.3±0.3 vs. 27.2±8.5, $P = 0.02$), compared to control WD group pups (1.0±0.2 vs. 9.9±3.4, $P = 0.02$) (Figure 7.4 B). Stimulation of splenocytes with ova elicited higher IgG2a levels (3.8±0.9 vs. 1.8±0.9; $P = 0.02$) in pups from DHA+ARA SPD vs. control, whereas WD showed no significant effect (Figure 7.4 C). Additionally, Ova OT group pups showed was higher IgG2a than sucrose group (3.5±1.0 vs. 2.1±0.3; $P = 0.03$) (Figure 7.4 C).

LPS stimulation: The *ex-vivo* production of total IgG and IgG1 by splenocytes after stimulation with LPS was significantly lower in Ova exposed pups compared to sucrose group (both $P < 0.05$), irrespective of SPD or WD effect (shown in Figure 7.5 A and 7.5 B respectively). Note, the IgG1 also showed a significant SPD × WD × OT interaction ($P = 0.02$), but only relevant findings are shown in figure. On the contrary, the IgG2a production with LPS stimulation showed no significant difference between OT group pups (4.2±0.4 vs. 6.0±1.7, $P = 0.27$) (Figure 7.5 C).

PMAi stimulation: The *ex-vivo* production of total IgG, and IgG1 by splenocytes after stimulation with PMAi was significantly lower in Ova exposed pups compared to sucrose group (all $P < 0.001$) (shown in Figure 7.5 D and 7.5 E). The *ex-vivo* production of IgG2a also showed a significant SPD effect where DHA+ARA SPD fed pups showed lower response compared to control SPD fed pups (1.3±0.2 vs. 3.0±0.5; $P = 0.02$) (Figure 7.5 F). Additionally, Ova OT group pups showed higher IgG2a levels than sucrose group (Figure 7.5 F).

7.3.4. *Ex-vivo* cytokine production by splenocytes with different stimuli.

Table 7.2 provides data on *ex-vivo* cytokine production by different mitogen.

Ova stimulation:

The IL-2 production by ova stimulated splenocyte showed significant OT effect, where ova exposed OT group was lower than sucrose (24.5±1.4 vs. 34.4±2.9; $P < 0.001$) (shown in Figure 7.4 D). Similarly, IL-6 production showed significant OT effect, in which ova OT group was lower than sucrose (912.9±61.5 vs. 2165.5±168; $P < 0.001$), irrespective of SPD or WD (Figure 7.4 E). Additionally, a significant SPD × WD indicated that IL-6 production to ova stimulation *ex-vivo* was lower in pups from DHA+ARA SPD + DHA+ARA WD group compared to DHA+ARA SPD + control WD (842.5±126.1 vs 1997±282.2; $P = 0.03$) (Figure 7.4

E). Finally, IL-10 production was also significantly lower in pups exposed to ova OT compared to sucrose OT group (102.7±4.1 vs 128.4±5.6; $P < 0.001$) irrespective of SPD or WD (Figure 7.4 F).

The IL-4 production by splenocytes stimulated with ova showed a significant SPD × WD × OT interaction ($P = 0.03$, described in Figure 7.6 A). The *post-hoc* analysis indicated that Ova OT group was significantly lower that sucrose OT group in all diet group combination except DHA+ARA SPD + DHA+ARA WD. Thus, providing DHA+ARA during SPD and WD lowered the IL-4 to Ova stimulation, making the OT group difference non-significant (Figure 7.6 A). The IL-5 production with ova stimulation showed significant SPD × OT interaction effect, in which ova OT group had lower IL-5 levels than sucrose group but only when pups were fed control SPD (Figure 7.6 B). This OT effect on IL-5 level was absent when pups were fed DHA+ARA SPD (Figure 7.6 B). IL-1β, IFN-γ and TNF-α production by splenocytes stimulated with ova showed no significant differences due to SPD, WD or OT. However, CXCL-1 and IL-12 showed significant SPD × OT effect in which ova exposed OT group was lower for CXCL-1 and IL-12 than sucrose but only when pups were fed DHA+ARA SPD (Figure 7.6 C and 7.6 D). Note, the cytokine production by splenocytes in unstimulated condition was found to be below detection in our preliminary analyses.

LPS stimulation:

For IL-4 and TGF-β *ex-vivo* response to LPS stimulation, ova OT group was lower than sucrose OT group irrespective of SPD or WD intervention (Figure 7.7 A and 7.7 B respectively). A significant WD × OT interaction effect showed that IL-6 response to LPS was lower in ova OT group than sucrose but only when pups were fed DHA+ARA WD (3210.0±388.8 vs. 4638.4±505.5; $P = 0.006$, shown in Figure 7.7 C).

The LPS stimulation of splenocytes from pups from DHA+ARA SPD elicited lower IFN-γ, TNF-α, CXCL-1 ($P = 0.07$) and TGF-β compared to control SPD (Figure 7.8 A – D). These SPD effects were observed independent of WD and OT intervention.

PMAi stimulation:

The IL-2 production showed marginal significant OT effect, in which ova OT group was lower than sucrose (9169.2±206.5 vs. 9312.6±190.0; $P = 0.049$), irrespective of SPD or WD. The IFN-γ production to PMAi stimulation showed significant SPD × WD effect but no *post-hoc* differences were found. Further, the cytokines IL-10, IL-12, IL-1β, IL-4, IL-5, IL-6, CXCL-1 and TNF-α showed no significant differences due to SPD, WD or OT intervention.

Anti-CD3/anti-CD28 stimulation:

The *ex-vivo* production of IL-2 and IFN-γ was significantly higher in pups fed DHA+ARA SPD compared to control (respectively 12481.3±119.0 vs. 11429.7±42.5; $P < 0.001$ and 4036.1±65.1 vs. 3007.7±56.5; $P < 0.001$), irrespective of WD and OT interventions (Figure 7.8 E and 7.8 F). Similarly, IL-1β was also significantly higher in DHA+ARA SPD group than control (12.1±0.6 vs. 8.7±0.4; $P < 0.001$) (Figure 7.8 G). There was a significant interaction between SPD, WD and OT suggesting the difference in IL- 1β associated with SPD changes was dependent on WD and OT intervention (for specific differences see Figure 7.8 G). The stimulation of splenocytes with anti-CD3/anti-CD28 resulted in significantly lower IL-4 and IL-6 when pups were fed DHA+ARA WD compared to control (respectively 363.0±24.5 vs. 471.4±33.5; $P = 0.01$ and 2615.9±254.2 vs. 2956.2±205.9; $P = 0.04$), irrespective of SPD or OT intervention. Finally, significant OT effect on IL-5 and CXCL-1 was observed in which ova exposed OT group pups were lower than sucrose OT group pups, respectively 162.4±17.2 vs.

198.0±10.2; $P = 0.02$ and 88.7±7.9 vs. 129.0±13.8; $P = 0.03$ (Figure 7.8 H and 7.8 I respectively). These OT effects were observed independent of SPD or WD.

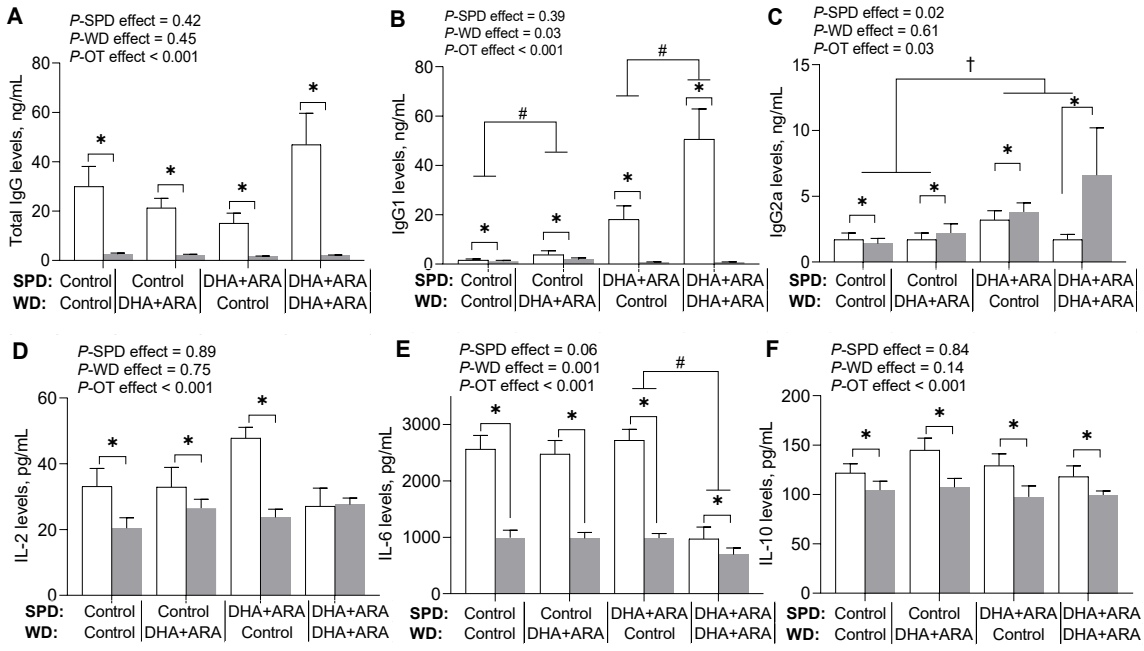

Figure 7.4. The SPD, WD and OT effects on *ex-vivo* antibody and cytokine responses by splenocytes with different stimuli. *Ex-vivo* ova stimulated splenocyte level of total IgG (A), IgG1 (B) and IgG2a (C). The production of IL-2 (D), IL-6 (E) and IL-10 (F) by splenocytes stimulated with ova. We reported difference between sucrose (white bars) and ova (gray bar) OT group (✱) at $P < 0.05$. Difference between DHA+ARA SPD and control SPD group (†) shown at $P < 0.05$. Difference between DHA+ARA WD and control WD group (#) shown at $P < 0.05$. Values are presented as mean ± SEM. Abbreviation; ARA, arachidonic acid; DHA, docosahexaenoic acid; IgG, immunoglobulin-G; IL, interleukin; LPS, lipopolysaccharides; Ova, ovalbumin; OT, oral tolerance; PMAi, phorbol–myristate–acetate and ionomycin, SPD, suckling period diet; WD, weaning diet.

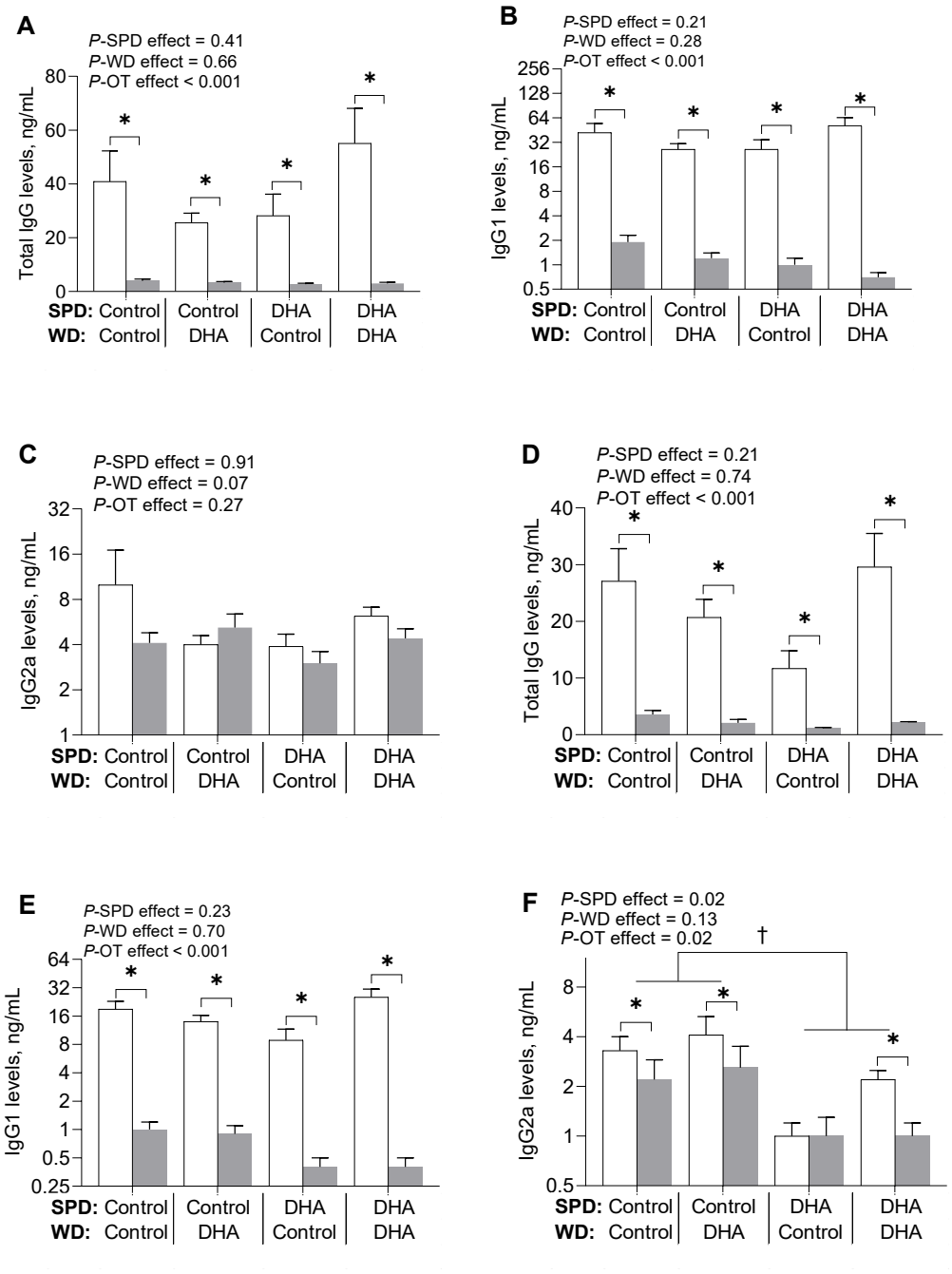

Figure 7.5. The SPD, WD and OT effects on *ex-vivo* antibody and cytokine responses by splenocytes with different stimuli. *Ex-vivo* LPS stimulated splenocyte level of total IgG (A), IgG1 (B) and IgG2a (C); and PMAi stimulated splenocyte levels of total IgG (D), IgG1 (E) and IgG2a (F).

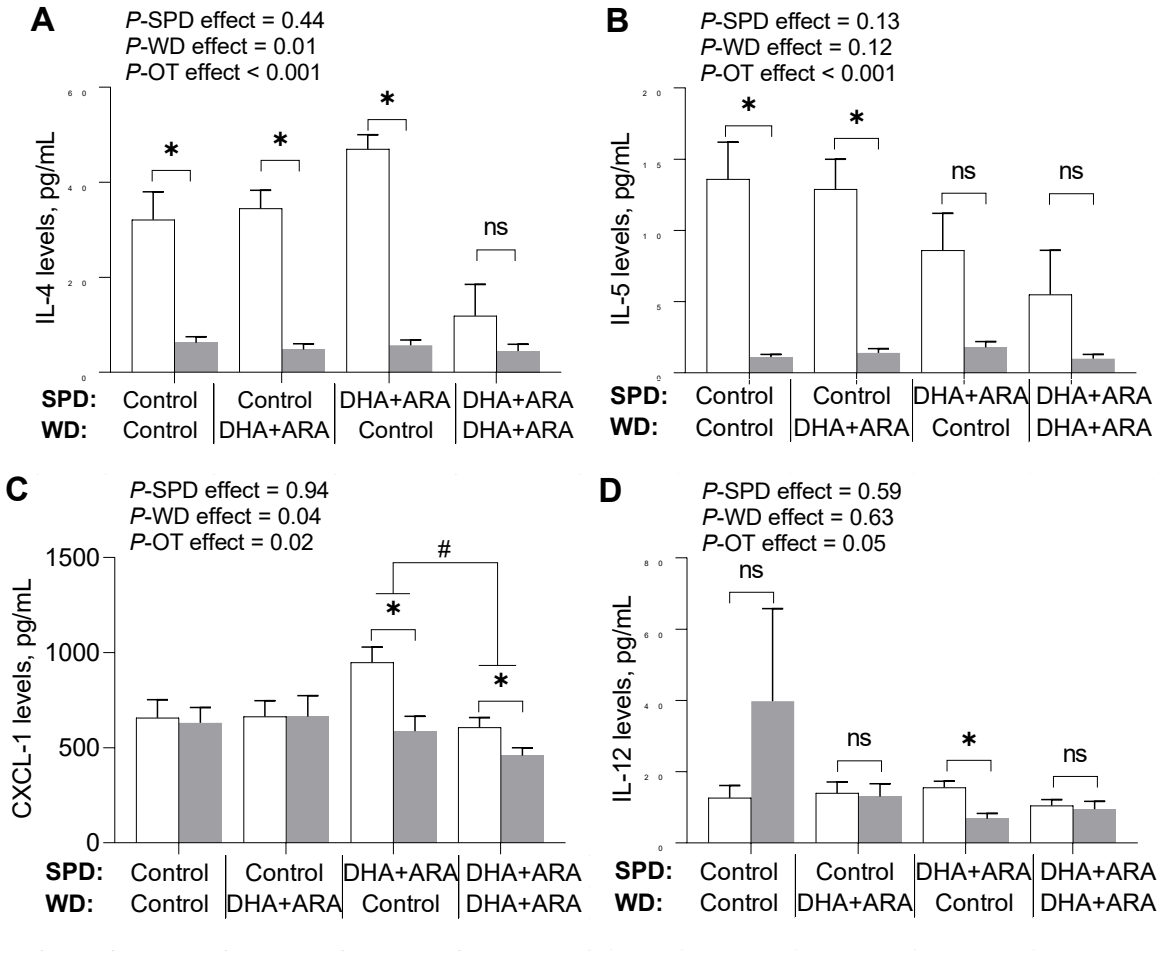

Figure 7.6. The SPD, WD and OT effects on cytokine production by splenocytes stimulated with different stimuli. The production of IL-4 (A) IL-5 (B), CXCL-1 (C) and IL-12 (D) by splenocytes stimulated with ova; Significant differences between sucrose (white bars) and ova (gray bar) OT group were reported (✱) at $P < 0.05$. Significant difference between DHA+ARA SPD and control SPD group were reported (†) at $P < 0.05$. Significant difference between DHA+ARA WD and control WD group were reported (#) at $P < 0.05$. Values are presented as mean ± SEM. Abbreviation: ARA, arachidonic acid; DHA, docosahexaenoic acid; CXCL1, C-X-C motif ligand 1; IL, interleukin; OT, oral tolerance; ova, ovalbumin; SPD, suckling period diet; WD, weaning diet

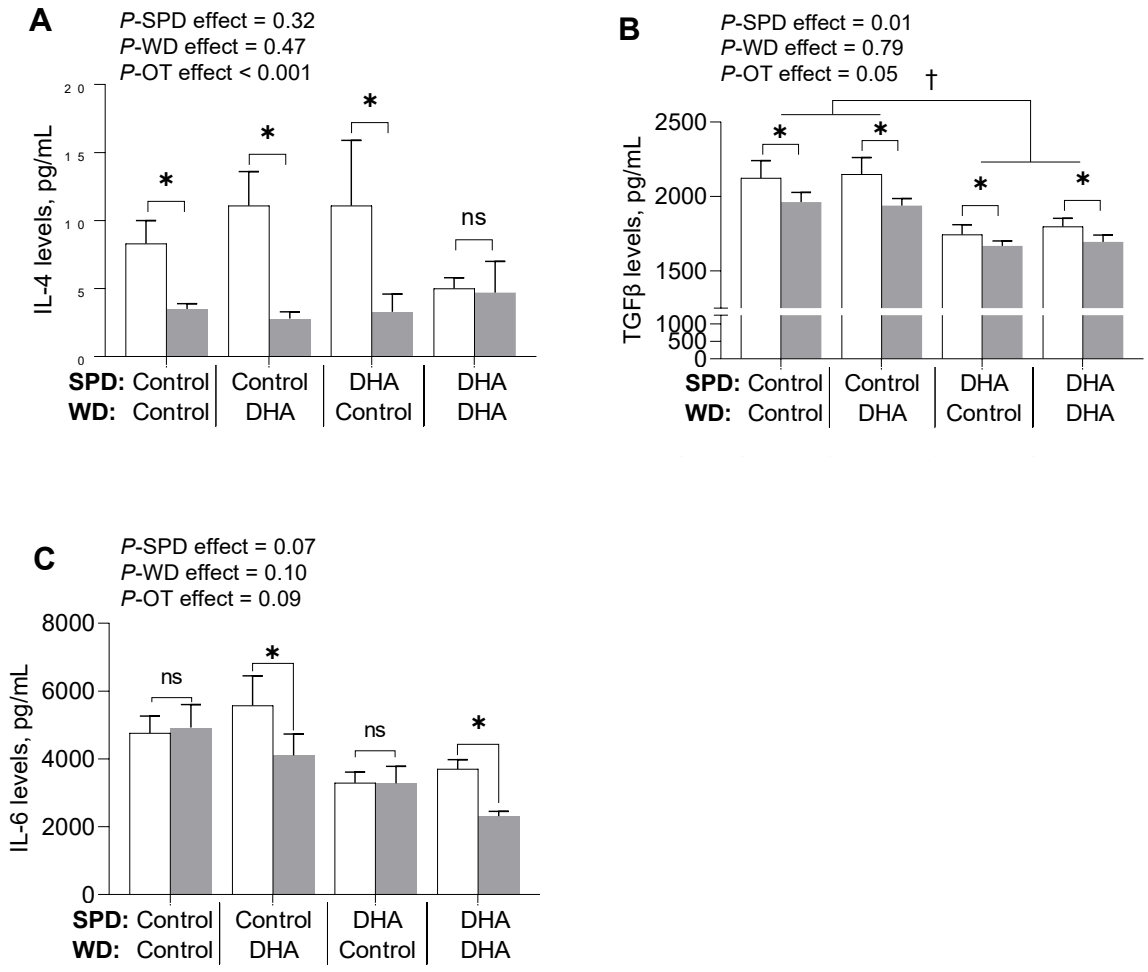

Figure 7.7. The SPD, WD and OT effects on cytokine production by splenocytes stimulated with different stimuli. The production of IL-4 (A), TGF-β (B) and IL-6 (C) production by splenocytes stimulated with LPS. Significant differences between sucrose (white bars) and ova (gray bar) OT group were reported (✱) at $P < 0.05$. Significant difference between DHA+ARA SPD and control SPD group were reported (†) at $P < 0.05$. Significant difference between DHA+ARA WD and control WD group were reported (#) at $P < 0.05$. Values are presented as mean ± SEM. Abbreviation: TGF-β, transforming growth factorβ; CXCL1, C-X-C motif ligand 1; IL, interleukin

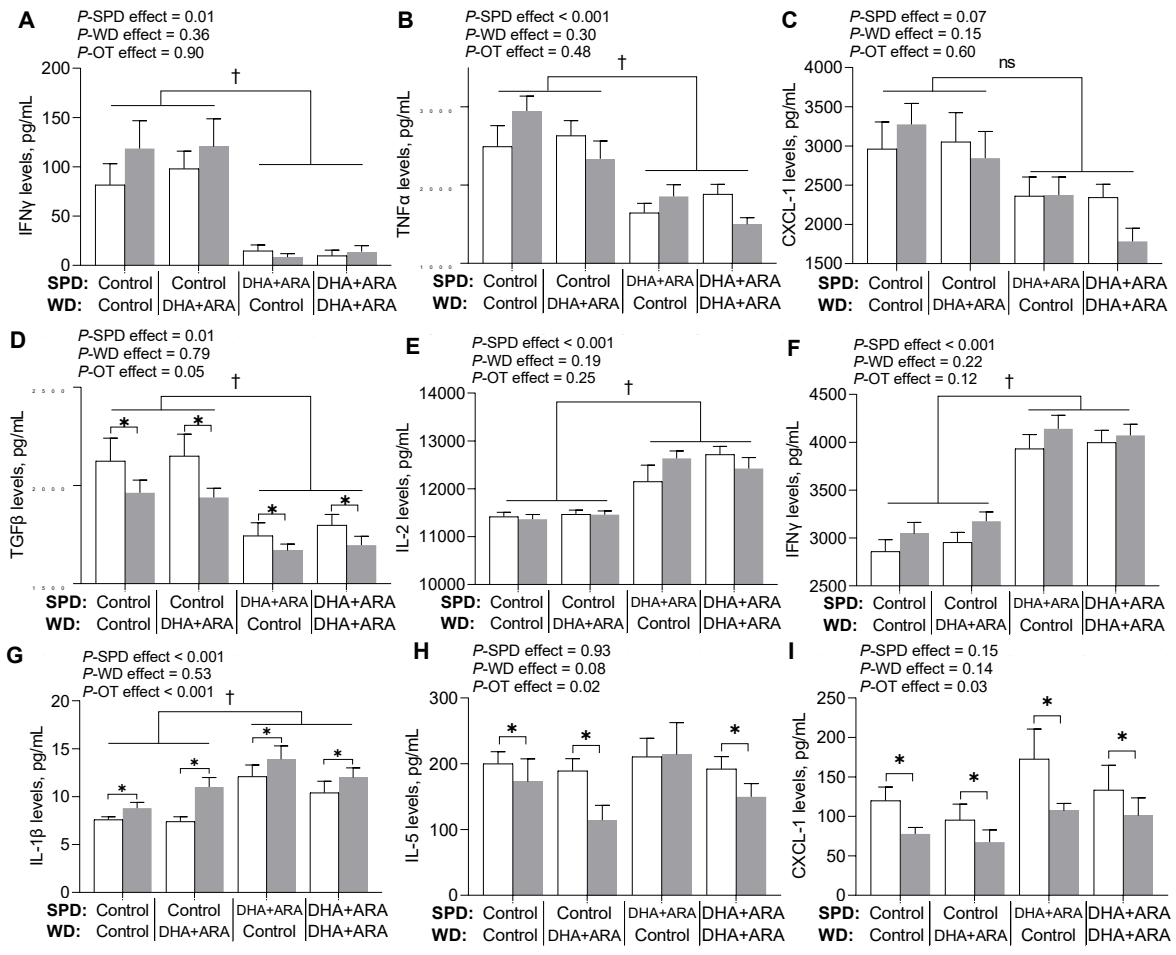

Figure 7.8. The SPD, WD and OT effects on *ex-vivo* cytokine response by splenocytes with different stimuli. The production of IFN-γ (A), TNF-α (B), CXCL-1 (C) and TGF-β (D) by splenocytes stimulated with LPS, and IL-2 (E), IFN-γ (F), IL-1β (G) IL-5 (H) and CXCL-1 (I) by splenocytes stimulated with anti-CD3/anti-CD28. The statistical analysis was conducted using 3-way ANOVA. Significant differences between sucrose (white bars) OT and ova (gray bar) OT group was reported (✱) at $P < 0.05$. Significant difference between DHA+ARA SPD and control SPD group was reported (†) at $P < 0.05$. Abbreviation; : ARA, arachidonic acid; DHA, docosahexaenoic acid; CXCL1, C-X-C motif ligand 1; IL, interleukin; IFN-γ, interferon-gamma; LPS, lipopolysaccharides; Ova; OT, oral tolerance; ova, ovalbumin; PMAi, phorbol–myristate–acetate and ionomycin; TNF-α, tumor necrosis factor-alpha; TGF-β, transforming growth factor-beta.

Table 7.2. The effect of SPD and WD on *ex vivo* cytokine production by splenocytes to different stimuli.

SPD WD	Control Control	Control DHA+ARA	DHA+ARA Control	DHA+ARA DHA+ARA	SPD ×WD *P* - value
Ova stimulation, pg/ml					
IFN-γ	21.5 ± 5.9	30.7 ± 9.4	11.1 ± 2.1	15.2 ± 4.1	0.75
IL-10	113 ± 6.6	126.1 ± 8.7	115.9 ± 9.3	109.3 ± 6.4	0.28
IL-12	26.2 ± 13.1	13.5 ± 2.2	11.9 ± 1.7	9.9 ± 1.3	0.43
IL-1β	12.5 ± 1.3	13.4 ± 1.4	14 ± 1.2	10.8 ± 1.3	0.01
IL-2	26.7 ± 3.4	29.6 ± 3.2	36.8 ± 4.3	27.3 ± 3	0.24
IL-4	19.1 ± 4.4	19.6 ± 4.2	29.8 ± 6.3	8.4 ± 3.5	0.04
IL-5	7.3 ± 2	7.1 ± 1.8	5.7 ± 1.8	3.4 ± 1.6	0.07
IL-6	1777.3 ± 244	1727.5 ± 231.2	1997.9 ± 282.2	842.5 ± 126.1	0.02
CXCL1	644 ± 60	663.6 ± 66.6	797.8 ± 76.5	538.1 ± 37.9	0.02
TNF-α	758.3 ± 57.5	786.3 ± 58.1	646 ± 21.4	543.5 ± 37.2	0.09
TGF-β	1288 ± 38	1343 ± 49	1258 ± 35	1221 ± 21	0.25
Anti-CD3/anti-CD28 stimulation, pg/ml					
IFN-γ	2946.4 ± 85.8	3065.2 ± 74	4036.5 ± 102.5	4035.5 ± 83.5	0.77
IL-10	2401.3 ± 331.9	2197 ± 341.2	3307 ± 524.7	2640.1 ± 295.9	0.13
IL-12	29.2 ± 2.6	28.3 ± 2.4	24.2 ± 2.2	23.9 ± 1	0.60
IL-1β	8.1 ± 0.3	9.2 ± 0.7	13 ± 0.9	11.1 ± 0.7	0.03
IL-2	11393.6 ± 64.5	11463.5 ± 56.6	12393.5 ± 192.2	12569 ± 143.3	0.59
IL-4	395.2 ± 32.6	319 ± 31.3	542.7 ± 52.1	406.9 ± 35.1	0.13
IL-5	187.2 ± 18.5	151.7 ± 17	212.4 ± 26.9	170.9 ± 14.4	0.98
IL-6	3175 ± 321.8	2943.3 ± 454.9	2751 ± 260.3	2288.5 ± 214.1	0.32
CXCL1	100.2 ± 11.1	81.3 ± 12.8	137.7 ± 19.6	117.1 ± 19	0.52
TNF-α	1191.4 ± 88.3	1243.5 ± 112.7	1395.5 ± 52	1337.1 ± 55.1	0.54
TGF-β	1234 ± 59	1367 ± 82	1350 ± 19	1402 ± 26	0.56
LPS stimulation, pg/ml					
IFN-γ	100.1 ± 17.8	109.2 ± 16.3	11.3 ± 3.4	11.9 ± 4	0.74
IL-10	1229.6 ± 97.9	1107.8 ± 57.3	1070.2 ± 95.7	1067.5 ± 70.4	0.65
IL-12	35 ± 5.2	33 ± 5.8	67.7 ± 36.8	25.2 ± 11.6	0.24
IL-1β	87.1 ± 12.2	78.8 ± 10.8	65.4 ± 10	59.3 ± 8	0.97
IL-2	5.9 ± 1	6.9 ± 1.6	7.2 ± 2.6	4.8 ± 1.2	0.61
IL-4	2.2 ± 0.2	2.2 ± 0.1	1.7 ± 0.3	1.5 ± 0.3	0.59
IL-5	4844.8 ± 407.8	4838.7 ± 555.3	3285 ± 287.2	3009.6 ± 234.1	0.71
IL-6	3118.5 ± 213.9	2948.2 ± 245.2	2368 ± 161.3	2045.2 ± 136.2	0.72
CXCL1	2719.8 ± 169.4	2483 ± 149.6	1750.8 ± 97	1691.9 ± 88.1	0.79
TNF-α	2044 ± 67	2045 ± 64	1707 ± 37	1747 ± 36.9	0.78
PMAi stimulation, pg/ml					
IFN-γ	1922.7 ± 398.9	1162.1 ± 291.7	603 ± 94.8	709.7 ± 84.8	0.01
IL-10	848.8 ± 179.8	863 ± 138.2	981.8 ± 148.3	1264 ± 182.9	0.69
IL-12	21.7 ± 2.7	19.6 ± 2.9	18 ± 1.9	19.6 ± 1	0.42
IL-1β	5.5 ± 0.6	6.1 ± 0.7	4.1 ± 0.3	5.2 ± 0.4	0.97
IL-2	9232.2 ± 385.7	8791 ± 462.9	9278.4 ± 94.2	9580.5 ± 49	0.17

IL-4	181.5 ± 26.3	146 ± 18.7	190.7 ± 25.1	203.5 ± 19.7	0.58
IL-5	94 ± 16.4	75.2 ± 11.1	80.5 ± 12.9	89.4 ± 17.2	0.52
IL-6	2297.3 ± 337.8	2367 ± 323.8	2286 ± 339.9	2606.4 ± 437.3	0.97
CXCL1	57.1 ± 7	50.5 ± 7.1	59.3 ± 7.3	57.3 ± 6.1	0.40
TNF-α	1280.7 ± 312.3	1305.7 ± 298.7	1419.9 ± 160.9	1746.1 ± 126.1	0.86
TGF-β	1738 ± 45	1712 ± 79	1393 ± 46.3	1393.7 ± 35	0.61

Abbreviation: ova, ovalbumin; LPS, lipopolysaccharide, PMAi, phorbol–myristate–acetate and ionomycin; IFN-γ, interferon-gamma; IL, interleukin; TNF-α, tumor necrosis factor-alpha; TGF-β, transforming growth factor-beta; CXCL-1, chemokine (C-X-C motif) ligand 1; DHA, docosahexaenoic acid; ova, ovalbumin; OT, oral tolerance

7.3.5. Immune cell phenotype of unstimulated splenocytes.

In summary, table 7.3 provides phenotype differences associated with SPD (irrespective of WD or OT); table 7.4 provides phenotype differences associated with SPD X OT interaction and table 7.5 provides phenotype differences associated with WD (irrespective of SPD or OT). Finally, table 7.9 provides a summary of the key findings of the current study. Overall, some changes in the relative proportion of immune cells were observed between different SPD, WD and OT groups. These changes are described below. In splenocytes, the proportion of total CD3+ cells, CD3+ CD4+ (Th) cells and CD3+ CD8+ (cytotoxic T) cells did not differ significantly between SPD group pups. However, some specific T cell phenotype were found to be significantly lower in splenocytes from pups fed DHA+ARA SPD compared to control SPD. These phenotypes include CD3+ CD45R+ (naïve T cells), CD3+ CD4+ CD25+ (CD25+ Th cells), CD3+ CD4+ CD152+ (CTLA-4 Th cells), and CD3+ CD8+ CD152+ (CTLA-4 CTL). A 3-way SPD × WD × OT interaction was observed for some T cell phenotype (see Figure 7.9). The *post-hoc* analysis showed that, when pups did not receive DHA+ARA in SPD and/or WD, the ova OT group had significantly higher total CD3+, CD3+ CD45RB+, CD3+ CD4+ CD27+ and CD3+ CD8+ CD27+ proportion in splenocytes than sucrose OT group pups (Figure 7.9 A – D). In other words, when pups received DHA+ARA during SPD and/or WD, these differences between OT groups were absent.

The splenocyte of pups from DHA+ARA SPD groups were associated with lower proportion of CD11c+ cells (total DC and some macrophage) and CD3- CD49b+ CD27+ (CD27+ NK cells) compared to control SPD (both P's < 0.05) (Table 7.3). Additionally, a significant SPD x OT effect was also observed for CD11c+ CD8a+ CD4- (enriched for conventional DC type-1 (cDC1)) cell proportion, in which ova group was significantly higher

than sucrose OT group but only when pups were fed control SPD (1.1±0.06 vs. 0.9±0.05; P = 0.002) (Table 7.4).

Some phenotypic changes were also observed for the B cell in splenocytes. The proportion IgM+ on CD45R+ CD19- CD138+ (IgM plasma cells) were found to be significantly lower in pups fed DHA+ARA SPD vs. control SPD (Table 7.3). The DHA+ARA WD group pups' splenocytes showed higher proportion of CD19+ IgM+ (primary B cell) than control WD, irrespective of SPD and OT (Table 7.5). The proportion of CD19+ IgG+ (secondary B cell) showed no significant difference due to WD intervention.

Table 7.3. The effect of suckling period diet on immune cell phenotype of splenocyte with or without stimulation.

Suckling period diet		**Control diet**	**DHA+ARA diet**	***P* - value**
Phenotype, *% splenocyte*	**Cell types[1]**			
Unstimulated condition				
CD3+ CD45RB+	Naïve T cells	23.6 ± 0.8	20.4 ± 0.6	0.003
CD25+ CD3+ CD4+	CD25+ Th	3.8±0.2	3.3±012	0.159
CD152+ CD3+ CD4+	CTLA-4+ Th	2.3±0.1	1.0±0.1	0.03
CD152+ CD3+ CD8+	CTLA-4+ CTL	0.6±0.03	0.3±0.02	0.002
CD3+ CD4+ CD183+ CD194-	Th1 cells	5.8±0.2	5.0±0.2	0.23
Total CD19+	Total B cells	52.3±0.9	53.4±0.5	0.59
CD19+ CD23+	Follicular B cells	37.2±0.9	38.1±0.6	0.65
CD19- CD45R+ CD138+	Total plasma cells	3.5±0.3	2.0±0.1	0.21
IgM+ on CD19- CD45R+ CD138+	IgM+ plasma cells	60.4±1.8	40.5±1.6	0.001
CD3+ CD49b+	NKT cells	6.1±0.6	4.6±0.6	0.43
CD3+ CD49b+ CD27+	CD27+ NKT cells	4.9±0.2	3.5±0.1	0.18
CD3- CD49b+	NK cells	10.7±0.5	8.5±0.3	0.19
CD3- CD49b+ CD27+	CD27+ NK cells	3.5±0.1	2.6±0.1	0.007
Total CD11c+	DC	16.0±0.5	10.7±0.3	0.002
CD11b+ CD68+	Macrophages	12.9±0.5	9.1±0.2	0.02
CD11b+ CD68+ CD163+	M2 type macrophage	3.9±0.2	3.4±0.2	0.87
LPS stimulation				
Total CD19+	Total B cells	52.9±0.8	55.6±0.8	0.96
CD19+ CD23+	Follicular B cells	29.8±0.8	35.2±0.9	0.01
CD19- CD45R+ CD138+	Total plasma cells	4.9±0.2	7.2±0.3	0.006
IgM+ on CD19- CD45R+ CD138+	IgM+ plasma cells	49.7±1.6	47.1±1.2	0.57
CD3+ CD49b+	NKT cells	12.7±0.01	6.6±0.01	0.005
CD3+ CD49b+ CD27+	CD27+ NKT cells	10.2±0.5	4.1±0.1	0.003
CD3+ CD49b+ CD335+	CD335+ on NKT	2.9±0.2	4.2±0.3	0.03
CD3- CD49b+	NK cells	9.4±0.1	8.9±0.1	0.45
Total CD11c+	DC	15.8±0.6	9.2±0.2	0.009
CD11b+ CD68+	Macrophages	12.5±0.5	8.4±0.3	0.02
CD11b+ CD68+ CD163+	M2 type macrophage	10.1±0.5	6.5±0.2	0.007

[1]The combinations of surface markers are suggestive of described specific immune cell subtypes. Note, alternative combinations of markers are also possible.

Abbreviation: Th, T helper cell; CTLA4, cytotoxic T-lymphocyte associated protein-4; DC, dendritic cells; NK, natural killer cells; NKT, natural killer T cells; IgM, immunoglobulin-A; DHA, docosahexaenoic acid

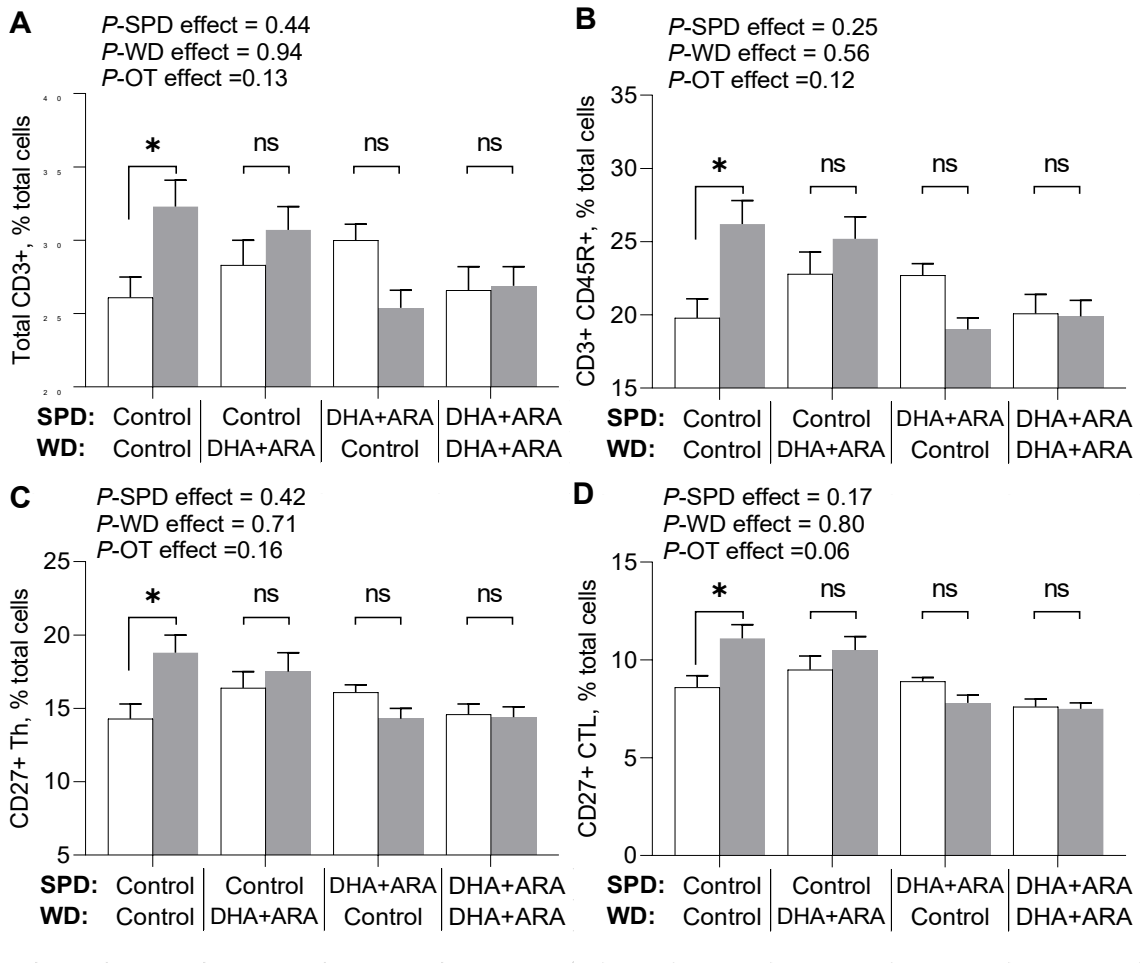

Figure 7.9. The SPD × WD × OT interaction effect on the T cell phenotypes in unstimulated splenocytes, total CD3+ (A), CD3+ CD45R+ (B), CD3+ CD4+ CD27+ (C) and CD3+ CD8+ CD27+ (D). The statistical analysis was conducted using 3-way ANOVA. Significant differences between sucrose (white bars) OT and ova (gray bar) OT group was reported (✱) at $P < 0.05$. Values are presented as means ± SEMs. Abbreviation: ARA, arachidonic acid; DHA, docosahexaenoic acid; CTL, cytotoxic T cell; OT, oral tolerance; Ova, ovalbumin; ns, non-significant; SPD, suckling period diet; Th, T helper; WD, weaning diet.

Table 7.4. The suckling period diet and oral tolerance interaction affect on immune cell phenotype of splenocytes with or without stimulation.

Suckling period diet		**Control diet**	**Control diet**	**DHA diet**	**DHA diet**	**SPD × OT**
OT group		**Sucrose**	**Ova**	**Sucrose**	**Ova**	***P* - value**
Phenotype, *% splenocytes*	**Cell type**[1]					
Unstimulated						
CD3+ CD49b+	NKT cells	5.8±0.6	6.0±0.6	4.6±0.6	4.7±0.6	0.83
CD3+ CD49b+ CD27+	CD27+ NKT	4.8±0.3	5.1±0.3	3.4±0.1	3.5±0.2	0.46
CD3- CD49b+	NK cells	10.8±0.01	10.5±0.01	8.4±0.03	8.5±0.03	0.62
CD3- CD49b+ CD27+	CD27+ NK cells	3.6±0.2	3.5±0.2	2.7±0.1	2.6±0.1	0.79
Total CD11c+	DC	16.2±0.7	15.8±0.6	10.7±0.4	10.7±0.4	0.94
CD11c+ CD4+ MHC-II+	pDC	5.4±0.3	5.7±0.3	3.5±0.1	3.4±0.1	0.22
[2]CD11c+ CD8a+ CD4-	cDC1	0.9±0.05 b	1.1±0.06 a	0.6±0.04 c	0.5±0.04 c	0.002
CD11b+ CD68+	Macrophage	13.2±1.1	12.2±1.1	8.7±1.1	9.7±1.1	0.07
PMAi stimulation						
Total CD3+	Total T cells	35.4±1.5 b	31.8±1.5 a	23.2±1.6 a	25.3±1.6 a	0.006
CD3+ CD49b+	NKT cells	8.2±1.1 †	10.3±1.1 †	6.6±1.1	5.9±1.1	0.007
CD3+ CD49b+ CD27+	CD27+ NKT	9.65±0.79 †	8.7±0.66 †	4.71±0.19	4.31±0.15	0.33
CD3- CD49b+	NK cells	11.9±0.01	10.5±0.01	9.1±0.01	9.2±0.01	0.20
CD3- CD49b+ CD27+	CD27+ NK cells	4.2±0.2	3.5±0.2	3.6±0.2	3.5±0.2	0.14
CD3+ CD4+ CD183+ CD196-	Th1	4.3±0.5	3.6±0.5	2.2±0.28	2.6±0.2	0.02
CD3+ CD4+ CD194+ CD196-	Th2	13.3±1.1 †	11.5±0.8 †	6.1±0.6	4.9±0.5	0.15
CD3+ CD4+ CD25+	CD25+ Th	6.9±0.9 b	9.9±0.9 a	4.4±0.6 b	4.2±0.5 b	0.04
CD3+ CD8+ CD25+	CD25+ CTL	1.8±0.2 b	2.7±0.2 a	1.2±0.1 b	1.1±0.2 b	0.01
Total CD19+	Total B cells	45.6±2.2	48.4±2.4	40.0±1.3	38.5±1.1	0.63
CD19+ CD23+	Follicular B cells	27.3±5.3	30.2±5.2	21.9±5.4	20.9±5.4	0.04
CD19+ CD23-	Marginal B cells	19.4±0	17.5±0.8	16.2±0.8	16.8±0.8	0.04

Suckling period diet		**Control diet**	**Control diet**	**DHA diet**	**DHA diet**	**SPD × OT**
OT group		**Sucrose**	**Ova**	**Sucrose**	**Ova**	***P* - value**
Phenotype, *% splenocytes*	**Cell type**[1]					
CD19+ IgM+	IgM+ B cells	20.1±1.2 †	20.3±1.1 †	12.3±0.6	12.4±0.5	0.59
CD19+ IgG+	IgG+ B cells	26.1±1.3	29.6±1.7	27.9±1.3	27.1±1.2	0.30

[1] The combinations of surface markers are suggestive of described specific immune cell subtypes. Note, alternative combinations of markers are also possible.

[2] In a row, means with uncommon letters or symbols are significantly different according to post hoc analysis with multiple groups comparison-adjusted.

Abbreviation: pDC, plasmacytoid DC; cDC1, Conventional DC type-1; CTL, cytotoxic T-lymphocyte; Ig, immunoglobulin-; DHA, docosahexaenoic acid; ova, ovalbumin; OT, oral tolerance.

Table 7.5. The effect of weaning diet on immune cell phenotype of the spleen with or without stimulation.

Weaning diet		**Control diet**	**DHA diet**	***P* - value**
Phenotype, *% splenocytes*	**Cell type**[1]			
Unstimulated				
Total MHC-II+	I-A^d/I-E^d APC	49.9±0.7	52.0±0.6	0.06
Total CD19+	Total B cells	52.3±0.8	53.4±0.6	0.09
CD19+ MHC-II+	I-A^d/I-E^d B cells	47.5±0.9	47.7±0.8	0.94
CD19+ IgM+	Primary B cells	22.0±0.7	24.2±0.5	0.01
CD19+ IgG+	Secondary B cells	41.2±1.0	42.4±0.7	0.27
CD19+ CD23+	Follicular B cells	36.6±0.8	38.7±0.6	0.11
CD19+ CD23-	Marginal B cells	15.7±0.8	14.6±0.6	0.12
LPS stimulation				
Total MHC-II+	I-A^d/I-E^d APC	53.3±2.6	60.3±2.7	0.01
Total CD19+	Total B cells	53.7±0.9	54.9±0.8	0.87
CD19+ MHC-II+	I-A^d/I-E^d B cells	42.7±2.8	45.5±2.8	0.04
CD19+ IgM+	Primary B cells	23.6±0.7	22.4±0.9	0.07
CD19+ IgG+	Secondary B cells	38.4±1.1	41.0±1.0	0.03
CD19+ CD23+	Follicular B cells	29.2±3.5	32.8±3.6	0.05
CD19+ CD23-	Marginal B cells	22.4±0.9	20.3±0.9	0.35

[1]The combinations of surface markers are suggestive of described specific immune cell subtypes. Note, alternative combinations of markers are also possible.

Abbreviation: LPS, lipopolysaccharide, MHC-II, major histocompatibility-II complex; Ig-, immunoglobulin-; DHA, docosahexaenoic acid; ova, ovalbumin; OT, oral tolerance

7.3.6. Immune cell phenotype of LPS stimulated splenocytes.

The stimulation of splenocytes with LPS can activate antigen presenting cells (macrophages, DC, and B cells) through toll-like receptor-4 (TLR-4, identified by CD284). Thus, we have reported phenotypic changes in the splenocytes associated with these cell type (see Table 7.3). In the splenocytes stimulated with LPS, the proportion of CD3+ CD49b+ (NKT cells), CD3+ CD49b+ CD27+ (CD27+ NKT cells) was significantly lower in DHA+ARA SPD compared to control SPD. However, the proportion of CD3+ CD49b+ CD335+ (CD335+ NKT cells) splenocytes after LPS stimulation was higher in DHA+ARA SPD group compared to control SPD (Table 7.3). The CD3- CD49b+ (NK cells) showed no difference due to SPD, WD or OT intervention. The proportion of total CD11c+ cells (DC), CD11b+ CD68+ (macrophages), and CD11b+ CD68+ CD163+ (M2 macrophage) in LPS stimulated splenocytes was significantly lower in pups from DHA+ARA SPD group compared to control SPD, irrespective of WD and OT interventions (Table 7.3). The proportion of CD11b+ MHC-II+ cells was found to be higher with DHA+ARA SPD versus control SPD (45.9±1.9 vs. 31.8±1.6; $P = 0.001$).

The splenocyte stimulation with LPS resulted in significant changes in the phenotype of B cells due to SPD and WD. First, the DHA+ARA SPD resulted in higher CD19+ CD23+ (follicular B cells), and CD19- CD45R+ CD138+ (enriched for plasma cells) compared to control SPD (Table 7.3). Next, pups from DHA+ARA WD showed that the splenocytes after LPS stimulation resulted in significantly higher proportion of CD19+ IgG+ (secondary B cells) than pups from control WD (Table 7.5). Furthermore, LPS stimulation of splenocytes also showed higher proportion of total MHC-II cells and CD19+ MHC-II+ in DHA+ARA WD group pups compared to control WD (Table 7.5).

7.3.7. Immune cell phenotype of PMAi stimulated splenocytes.

The SPD significantly affected the phenotype of splenocyte after stimulation with PMAi. Only the relevant changes are described here. A significant SPD × OT interaction was observed on the proportion of total CD3+, CD3+ CD4+ CD25+ and CD3+ CD8+ CD25+ splenocytes was found to be significantly lower in pups fed DHA+ARA SPD and ova OT group compared to control SPD and ova OT group (Table 7.4). The splenocyte proportion of CD196- CD194+ on CD3+ CD4+ (enriched for Th2) was lower in DHA+ARA SPD group compared to control SPD (5.5±0.4 vs. 12.4±0.7; $P < 0.001$) (Table 7.4).

In the pups from the DHA+ARA SPD group, we observed significantly lower proportion of CD3+ CD49b+ (NKT cell) and CD3+ CD49b+ CD27+ (CD27+ NKT cell) compared to control SPD (Table 7.4). However, the proportion of CD3+ CD49b+ CD335+ (CD335+ NKT cells; 0.86±0.05 vs. 0.55±0.03) splenocytes after PMAi stimulation was higher in DHA+ARA SPD group compared to control SPD (0.55±0.03 vs. 0.86±0.05; $P = 0.02$). The WD and OT intervention did not change these phenotypes. The proportion of CD3+ CD4+ CD28+ splenocytes were found to be significantly higher in ova OT group than sucrose, irrespective of SPD or WD (14.3±1.1 vs. 12.8±1.1; $P = 0.028$). Whereas the splenocytes from ova OT group pups' splenocytes after PMAi stimulation resulted in significantly lower proportion of CD3+ CD152+ (3.89±0.01 vs. 4.12±0.01; $P = 0.003$) and CD152+ on CD3+ CD4+ (8.8±0.6 vs. 9.8±0.8; $P = 0.021$). The DHA+ARA SPD group showed significantly lower CD19+ IgM+ (primary B cell, 12.3±0.4 vs. 20.2±0.8; $P = 0.001$) compared to control SPD group pups splenocytes after PMAi stimulation (Table 7.4). Lastly, pups from DHA+ARA WD group showed higher CD19+ CD23+ (enriched for follicular B cell, 60.6±1.2 vs. 55.9±1.5; $P = 0.006$) and lower proportion of CD19+ CD23- (enriched for marginal B cell, 39.4±1.2 vs. 44.1±1.5; $P =$

0.008) (Table 7.4). Similarly, CD19+ MHC-II+ was significantly higher in pups from DHA+ARA WD groups (49.1±0.7 vs. 46.1±0.8; $P = 0.05$) compared to control WD.

Table 7.6. The effect of SPD and WD on the phenotype of immune cells of the spleen without stimulation.

Suckling period diet	Control	Control	DHA	DHA	SPD ×WD
Weaning diet	Control	DHA	Control	DHA	*P* - value
T cell phenotype					
Total CD3+	32.09 ± 1.41	31.59 ± 1.25	29.48 ± 1.02	28.68 ± 0.87	0.72
CD4+ in CD3+	59.59 ± 0.74	60.26 ± 0.84	59.74 ± 0.28	61.91 ± 0.56	0.41
CD25+ CD4+ CD3+	3.83 ± 0.28	3.85 ± 0.13	3.3 ± 0.16	3.23 ± 0.08	0.91
CD28+ CD4+ CD3+	18.22 ± 0.95	18.2 ± 0.95	17.23 ± 0.77	16.9 ± 0.55	0.86
CD86+ CD4+ CD3+	11.07 ± 0.6	10.55 ± 0.47	9.27 ± 0.43	8.82 ± 0.32	0.60
CD152+ CD4+ CD3+	2.28 ± 0.22	2.32 ± 0.12	0.94 ± 0.08	1.11 ± 0.09	0.51
CD8+ in CD3+	31.32 ± 0.73	30.23 ± 0.48	33.34 ± 0.38	31.51 ± 0.37	0.46
CD25+ CD8+ CD3+	0.86 ± 0.07	0.8 ± 0.03	0.74 ± 0.05	0.66 ± 0.03	0.83
CD28+ CD8+ CD3+	8.32 ± 0.51	7.67 ± 0.3	8.01 ± 0.47	7.22 ± 0.24	0.92
CD86+ CD8+ CD3+	7.6 ± 0.42	7.11 ± 0.36	6.78 ± 0.36	6.09 ± 0.31	0.79
CD152+ CD8+ CD3+	0.61 ± 0.04	0.62 ± 0.02	0.35 ± 0.03	0.29 ± 0.01	0.34
CD25+ in CD3+	15.24 ± 0.47	16.13 ± 0.31	14.86 ± 0.45	15.05 ± 0.38	0.61
CD28+ in CD3+	80.4 ± 1.07	80.57 ± 0.67	81.64 ± 1.09	80.35 ± 0.52	0.35
CD86+ CD3+	18.68 ± 1	17.67 ± 0.78	16.31 ± 0.84	15.11 ± 0.58	0.74
CD152+ in CD3+	12.94 ± 0.78	13.28 ± 0.5	8.77 ± 1.03	7.21 ± 0.42	0.69
CD196+ CD194+ in CD4+ CD3+	7.89 ± 0.54	7.63 ± 0.48	5.58 ± 0.26	6.21 ± 0.19	0.02
CD196- CD194+ in CD4+ CD3+	4.45 ± 0.24	5.05 ± 0.32	5.31 ± 0.5	6.04 ± 0.29	0.84
CD196-CD183+ in CD4+ CD3+	8.53 ± 0.35	9.59 ± 0.3	7.81 ± 0.46	8.08 ± 0.4	0.04
B cell phenotype					
Total CD19+	51.25 ± 1.48	54.57 ± 1.24	58.35 ± 0.77	53.05 ± 0.9	0.00
MHC-II+ CD19+	45.7 ± 1.45	49.07 ± 1.12	49.27 ± 1	46.23 ± 0.91	0.00
CD23+ in CD19+	70.26 ± 1.28	71.99 ± 1.07	69.7 ± 1.93	73.13 ± 1.48	0.61
CD45R+ CD23+ CD19+	35.42 ± 1.53	38.11 ± 1.11	36.68 ± 0.95	38.31 ± 0.93	0.37
CD23- in CD19+	29.71 ± 1.28	28 ± 1.07	30.23 ± 1.93	26.78 ± 1.48	0.60
CD45R+ CD23- CD19+	7.82 ± 0.37	8.14 ± 0.33	8.96 ± 0.76	8.08 ± 0.62	0.07
IgG+ CD19+	40.11 ± 1.65	42.14 ± 1.2	42.21 ± 1.08	42.68 ± 0.69	0.41
IgM+ CD19+	23.87 ± 1.1	25.83 ± 0.61	20.18 ± 0.52	22.6 ± 0.56	0.72

Suckling period diet Weaning diet	Control Control	Control DHA	DHA Control	DHA DHA	SPD ×WD *P* - value
CD19+ CD45R+	42.46 ± 1.6	45.38 ± 1.16	44.26 ± 0.72	44.37 ± 0.67	0.11
CD19- CD45R+	4.33 ± 0.33	4.48 ± 0.35	6.11 ± 0.3	7.37 ± 0.34	0.04
CD138+ in CD19- in CD45R+	3.42 ± 0.53	3.48 ± 0.41	2.01 ± 0.14	1.88 ± 0.14	0.36
IgG+ CD138+ CD19- in CD45R+	0.06 ± 0.0	0.07 ± 0.0	0.06 ± 0.0	0.07 ± 0.0	0.81
IgM+ CD138+ CD19- in CD45R+	0.17 ± 0.02	0.17 ± 0.01	0.09 ± 0.0	0.1 ± 0.0	0.72
Dendritic cell phenotype					
Total CD11c+	15.93 ± 0.67	16.06 ± 0.63	11.14 ± 0.44	10.24 ± 0.31	0.47
CD11c+ MHC-II+ CD4+	5.5 ± 0.32	5.54 ± 0.22	3.52 ± 0.12	3.41 ± 0.13	0.93
CD11c+ CD4- CD8a+	1.03 ± 0.08	1.01 ± 0.04	0.58 ± 0.04	0.57 ± 0.04	0.63
CD11c+ CD8a-	11.78 ± 0.56	11.17 ± 0.44	8.05 ± 0.38	7.5 ± 0.34	0.72
Macrophage phenotype					
Total CD11b+	17.91 ± 0.69	16.78 ± 0.82	14.1 ± 0.36	13.51 ± 0.5	0.94
CD68+ CD11b+	13.12 ± 0.55	12.59 ± 0.8	9.49 ± 0.36	8.73 ± 0.29	0.68
CD163+ CD68+ CD11b+	4.11 ± 0.36	3.73 ± 0.26	3.62 ± 0.4	3.17 ± 0.25	0.69
Total CD68+	15.05 ± 0.64	14.83 ± 0.8	11.73 ± 0.58	10.45 ± 0.39	0.10
CD68+ MHC-II+ CD86+	6.5 ± 0.32	6.9 ± 0.42	4.53 ± 0.29	4.14 ± 0.26	0.16
CD284+ MHC-II+ CD86+	2.61 ± 0.21	2.76 ± 0.27	1.51 ± 0.15	1.44 ± 0.22	0.77
Total CD163+	5.17 ± 0.38	4.69 ± 0.3	4.81 ± 0.48	4.36 ± 0.29	0.79
CD68+ CD163+	4.49 ± 0.33	4.1 ± 0.29	3.97 ± 0.45	3.51 ± 0.28	0.52
Total MHC-II+	49.74 ± 1.23	53.28 ± 0.97	50.07 ± 0.73	50.78 ± 0.74	0.05
Natural Killer cell phenotype					
CD27+ CD4+ CD3+	16.66 ± 0.98	16.93 ± 0.83	15.2 ± 0.47	14.5 ± 0.46	0.34
CD27+ CD8+ CD3+	9.89 ± 0.56	9.98 ± 0.47	8.36 ± 0.25	7.56 ± 0.24	0.22
CD45RB+ CD3+	23.2 ± 1.32	23.98 ± 1.08	20.84 ± 0.74	20 ± 0.82	0.27
CD49b+ in CD3+	21.95 ± 0.7	20.78 ± 0.95	18.85 ± 1.04	16.6 ± 0.62	0.61
CD27+ CD49b+ CD3+	4.83 ± 0.29	5.01 ± 0.24	3.79 ± 0.21	3.12 ± 0.05	0.09
CD335+ CD49b+ CD3+	0.36 ± 0.02	0.33 ± 0.02	0.51 ± 0.06	0.3 ± 0.02	0.15
CD45RB+ in CD3-	70.34 ± 3.64	72.25 ± 2.07	76.5 ± 1.59	77.91 ± 1.18	0.74
CD49b+ in CD3-	15.28 ± 0.82	14.5 ± 0.97	12.17 ± 0.4	10.67 ± 0.19	0.24
CD27+ in CD49b+ CD3-	3.59 ± 0.16	3.43 ± 0.15	2.63 ± 0.08	2.65 ± 0.07	0.29

Suckling period diet Weaning diet	Control Control	Control DHA	DHA Control	DHA DHA	SPD ×WD *P* - value
CD335+ CD49b+ CD3-	2.43 ± 0.11	2.26 ± 0.13	2.64 ± 0.13	2.24 ± 0.09	0.92

Table 7.7. The effect of SPD and WD on the phenotype of immune cells of the spleen with LPS stimulation.

Suckling period diet Weaning diet	Control Control	Control DHA	DHA Control	DHA DHA	SPD × WD *P* - value
T cell phenotype					
Total CD3+	28.93 ± 1.57	28.31 ± 1.6	29.56 ± 0.95	28.76 ± 0.86	0.59
CD4+ in CD3+	65.83 ± 0.98	64.83 ± 1.55	61.13 ± 0.76	62.62 ± 0.81	0.26
CD25+CD4+ CD3+	2.31 ± 0.32	2.31 ± 0.29	2.17 ± 0.08	2.28 ± 0.08	0.88
CD28+CD4+ CD3+	12.27 ± 2.31	13.68 ± 2.58	16.81 ± 0.55	16.17 ± 0.94	0.43
CD86+CD4+ CD3+	4.8 ± 0.69	4.81 ± 0.68	5.05 ± 0.16	4.77 ± 0.24	0.55
CD152+CD4+ CD3+	2.34 ± 0.32	2.19 ± 0.3	1.63 ± 0.08	1.59 ± 0.1	0.48
CD8+ in CD3+	23.98 ± 0.71	23.89 ± 0.76	21.74 ± 0.34	21.22 ± 0.49	0.83
CD25+CD8+ CD3+	0.55 ± 0.05	0.55 ± 0.02	0.57 ± 0.02	0.58 ± 0.03	0.97
CD28+CD8+ CD3+	5.2 ± 0.52	5.37 ± 0.36	5.15 ± 0.24	4.69 ± 0.23	0.15
CD86+CD8+ CD3+	4.16 ± 0.33	3.85 ± 0.31	3.84 ± 0.15	3.72 ± 0.18	0.66
CD152+CD8+ CD3+	0.6 ± 0.05	0.61 ± 0.04	0.49 ± 0.06	0.42 ± 0.03	0.99
CD25+ in CD3+	15.92 ± 1.11	16.18 ± 1.1	20.35 ± 0.89	20.48 ± 0.99	0.87
CD28+ in CD3+	62.5 ± 6.56	60.74 ± 7.07	75.93 ± 0.79	77.74 ± 0.82	0.01
CD86+ in CD3+	45.23 ± 2.13	44.15 ± 2.31	52.66 ± 0.73	52.46 ± 0.99	0.43
CD152+ in CD3+	15.07 ± 1.1	15.28 ± 1.15	12.34 ± 1.13	12.5 ± 1.37	0.52
CD196-CD183+ in CD4+ CD3+	5.82 ± 0.48	6.17 ± 0.58	6.63 ± 0.39	5.8 ± 0.31	0.05
CD196+ CD194+ in CD4+ CD3+	8.3 ± 1.25	7.89 ± 1.75	10.25 ± 0.8	8.96 ± 0.74	0.89
B cell phenotype					
Total CD19+	52 ± 1.08	53.83 ± 1.14	55.2 ± 1.22	56.03 ± 1.12	0.01
CD23+ in CD19+	53.92 ± 1.64	58.45 ± 2.09	62.5 ± 1.46	64.12 ± 0.96	0.33
CD45R+ CD23+ CD19+	28.08 ± 1.3	30.92 ± 1.3	34.38 ± 1.26	35.85 ± 1.12	0.06
CD23- in CD19+	46.06 ± 1.64	41.53 ± 2.09	37.48 ± 1.46	35.86 ± 0.96	0.33
CD45R+ CD23- CD19+	16.66 ± 1.05	15.23 ± 1.28	13.9 ± 0.56	14.51 ± 0.53	0.42
IgG+ CD19+	36.32 ± 1.57	37.83 ± 1.44	40.41 ± 1.25	44.11 ± 1.04	0.99
IgM+ CD19+	23.14 ± 1.07	22.96 ± 1.57	24.07 ± 0.96	21.9 ± 0.74	0.08
CD19+ CD45R+	42.45 ± 0.64	43.51 ± 1.06	45.73 ± 0.76	46.65 ± 0.98	0.12
CD19- CD45R+	21.29 ± 0.61	21.09 ± 0.63	21.76 ± 1.12	20.95 ± 0.75	0.26

Suckling period diet Weaning diet	Control Control	Control DHA	DHA Control	DHA DHA	SPD × WD *P* - value
CD138+ CD19- in CD45R+	4.94 ± 0.36	4.81 ± 0.32	7.56 ± 0.3	6.89 ± 0.41	0.31
IgG+ CD138+ CD19- in CD45R+	0.39 ± 0.04	0.49 ± 0.07	0.81 ± 0.09	0.68 ± 0.09	0.40
IgM+ CD138+ CD19- in CD45R+	0.8 ± 0.09	0.84 ± 0.08	1.15 ± 0.06	0.98 ± 0.07	0.19
Dendritic cells phenotype					
CD11c+ MHC-II+ CD4+	2.88 ± 0.26	3.16 ± 0.19	1.7 ± 0.07	1.44 ± 0.06	0.12
Total CD11c+	15.73 ± 0.91	15.91 ± 0.83	9.41 ± 0.28	9.05 ± 0.16	0.84
Total CD19+	51.04 ± 1	52.85 ± 0.89	53.19 ± 0.72	52.91 ± 0.76	0.10
MHC-II+ CD19+	41.33 ± 1.36	43.56 ± 1.22	45.39 ± 0.89	47.14 ± 0.77	0.47
CD11c+ CD4- CD8a+	0.45 ± 0.02	0.42 ± 0.02	0.4 ± 0.01	0.44 ± 0.02	0.27
CD103+ CD8a+	14.26 ± 0.72	13.85 ± 0.82	8.32 ± 0.2	7.08 ± 0.15	0.30
CD11c+ in CD8a-	4.05 ± 0.31	4.02 ± 0.3	3.24 ± 0.1	3.24 ± 0.19	0.63
CD11c+ CD8a-	3.25 ± 0.23	3.23 ± 0.23	2.78 ± 0.08	2.82 ± 0.16	0.49
Macrophages phenotype					
Total CD11b+	15.21 ± 0.7	15.36 ± 0.79	12.16 ± 0.4	11.16 ± 0.44	0.14
CD68+ CD11b+	12.17 ± 0.67	12.72 ± 0.86	9 ± 0.43	7.75 ± 0.31	0.07
CD163+ CD68+ CD11b+	10.09 ± 0.63	10.14 ± 0.71	6.78 ± 0.22	6.27 ± 0.31	0.31
Total CD68+	16.17 ± 0.76	16.61 ± 0.93	14.05 ± 0.52	13.05 ± 0.63	0.34
CD68+ MHC-II+ CD86+	6.22 ± 0.35	6.48 ± 0.26	6.62 ± 0.29	5.86 ± 0.39	0.08
CD68+ CD163+	11.59 ± 0.58	11.77 ± 0.67	7.71 ± 0.46	7.48 ± 0.42	0.22
CD284+ in CD163+	59.89 ± 1.97	61.45 ± 1.58	41.44 ± 1.06	40.33 ± 1.03	0.18
CD284+ CD163+	8.57 ± 0.53	8.83 ± 0.55	4.35 ± 0.21	4.43 ± 0.2	0.38
Natural Killer cell phenotype					
CD27+ CD4+ CD3+	20.3 ± 1.17	21.1 ± 1.18	16.68 ± 0.48	15.7 ± 0.51	0.19
CD27+ CD8+ CD3+	12.96 ± 0.75	13.53 ± 0.91	8.04 ± 0.25	7.65 ± 0.34	0.15
CD45RB+ CD3+	18.1 ± 1.05	17.31 ± 0.99	17.25 ± 0.94	17.75 ± 0.81	0.55
CD49b+ in CD3+	37.36 ± 1.8	38.2 ± 1.48	23.33 ± 1.42	22.43 ± 0.73	0.61
CD27+ CD49b+ CD3+	9.86 ± 0.62	10.53 ± 0.71	4.33 ± 0.25	3.91 ± 0.09	0.12
CD335+ CD49b+ CD3+	0.91 ± 0.1	1.12 ± 0.1	1.45 ± 0.07	0.94 ± 0.09	0.00
CD49b+ in CD3-	14.78 ± 0.55	13.86 ± 0.67	12.66 ± 0.32	12.35 ± 0.25	0.15
CD27+ in CD49b+ CD3-	2.17 ± 0.17	2.33 ± 0.17	1.97 ± 0.21	1.86 ± 0.2	0.72

Suckling period diet Weaning diet	Control Control	Control DHA	DHA Control	DHA DHA	SPD × WD *P* - value
CD335+ in CD49b+ CD3-	2.28 ± 0.11	2.47 ± 0.14	2.96 ± 0.15	2.62 ± 0.15	0.15

Table 7.8. The effect of SPD and WD on the phenotype of immune cells of the spleen with PMAi stimulation.

Suckling period diet Weaning diet	Control Control	Control DHA	DHA Control	DHA DHA	SPD × WD *P* - value
T cell phenotype					
Total CD3+	35.4 ± 1.5	31.8 ± 1.5	23.2 ± 1.6	25.3 ± 1.6	0.006
CD4+ in CD3+	58.08 ± 2.23	58.02 ± 2.23	56.04 ± 1.67	51.93 ± 2.68	0.17
CD25+ CD4+ CD3+	8.29 ± 0.99	8.55 ± 1.01	4.24 ± 0.51	4.34 ± 0.6	0.63
CD28+ CD4+ CD3+	16.41 ± 1.56	16.35 ± 1.48	11.52 ± 1.19	8.72 ± 1.03	0.19
CD86+ CD4+ CD3+	8.21 ± 0.72	8.01 ± 0.67	4.43 ± 0.33	3.82 ± 0.33	0.69
CD152+ CD4+ CD3+	3.52 ± 0.38	3.58 ± 0.41	1.81 ± 0.25	1.62 ± 0.19	0.29
CD8+ in CD3+	18.11 ± 0.97	18.21 ± 1.21	21.01 ± 1.49	17.72 ± 1.22	0.16
CD25+ CD8+ CD3+	2.39 ± 0.27	2.15 ± 0.21	1.11 ± 0.16	1.16 ± 0.14	0.55
CD28+ CD8+ CD3+	4.65 ± 0.49	4.73 ± 0.43	4.11 ± 0.54	2.94 ± 0.32	0.12
CD86+ CD8+ CD3+	3.65 ± 0.38	3.72 ± 0.34	3.19 ± 0.38	2.2 ± 0.24	0.12
CD152+ CD8+ CD3+	0.65 ± 0.06	0.64 ± 0.05	0.68 ± 0.11	0.49 ± 0.06	0.60
CD25+ in CD3+	45.21 ± 4.44	46.7 ± 4.55	33.78 ± 5.96	43.25 ± 5.12	0.56
CD28+ in CD3+	76.81 ± 1.43	78.51 ± 1.21	67.16 ± 1.99	63.87 ± 1.88	0.22
CD86+ in CD3+	58.48 ± 1.56	58.2 ± 1.32	48.79 ± 1.54	46.77 ± 1.21	0.76
CD152+ in CD3+	16.84 ± 1.13	16.66 ± 1.01	12.83 ± 0.99	12.32 ± 0.91	0.48
CD196- CD183+ in CD4+ CD3+	4.15 ± 0.55	3.74 ± 0.49	2.54 ± 0.23	2.3 ± 0.25	0.83
CD194+ in CD4+ in CD3+	50.33 ± 3.21	50.77 ± 4.52	25.36 ± 3.22	37.43 ± 4.91	0.13
CD196+ CD194+ CD4+ in CD3+	7.98 ± 0.74	7.93 ± 0.46	4.68 ± 0.3	5.52 ± 0.4	0.41
CD196- CD194+ CD4+ in CD3+	12.72 ± 1	12.11 ± 0.97	4.31 ± 0.3	6.69 ± 0.57	0.01
B cell phenotype					
Total CD19+	45.94 ± 2.27	48.11 ± 2.3	38.3 ± 1.15	40.22 ± 1.19	0.85
CD23+ in CD19+	56.97 ± 2.53	61.57 ± 2.25	54.83 ± 1.54	59.69 ± 1.08	0.80
CD45R+ CD23+ CD19+	26.21 ± 2.22	29.74 ± 2.4	20.04 ± 1.24	23.53 ± 1.02	0.82
CD23- in CD19+	42.98 ± 2.51	38.41 ± 2.25	45.13 ± 1.53	40.3 ± 1.08	0.80
CD45R+ CD23- CD19+	10.95 ± 0.65	11.29 ± 0.69	7.66 ± 0.4	8.4 ± 0.45	0.40
MHC-II+ CD19+	46.05 ± 1.37	48.52 ± 0.97	46.16 ± 0.99	49.66 ± 1.13	0.47
IgG+ CD19+	26.96 ± 1.45	28.75 ± 1.67	26.26 ± 1.13	28.75 ± 1.24	0.48

Suckling period diet Weaning diet	Control Control	Control DHA	DHA Control	DHA DHA	SPD × WD *P* - value
IgM+ CD19+	19.9 ± 1.13	20.54 ± 1.08	12.57 ± 0.41	12.07 ± 0.64	0.59
Total CD23+	38.81 ± 1.36	42.09 ± 0.88	37.91 ± 0.92	41.99 ± 0.78	0.74
Total CD45R+	54.85 ± 1.15	57.96 ± 1	51.35 ± 1.02	57.36 ± 0.92	0.16
CD19+ CD45R+	35.61 ± 1.83	37.87 ± 2.34	31.35 ± 1.36	35.83 ± 1.24	0.46
CD19- CD45R+	19.2 ± 1.1	20.08 ± 1.46	19.98 ± 0.6	21.5 ± 0.81	0.64
CD138+ in CD19- in CD45R+	2.7 ± 0.22	2.57 ± 0.2	2.94 ± 0.4	2.24 ± 0.29	0.93
IgG+ CD138+ CD19- in CD45R+	0.31 ± 0.05	0.29 ± 0.05	0.43 ± 0.08	0.33 ± 0.07	0.57
IgM+CD138+ CD19- in CD45R+	0.43 ± 0.04	0.45 ± 0.05	0.4 ± 0.07	0.27 ± 0.03	0.50
Dendritic cells phenotype					
CD11c+ MHC-II+ CD4+	5.6 ± 0.45	5.97 ± 0.4	3.99 ± 0.26	3.42 ± 0.15	0.20
Total CD11c+	16.18 ± 0.93	16.18 ± 0.79	9.84 ± 0.26	9.21 ± 0.22	0.49
CD11c+ CD4- CD8a+	0.77 ± 0.08	0.83 ± 0.07	0.67 ± 0.03	0.65 ± 0.04	0.37
CD103+ CD8a+	14.32 ± 0.52	13.45 ± 0.72	8.86 ± 0.31	7.72 ± 0.21	0.69
MHC-II+ CD8+	7.19 ± 0.41	6.86 ± 0.41	4.72 ± 0.33	4.46 ± 0.31	0.71
Macrophage phenotype					
Total CD11b+	19.84 ± 1.11	19.13 ± 1.05	14.04 ± 0.39	13.24 ± 0.53	0.91
CD68+ CD11b+	17.59 ± 1.1	16.69 ± 1.13	11.16 ± 0.37	10.47 ± 0.33	0.93
CD163+ CD68+ CD11b+	15.59 ± 1.12	14.47 ± 1.06	9.66 ± 0.36	8.98 ± 0.31	0.85
CD68+ MHC-II+ CD86+	29.22 ± 1.6	34.43 ± 1.53	22.85 ± 1.2	26.93 ± 1.2	0.50
CD68+ CD163+	16.21 ± 1.22	15.35 ± 1.23	10.27 ± 0.55	9.46 ± 0.34	0.02
CD284+ CD163+	9.59 ± 0.73	8.84 ± 0.69	5.83 ± 0.41	5.09 ± 0.17	0.79
Natural killer cell phenotype					
CD27+ CD4+ CD3+	18.73 ± 1.27	19.17 ± 1.3	14.55 ± 0.54	11.37 ± 0.85	0.01
CD27+ CD8+ CD3+	15.19 ± 0.95	15.4 ± 0.75	8.37 ± 0.23	7.1 ± 0.27	0.16
CD45RB+ CD3+	23.67 ± 1.14	24.2 ± 0.67	21.47 ± 1.17	20.49 ± 0.93	0.28
CD49b+ in CD3+	30.46 ± 1.87	28.35 ± 1.51	22.48 ± 0.73	19.35 ± 0.75	0.78
CD27+ CD49b+ CD3+	9.26 ± 0.77	9.08 ± 0.69	4.87 ± 0.17	4.1 ± 0.09	0.78
CD335+ CD49b+ CD3+	0.57 ± 0.04	0.53 ± 0.03	0.91 ± 0.06	0.81 ± 0.06	0.62
Total CD3-	60.92 ± 1.07	61.53 ± 1.02	67.11 ± 1.28	69.21 ± 1.14	0.13
CD45RB+ in CD3-	78.8 ± 2.22	83.18 ± 1.82	79.44 ± 2.07	84.63 ± 1.69	0.95

Suckling period diet Weaning diet	Control Control	Control DHA	DHA Control	DHA DHA	SPD × WD *P* - value
CD49b+ in CD3-	18.1 ± 0.86	18.45 ± 1.18	14.1 ± 0.29	12.5 ± 0.37	0.06
CD27+ in CD49b+ CD3-	3.77 ± 0.22	3.89 ± 0.19	3.6 ± 0.21	3.45 ± 0.16	0.98
CD335+ in CD49b+ CD3-	2.15 ± 0.14	2.24 ± 0.13	2.49 ± 0.1	2.19 ± 0.12	0.23

7.4. Discussion

We evaluated the effect of plant-based DHA from a novel canola oil, along with the same amount of ARA, on the immune system and OT development in Th2 skewed BALB/c mouse pups (summarized in Table 7.9). The changes in fatty acid composition for 3-week pups have been reported elsewhere (manuscript submitted) (D Patel et al., 2022). Briefly, the DHA composition of splenocytes was higher in the DHA+ARA group compared to the control (5.8+0.2 vs. 3.5+0.2; $P < 0.001$), whereas the ARA composition showed no significant difference (13.9+0.3 vs. 12.3+0.5; $P = 0.22$). Through the addition of 1% DHA and 1% ARA of total fat in the SPD and WD, we expected to improve DHA status of 6-week pups without affecting ARA based on the previous experiments from our lab (Dhruvesh Patel et al., 2022; Patel et al., 2021; Caroline Richard et al., 2016e; C. Richard et al., 2016). Infant immune system continues to develop after birth and the role of LCPUFA in diet plays a vital role in immune cell functions (Miles et al., 2021; Nettleton & Salem Jr., 2019; West, 2002). Past studies from our group using DHA and ARA, respectively from algae and fungus single cell sources, have shown to be beneficial for OT induction in rodent models, Th1 skewed Sprague Dawley and Th2 skewed Brown Norway rats (Dhruvesh Patel et al., 2022; C. Richard et al., 2016). However, these LCPUFA single cell sources and fish oil are not found in commonly used dietary fat source in western diet (Papanikolaou et al., 2014). Since canola oil, in parts of North America, is one of the most commonly used cooking oil, the use of genetically modified high-DHA canola oil, tested in the current study, can be used to improve DHA status at population level. Thus, through novel high-DHA canola oil we hypothesized to provide beneficial OT outcomes in Th2 skewed BALB/c mouse offspring associated with DHA.

In the current study, sucking period DHA+ARA supplementation was associated with higher *ex-vivo* cytokine (IL-2, IFN-γ and IL-1β) responses by splenocytes after stimulation with

anti-CD3/anti-CD28. Additionally, weaning period DHA+ARA supplementation promoted suppression of Th2 responses towards ova. This can also be partly attributed to higher proportion of mature B cells associated with DHA+ARA WD. Finally, the current study also provides insight on selective suppression of immune response (Th2 type cytokines and IgG-subtypes) towards ova in tolerized pups.

Table 7.9. Summary of important findings based on the intervention in 6-week BALB/c pups.

OT intervention groups (ova compared to sucrose[1])	Suckling diet (DHA compared to control[2])	Weaning diet (DHA compared to control[3])
↑ plasma ova-IgG levels	↓ plasma ova-IgE	↓ IL-4 to ova stimulation
↔ plasma ova-IgE levels	↑ IL-2, IL-1β and IFN-γ to anti-CD3/anti-CD28 stimulation	↓ IL-6 to ova stimulation
↓ total IgG and IgG1 to ova stimulation	↓ IFN-γ, TNF-α, IL-6 and CXCL-1 to LPS stimulation	↓ IL-6 to LPS stimulation (only in pups from the ova OT group)
↓ total IgG and IgG1 to LPS or PMAi stimulation	↓ proportion of CD25+Th, CD183+ Th, CD86+ Th cells in splenocytes (unstimulated)	↓ IL-6 to anti-CD3/anti-CD28 stimulation
↑ IgG2a to ova or PMAi stimulation	↓ proportion of CD11b+ CD68+, CD11c+ DC, CD3- CD49b+ CD27+ NK cells in splenocytes (unstimulated)	↑ proportion of CD19+ B cells, CD19+ CD23+ follicular B cells and CD19+ IgG+ B cells in splenocytes (unstimulated)
↓ IL-2, IL-4 (not with SPD+WD DHA), IL-6 and IL-10 to ova stimulation	↓ proportion of CD3+ CD49b+ NKT, CD27+ NKT, CD11c+ DC, CD11b+ CD68+ macrophage and CD163+ M1 macrophage in LPS stimulated splenocytes	↑the proportion of CD19+ MHC-II+ B cells in LPS-stimulated splenocytes
↓ IL-4 and TGF-β to LPS stimulation		↑ the proportion of CD19+ MHC-II+ B cells in PMAi-stimulated splenocytes
↓ IL-5 and CXCL-1 to anti-CD3/anti-CD28		
↑ CD11c+ CD8a+ DC in splenocytes (unstimulated)		

[1] Describes the difference between the Ova group compared to the sucrose group showing statistical significance due to OT intervention.
[2] Describes the difference between the DHA group compared to the control group showing statistical significance due to suckling diet intervention.
[3] Describes the difference between the DHA group compared to the control group showing statistical significance due to weaning diet intervention.
Abbreviation: ↑, higher; ↓, lower; ↔, no change; ova, ovalbumin; OT, oral tolerance; DHA, docosahexaenoic acid; ova-Ig, ova specific immunoglobulin; LPS, lipopolysaccharide; PMAi, phorbol–myristate–acetate and ionomycin; DC, dendritic cell; Th, T helper cell; IL, interleukin; NK, natural killer cell; MHC-II; major histocompatibility complex-II.

7.4.1. OT development and immune responses associated with ova stimulation in DHA+ARA fed 6-week pups

We investigated the DHA+ARA supplementation effect on OT development in 6-week pups through repetitive exposure of orally fed low-dose of ova between 21 d to 25 d. The *in-vivo* response towards ova did not show a significant OT effect based on the plasma levels of ova-IgG and ova-IgE in 6-week BALB/c mouse (Figure 7.3). These findings are different from previous studies from our group, where we showed LCPUFAs induced *in-vivo* suppression of plasma ova-specific-Ig's response (Patel et al., 2019; Dhruvesh Patel et al., 2022). The *in-vivo* production of antibodies towards ova may be affected by maternal transfer of specific antibodies (Chen et al., 2016; Yamamoto et al., 2012). Since, the dams obtained from the commercial source were not from ova-free colony, it is likely to interfere with the *in-vivo* ova-specific antibody response. Nevertheless, the splenocytes showed *ex-vivo* suppression of immune responses towards ova (Figure 7.4). For instance, in ova tolerized pups , ova stimulated splenocytes resulted in nearly 10-fold reduction in IgG1 (associated with Th2 response) (Firacative et al., 2018), production compared to non-tolerized pups. Whereas Th1 type response showed higher production of IgG2a by pups from ova OT group compared to sucrose exposed pups. Together, a lower Th2 and higher Th1 response towards ova, suggests that the OT induction in Th2 skewed BALB/c model may involve Th2 specific suppression (Nagatani et al., 2006; Noval Rivas et al., 2015; Russo et al., 2001). OT induced suppression of immune response was also observed in *ex-vivo* cytokine production by ova stimulated splenocytes . Ova tolerized pups showed lower *ex-vivo* IL-2 (surrogate marker of proliferation), IL-6 and IL-10 production than sucrose OT group (Figure 7.4). IL-2 acts as a growth factor and promote lymphocyte proliferation through CD25 (IL-2 receptor) whereas IL-10 and IL-6 are involved in antibody production by B cells (Chen et al., 2014). The reduction of IL-2 showed no significant change in splenocyte proportion of T cells or

B cells. However, a lower IL-6 and IL-10 may have contributed towards lower B cell IgG1 production by ova tolerized pups. Since, elevated IL-6 is observed in food hypersensitivity, this finding may support OT development (C. Kuhn et al., 2017). Previous reports using allergy prone Brown Norway (Th2-biased) model, supported a lower IL-6 production by splenocytes stimulated with Ova in tolerized pups (ova exposed) (Dhruvesh Patel et al., 2022), however, similar findings were not seen with Sprague Dawley (Th1-biased) rat offspring (C. Richard et al., 2016). Other studies with allergy-prone mouse models also have observed a reduction in the expression of IL-6 and elevation of IL-10 was associated with OT (Paiatto et al., 2017).

The anti-inflammatory properties of DHA and immunosuppression associated with OT are known to suppress inflammatory cytokine response (Calder, 2016). However, the current study showed no differences in IL-1β, IFN-γ and TNF-α production by splenocytes stimulated with ova from ova tolerized groups or DHA+ARA supplemented group. Thus, suggesting development of OT led to a lower Th2 (IgG1, IL-6, IL-10) responses without affecting inflammatory cytokine responses to ova stimulation. Although diet did not show a significant change in the *in-vivo* OT outcomes (plasma ova-IgE or ova-IgG), there was a significant reduction in *in-vivo* ova-IgE plasma levels associated with DHA+ARA SPD compared to control (Caroline Richard et al., 2016f).

7.4.2. Programming effect of suckling period DHA supplementation on T cell and innate immune cell responses.

Early life nutrition can have a programming effect on the immune system of infants, particularly during suckling period when infants consume their mother's breastmilk (Field, 2005). This period also corresponds with the development of robust adaptive immune system (B and T cells) and switching of Th2 dominant infant immune response to more balanced Th1/Th2 responses (Lewis et al., 2019). In the current study, providing DHA+ARA in SPD showed significant

increase in pro-inflammatory cytokines (IL-2, IL-1β and IFN-γ) without affecting IL-4, IL-5, IL-6 and IL-10 cytokine response by anti-CD3/anti-CD28 stimulated splenocytes. These outcomes were observed despite a small decrease in the proportion of CD25+ Th cells involved in pro-inflammatory response (characterizes activation phenotype) associated with DHA+ARA SPD. Therefore, this can be considered a more efficient T cell response (Gottrand, 2008; Granot et al., 2011).

Additionally, providing DHA+ARA during suckling period may support a reduction in pro-inflammatory responses by splenocytes upon stimulation with LPS, a bacterial component that can activate innate immune cells (Honda et al., 2015a, 2015b). DHA+ARA during suckling period led to lower production of IFN-γ, TNF-α, IL-6 and CXCL-1 towards LPS compared to control group pups. This may be due to lower proportion of CD11b+ CD68+ macrophages, CD11c+ DC and CD3- CD49b+ CD27+ NK cells. Immune cells with TLR-4 (receptor for LPS); macrophages, DC, natural killer cells and some B cells can be activated with LPS stimulation. However, macrophages are the key producers of inflammatory cytokines in spleen and their polarization towards M1 or M2 phenotype can modulate their function (Arango Duque & Descoteaux, 2014). In the presence of Th1 cytokines (IFN-γ and TNF-α), the naïve macrophages polarize towards M1 phenotype, which are involved in the production of strong inflammatory response and recruitment of cytotoxicity producing NK cells and Th1 cells (Arango Duque & Descoteaux, 2014). Furthermore, the phenotype of LPS stimulated splenocytes also showed some changes in DHA+ARA SPD that may contribute to the suppression of pro-inflammatory response. This includes lower proportion of CD3+ CD49b+ NKT cells, CD27+ NKT cells, and CD163+ macrophage (associated with M1 phenotype) in LPS stimulated splenocytes with DHA+ARA SPD supplementation. Similar anti-inflammatory properties of DHA (and n-3

PUFA) have also been observed in the past studies from our group and others using allergy prone and healthy models (Allam-Ndoul et al., 2017; Chang et al., 2015; Patel et al., 2019; Dhruvesh Patel et al., 2022; C. Richard et al., 2016; Titos et al., 2011).

7.4.3. DHA in the weaning period modulated B cell phenotype and induced lower Th2 cytokines. The DHA+ARA supplementation of WD resulted in lower IL-6 production by splenocytes upon stimulation with ova, anti-CD3/anti-CD28 as well as LPS (only in ova tolerized OT group). Additionally, providing DHA+ARA during suckling followed by DHA+ARA in WD, results in lower IL-4 by ova stimulated splenocytes from ova exposed pups compared to other diet combinations (Figure 7.6 A). DHA+ARA WD associated decrease in IL-6 production towards ova may be beneficial for OT development, since elevated IL-6 have been observed in atopic conditions including food allergies (C. Kuhn et al., 2017). Food allergen specific IgE production is induced by IL-4 and IL-13 and can be enhanced by IL-6 resulting in the mast cell activation and anaphylactic response (Johnston et al., 2014). Therefore, DHA+ARA supplementation in weaning period may induce changes in cytokine production towards ova stimulation that can be considered beneficial for OT response. Past studies form our lab, suggested the beneficial effect of supplementation on OT (lower ova-specific Ig) may be due to decrease in IL-6 and increase in IL-10 associated with DHA (Dhruvesh Patel et al., 2022; C. Richard et al., 2016; Caroline Richard et al., 2016f). The current study found no differences in the OT outcomes (*in-vivo* and *ex-vivo*) due to WD supplementation, although, there were significant phenotypic changes in B cells from splenocytes. In the DHA+ARA WD group pups, the proportion of LPS stimulated splenocytes showed higher, CD19+ CD23+ follicular B cells and CD19+ IgG+ B cells, than control (Chung et al., 2002). Note, the total proportion of CD19+ B cells were not different in unstimulated or LPS stimulated splenocytes between WD groups. Additionally, the proportion of

CD19+ MHC-II+ B cells was also higher in splenocytes stimulated with LPS or PMAi. The expression of MHC-II on B cells may be important for providing costimulatory signal, akin to antigen presenting cells, in the activation of CD4+ T cells which in turn promotes B cells Ig production function (Linton et al., 2000). Although, we did not observe any Ig functional changes associated with DHA+ARA supplementation of WD, the higher proportion of B cells in 6-week pups can be associated with the maturation of adaptive immunity.

The study has some limitations. The current study used a previously developed and validated protocol of OT induction in Brown Norway and Sprague Dawley rat models. This may have resulted in a non-significant difference in *in-vivo* OT outcomes. Therefore, future studies should validate ova tolerization and immunization protocols to measure significance in the *in-vivo* responses. Nevertheless, we induced OT towards ova (suppression of *ex-vivo* antibody response with ova stimulation). Secondly, the use of multiple surface mAb in combination to identify specific immune cell subtype is common in immunology literature. However, we recognize the limitation with using surface mAb in identifying immune cell type and suggest the use of functional changes to support immune cell phenotype data. For instance, the current study uses CD183+ markers on Th cells and CD194+ CD196- on Th cells to identify Th1 and Th2 cells. This should be supported by Th1 and Th2 specific cytokines, such as IFN-γ and IL-4 respectively. Additionally, the use of transcription factor can also provide further evidence to support the changes observed in immune cell phenotype. The development of OT is initiated in the gut, therefore, future studies should evaluate immune cells from mesenteric lymph nodes and Peyer's patches. Last, the current study explored the functional and phenotypic changes in the immune system and did not study the mechanism of actions involving OT or immune system

development. Future studies should explore the mechanism of action behind the observed changes associated with DHA+ARA supplementation.

7.5. Conclusion

In conclusion, this study provides evidence supporting the beneficial effects of DHA (from novel canola-oil), alongside ARA, on immune system development, including OT towards ova (egg protein), using Th2 dominant BALB/c mouse. The programming effect of DHA+ARA in suckling period resulted in better T cell response (IL-2 and IFN-γ to anti-CD3/anti-CD28 stimulation) to enhance select Th1 specific responses. Furthermore, DHA+ARA SPD promoted a lower inflammatory response toward LPS stimulation, supporting the anti-inflammatory properties of DHA. The development of OT towards ova was suggested with specific suppression of IgG responses towards *ex-vivo* ova stimulation. Supplementing DHA+ARA during suckling may support *in-vivo* OT outcome (lower plasma ova-IgE levels). Lastly, providing DHA+ARA during weaning period was associated with lower Th2 response (IL-4 and IL-6) towards ova stimulation and higher proportion of B cell with class-switched IgG+ cells and antigen presenting marker (MHC-II+) cells, which may support adaptive immune system maturation in 6-week pups. Overall, our findings suggests that the supplementation of plant-based DHA, along with ARA, can induce some Th1 specific responses and reduce the Th2 response and can be beneficial for the development of OT and the immune system of allergy prone BALB/c pups.

7.6. References

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Chapter 8. Overall discussion and future direction

The overall objective of this thesis is to understand the role of LCPUFAs during early life on the development of the immune system and OT towards food protein. First, we used SDA (precursor to DHA) supplementation to study the effect on immune system development in Sprague Dawley rat offspring. Second, we identified two Th2-biased allergy-prone rodent models, Brown Norway rat and BALB/c mice, to study the effect of early-life dietary supplementation of LCPUFA. Last, we determined the effect of DHA and ARA supplementation during early life (suckling and weaning periods) on immune system development. Additionally, we evaluated the role of these LCPUFAs on the induction of OT towards food protein and associated immune responses in Th2-biased Brown Norway and BALB/c offspring.

8.1. Executive summary of findings

8.1.1. Objective 1: SDA-enriched bioactive oil improved n-3 LCPUFAs (EPA and DPA) status but not DHA in the immune cells of Sprague Dawley rat offspring (3-wk and 6wk).

In chapter 3, we reported the findings from the SDA supplementation experiment in Sprague-Dawley rat offspring at 3wk and 6 wk. Feeding only 3% of total fat as SDA enriched maternal diet resulted in higher n-3 LCPUFA status (significantly more EPA, DPA and DHA) in the breastmilk of dams and plasma phospholipids. In 6-wk pups, the SDA-supplemented diet showed a significant increase in plasma phospholipid levels of EPA and DPA but not DHA. Additionally, SDA showed no significant changes in the DHA composition of splenocyte phospholipids. The suckling period supplementation resulted in higher adaptive immune cells (Th cells) and lower innate immune cells (NK cells and macrophages) in splenocytes. At the same time, no changes in the *ex-vivo* cytokine production to LPS or ova were observed. In 6-wk

pups, SDA supplementation during the suckling and weaning period resulted in a lower amount of pro-inflammatory cytokines (TNF-α and IL-6) and higher anti-inflammatory cytokine (IL-10) by splenocytes stimulated with LPS or ova. The supplementation of SDA resulted in no effect on the development of OT. This chapter was published in the Journal of Nutrition (Patel et al., 2019).

8.1.2. Objective 2.1: Identification of rodent model to study the effect of dietary interventions on allergy prevention.

The immune system generates an abnormal Th2-biased immune response in atopic conditions, such as food allergies. The development of OT is a multifactorial process and can be influenced by genetics, age, dietary antigen (dose and frequency used for primary exposure), immunization (adjuvant and injection site), etc. We identified that Brown Norway rat and BALB/c mouse could be helpful to model the Th2 skewed immunity associated with food allergies during early life. These animal models were used for supplementation studies for objectives 2.2 and 2.3.

8.1.3. Objective 2.2: DHA and ARA promoted immune system maturation and benefited OT in allergy-prone Brown Norway rat offspring (8-wk).

The combined supplementation of DHA and ARA during the suckling and weaning period resulted in higher DHA, without affecting ARA, composition in splenocytes from 8-week Brown Norway offspring. This was associated with significantly higher adaptive immune cells in the spleen, mesenteric lymph nodes and Peyer's patches. In pups weaned to the supplemented diet, the splenocytes stimulated with a lymphocyte stimulant (PMAi) produced higher Th1 cytokine, TNF-α and IFN-γ. The plasma level of ova-IgG1 and ova-IgE was lower in groups that were exposed to ova compared to sucrose, indicating the development of OT. The supplementation of

the suckling period diet further promoted the development of OT by suppressing ova-IgG1 in plasma. The findings for objective 2.2 are reported in Chapters 5 and 6 and published, respectively, in Frontiers in Nutrition and the Journal of Nutrition (Dhruvesh Patel et al., 2022; Patel et al., 2021).

8.1.4. Objective 2.3: The supplementation of DHA from novel canola oil, along with ARA, promoted Th1 immune response in BALB/c mouse offspring (3-week and 6-week)

The supplementation of DHA, alongside ARA, in the maternal diet during lactation improved DHA composition in splenocytes without affecting ARA. The suckling period supplementation resulted in higher proportion of mature immune cell phenotypes in the spleen of 3-wk offspring, especially B cells (identified with CD19 and IgG surface markers). Further, providing DHA and ARA also improved the inflammatory response (IFN-γ, IL-1β and TNF-α) by splenocytes upon stimulation with LPS. At 6-wk, offspring from supplemented suckling period diet showed lower plasma ova-IgE and higher inflammatory cytokines (IL-2, IFN-γ and IL-1β) by T cells, which benefited OT. Providing DHA in suckling also had an anti-inflammatory effect (lower IFN-γ, TNF-α, IL-6 and CXCL-1) in splenocytes stimulated by LPS at 6-wk. Additionally, providing DHA and ARA during weaning lowered Th2 cytokine (IL-4) production by splenocytes stimulated with ova. The findings from this experiment suggested supplementation of suckling and weaning period can have different effects on immune system development. The findings for objective 2.3 are reported in chapters 7 and 8 and submitted for publication (under review), respectively, in the European Journal of Nutrition and Journal of Nutrition.

8.2. General discussion and future directions

In a series of experiments, we showed that supplementing the diet with LCPUFAs (DHA and ARA) can positively modulate immune system development during early life, particularly in allergy-prone conditions. We evaluated the effect of different dietary sources of LCPUFA, bioactive flaxseed enriched in SDA, DHASCO and high-DHA canola oil on DHA status in neonates. Further, in the experiments conducted using allergy-prone BALB/c mouse, addition of 1% DHA and 1% ARA of total fat was found to modulate the immune responses that can be considered beneficial for infant immune system. However, more research is required to test the effects of supplementing LCPUFAs at higher concentrations on neonatal immunity. Future studies evaluating the dose-response of DHA+ARA on OT outcomes may estimate the optimal dietary requirement of these LCPUFAs in allergy prone conditions. Providing DHA in the suckling period can have a programming effect on the neonatal immune system (Patel et al., 2019; Dhruvesh Patel et al., 2022; D Patel et al., 2022; Patel et al., 2021). This includes promoting OT development and suppressing Th2 dominant immune response in allergy-prone conditions. Previous studies from our lab have shown similar findings using healthy Sprague Dawley rodent models (Caroline Richard et al., 2016c, 2016e; C. Richard et al., 2016; Caroline Richard et al., 2016f). Overall, evidence from this research supports the need for supplementing the early life diet with a balance of DHA and ARA to promote desirable changes in the immune system of neonates (Caroline Richard et al., 2016a).

8.2.1. The increased requirement of n-3 LCPUFA (DHA) during early life may need to be fulfilled by supplementing preformed DHA.

ALA and LA are considered essential nutrients as they can not be synthesized in our bodies. These fatty acids are also substrate for the biosynthesis of LCPUFA, such as DHA and ARA. The conversion rate is so low that it is insufficient to meet the requirement, particularly in early

life (Carnielli et al., 2007; Gibson et al., 2011; Pawlosky et al., 2006). In the growing fetus and later infant, the role of LCPUFAs is critical for normal growth and development, and dietary ALA or LA can not meet the increased requirements (Agostoni & Mazzocchi, 2016). We showed that supplying SDA at 3% during suckling and weaning was insufficient to improve DHA incorporation in immune cells. A clinical trial conducted by E Miles et al. (2004) using SDA and other precursor PUFA has also reported similar findings (E A Miles et al., 2004). Therefore, supplementing the infant diet with DHA is a more practical and physiologically reliable way to meet the increased DHA requirement in neonates compared to precursor ALA and SDA supplementation (Gottrand, 2008; Harbige, 2003). Future research should aim to determine the concentration of DHA in the diet that is optimal to meet the increased requirements during early life while fulfilling maternal needs. The current thesis uses DHA supplementation at 1% of total fat as DHA alongside ARA and report significant improvements in the DHA status and neonatal immune system development. However, DHA supplementation at higher concentrations, along side ARA, remains to be tested.

Past research has used the tissue accretion rates of various fatty acids (i.e., DHA and ARA) to determine the optimal requirement of LCPUFAs in the diet (Brenna & Lapillonne, 2009; Brenna et al., 2009; Gázquez & Larqué, 2021; Lien et al., 2018; Van Houwelingen et al., 1995). However, more versatile methods are available and should be the basis for estimating the functional importance of DHA in not just brain and neural tissues but also immune organs (Hachem & Nacir, 2022). Additionally, the advancement in the field of lipidomic (Lam et al., 2020) and our understanding of specialized lipid mediators derived from these LCPUFAs (Gabbs et al., 2015) should inform the physiological requirement of LCPUFAs for optimal functioning. Overall, future research, especially in allergy-prone conditions, should aim at understanding the

functional importance of LCPUFAs and their interaction with all organ systems (neural, immune, cardiovascular, etc.).

8.2.2. A combination of DHA and ARA promotes Th1 immunity in allergy-prone (Th2 skewed) condition.

The essentiality of dietary ARA in infant development is well documented (Hadley et al., 2016) and advocated when the diet contains n-3 LCPUFAs such as EPA and DHA (Harbige, 2003; Koletzko et al., 2019). Experts in the field of infant nutrition have supported the combined supplementation of DHA and ARA during early life due to their role in the development and maturation of various body systems, such as neural, visual, psychomotor, immune, etc. (Nettleton & Salem Jr., 2019; Tounian et al., 2021). Such combined supplementation aims to increase the composition of DHA while not changing the ARA levels, especially in cell membranes (Caroline Richard et al., 2016a). Several clinical trials have reported positive outcomes (developmental and clinical) associated with the immune system with LCPUFAs (EPA, DHA and ARA) during early life (Birch et al., 2010; Clausen et al., 2018; D'Vaz et al., 2012; Field et al., 2010; Field et al., 2008a, 2008b; Foiles et al., 2016; Lapillonne et al., 2014; Lauritzen et al., 2005; Manley et al., 2011; Pastor et al., 2006). These beneficial effects of a balanced LCPUFA supplementation on immune functions can be explained by different mechanisms (Calder, 2008, 2012; Fussbroich et al., 2020). One such mechanism has been eluded in figure 8.1.

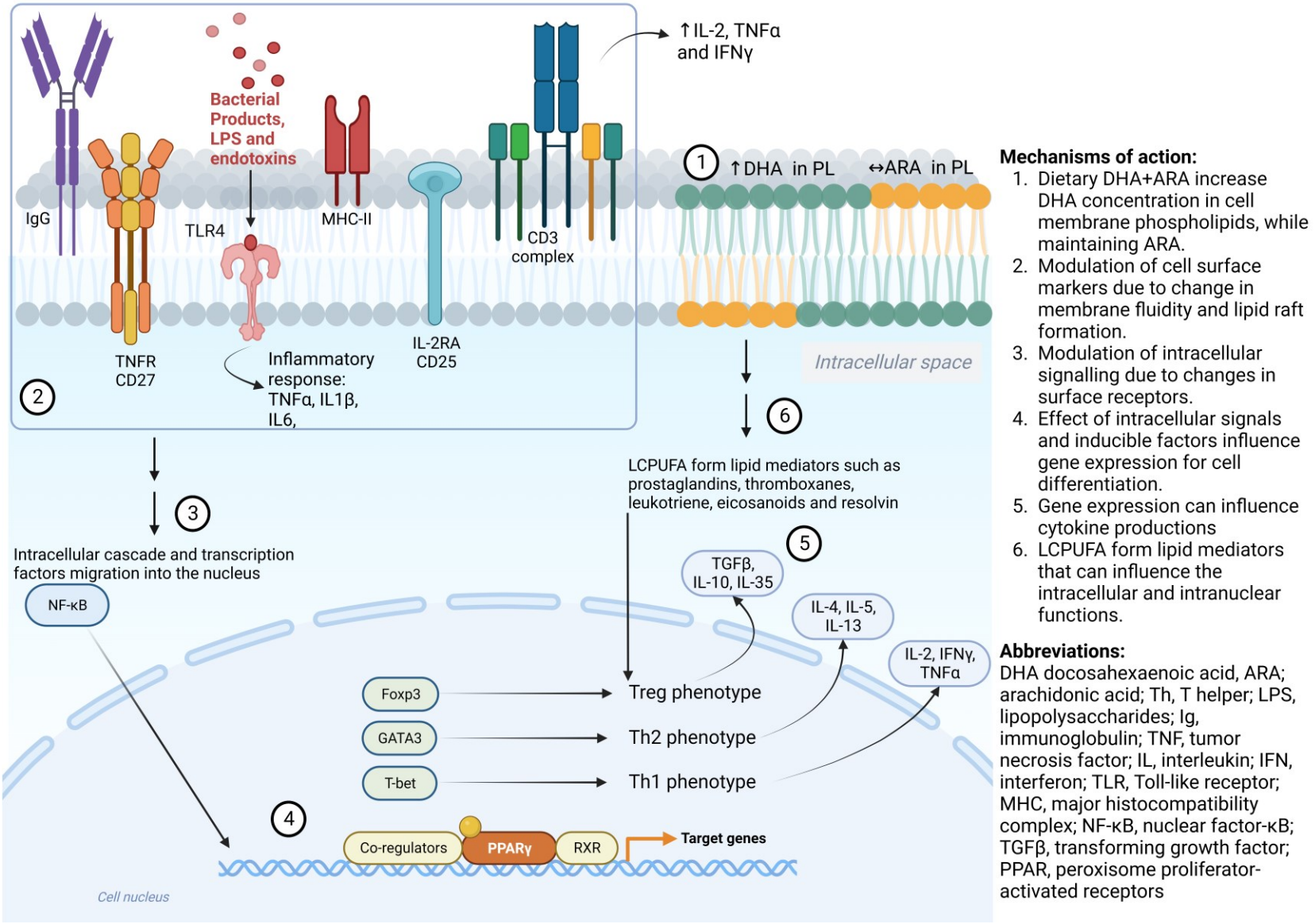

Figure 8.1. Mechanism by which LCPUFA (DHA and ARA) may have influenced immune cell functions.

Infants prone to allergies have immune system dysfunction where they generate a Th2-skewed immune response to foreign antigens, such as food or innocuous antigens. As the immune system matures and becomes more adult-like, the Th1/Th2 immune response is restored. The immunomodulatory properties of DHA and ARA are thought to help promote this balance by inducing Th1 and suppressing Th2 responses. The lipid mediators derived from DHA and ARA have different and opposing roles in the inflammatory processes, which can influence the risk of developing inflammatory diseases (Miles et al., 2021). DHA is thought to be anti-inflammatory, but more complex properties of this n-3 LCPUFA are also being reported (Serhan & Recchiuti, 2012; Serhan, 2014; Serhan et al., 2008). Similarly, the pro-inflammatory properties of ARA due to its role in the synthesis of prostaglandin and leukotriene (biomarkers of inflammation) are also being challenged (Hadley et al., 2016; Hanna & Hafez, 2018). Additionally, the preferential localization of LCPUFAs in cell membranes can influence membrane fluidity, signal transduction and lipid raft formation. The extra-cellular and intracellular signalling through cell surface receptors are also subject to membrane composition and can influence overall cell functions (Harbige, 2003; Simopoulos, 2002). Systematic reviews and clinical trials have reported emerging evidence supporting the role of DHA supplementation in suppressing allergic symptoms and preventing the development of atopic conditions (Alm et al., 2009; Gunaratne et al., 2015; Kull et al., 2006; Middleton et al., 2018; Nagakura et al., 2000; Schubert et al., 2009). These findings suggest that the immunomodulatory properties of LCPUFA can be beneficial in promoting Th1, and suppressing Th2, type response by inducing phenotypic and functional changes in immune cells. Therefore, future research should aim to demystify these complex immunomodulatory properties in Th2 dominant conditions.

8.2.3. Strengths and limitations

The diet used in animal experiments can influence the findings and their translation into humans. Therefore, it is essential to use animal diets that resemble human diets. Further, experiments evaluating the effect of a bioactive component should account for other nutrients in the diet that can influence the primary outcome. The animal experiments reported in this thesis research have tried to account for these pitfalls. The key strength of our animal experiments is the balanced and representative composition of the animal diets. Not only did we ensure the total SFA, MUFA and PUFA are representative of the human diet, but we also ensured it is closely matched between the experimental and control diets. This resulted in the first set of experiments conducted with a physiologically achievable dose of DHA and ARA while meeting the current dietary recommendations (Koletzko et al., 2014).

Another strength of our experiments involves using relatively low doses of supplementation to show programming effects that are visible later in life. We identified the suckling period as a critical window of opportunity and the ability of LCPUFAs to have a programming effect on the developing immune system of neonates (Philip C. Calder et al., 2010; Gutiérrez et al., 2019). Further, we explored this critical period to show that supplementation with DHA and ARA can enhance OT development in allergy-prone conditions. We used two rodent models with a Th2-biased immune system to evaluate the immunomodulatory effects of combined supplementation of DHA and ARA during early life (suckling and weaning). This phenomenon can be helpful in preventing the onset of food allergies seen in childhood (Philip Calder, 2013; Philip C. Calder, 2013b; Elghoudi & Narchi, 2022).

The biological differences between males and females also interact with nutrition supplementation and influence the biomarkers of immune outcomes (Chen et al., 2008; Christoforidou et al., 2019; Dor-Wojnarowska et al., 2017; Klein & Flanagan, 2016). The animal

experiments conducted as part of this thesis reported on the effect of sex on the immune system markers. However, our experiments were not sufficiently powered to understand the role of sex in immune system development and OT outcomes. This is a limitation that needs to be addressed in future animal experiments.

The experimental assays involving immune cell phenotype analysis are based on single-cell staining with fluorescently labelled antibodies. We used combinations of two or more antibodies (commercially available) to identify the various type of immune cells. However, some cell markers are shared between different immune cells (i.e., CD11b expression on DC and macrophages) (Ginhoux & Jung, 2014). Although we have used the current literature to determine the panel of antibodies used for the identification of immune cells, there are possibilities of misidentification. Therefore, future studies should verify the identified immune cells with functional markers exclusive for specific immune cells, such as transcription factors or unique cell surface markers.

Immune system development is an extended process that begins in fetal stage and extends into early life after birth. This involves various changes in the composition and function of immune cells of immune organs such as thymus, spleen, lymph nodes, etc. Our animal experiments mostly used spleen and lymph nodes collected from rodent offspring to isolate and analyze immune cells. The use of the spleen is common in the literature to determine the maturation of systemic immune cells, but it has limitations. Future studies should analyze immune cells and their functions from different immune organs. For instance, the development of OT is initiated in the gut where GALT is present. Therefore, analyzing APCs and their ability to influence adaptive immune cells to induce tolerogenic response can be very useful, especially in the context of dietary interventions.

OT development can be influenced by multiple factors (ova dosage during primary exposure, frequency of exposure, immunization protocol, and more). Therefore, experiments aimed to measure OT outcomes need to optimize these factors. The OT induction protocol used in animal experiment for chapter 8 may not be optimal. A successful development of OT is described by a reduction in plasma level of ova-specific immunoglobulins in ova-exposed pups than controls. Our findings did not support this hypothesis and we believe the OT induction protocol used for the animal experiments presented this limitation. Future studies should perform a pilot experiment to optimize important factors that can influence OT outcomes.

Another limitation pertaining to animal experiment conducted for chapter 9 arise from the calculation of observational unit. Generally, experiments evaluating dietary interventions on young pups, the observational unit is based on the number of dams per group used in the experiment and not the total number of pups per group. All the pups born to the same dam, forming a litter, are genetically identical. Therefore, dietary interventions that are targeted at the lactating dams should consider dams as observation unit, even if the outcomes are measure in pups. The experiments conducted for chapter 8 faced a challenge of small litter size born to BALB/c dams, making it difficult to evenly distribute the pups from each litter between the designated diet groups. Therefore, we used extra dams to attain the required number of pups and cross-fostered to match the number of pups per diet group. Future experiments should account for the smaller litter size and design animal experiments to avoid this limitation.

8.3. Conclusion

Overall, our findings have important considerations for the role of LCPUFA in immune system development and the prevention of food allergies during early life. We identified that the DHA, in the context of ARA, is essential for immune system development and providing n-3 SDA as a

precursor to DHA was insufficient for DHA. Using allergy-prone rodent models, we showed that the combined supplementation of DHA and ARA could be beneficial for the development of OT, potentially by restoring the balance between Th1/Th2 immune responses. Dietary supplementation of DHA and ARA during early life is essential for the optimal development of the immune system. However, due to the lack of precise understanding of the mechanism behind these beneficial changes, we need more research to explore the immunomodulatory properties of LCPUFAs and its implications in the development of the immune system during early life.

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LIST OF APPENDICES

Appendix A

Regulation of immune function in healthy adults: a one-stop guide on the role of dietary fatty acids, gut microbiota-derived metabolites and select micronutrients in combination with physical activity. [1]

Abstract

The immune system requires an adequate supply of nutrients, although current dietary recommendations may not account for optimal immune function in healthy adults. Nutrient inadequacies due to the growing influence of the western diet pose a risk for immune dysfunction. This review aims to determine the beneficial effects of supplementing: dietary fats, nutrients that modulate gut microbiota, and specific micronutrients, on systemic immune functions (concentrations of plasma cytokines, antibodies and acute phase proteins) during health and acute inflammatory conditions, including COVID-19. We discussed micronutrients (selenium, zinc and vitamin D) with compelling evidence supporting immunomodulatory properties. Additionally, the synergistic effects of physical activity and dietary interventions on systemic immune markers are explored. Briefly, evidence suggests that dietary consumption of monounsaturated (oleic and palmitoleic acids) and omega-3 polyunsaturated fatty acids (eicosapentaenoic and docosahexaenoic acids) promotes anti-inflammatory properties. Food sources (fiber, prebiotics, probiotics, omega-3) and patterns (Mediterranean diet) increase the production of short-chain fatty acids, beneficially altering gut microbiota composition, which

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Patel D, Evanchuk J, Wang R, Dunbar C, Munhoz J, Field C. (2023) Regulation of immune function in healthy adults: one-stop guide on the role of dietary fatty acids, gut microbiota-derived metabolites and select micronutrients in combination with physical activity. *Applied Physiology Nutrition and Metabolism* (under review).

subsequently enhances the immunomodulatory properties of circulating immune cells. A positive synergistic role of nutrient supplementation (omega-3 and fiber) and physical activity on circulating C-reactive protein and interleukin-6 levels have been observed . Lastly, omega-3 supplementation during COVID-19 infection may reduce circulating C-reactive protein and pro-inflammatory cytokines and improves pain and fatigue symptoms. This review highlights recent findings that support the beneficial role of specific nutrients in promoting systemic immune function in healthy adults. However, to establish specific dietary recommendations to support optimal immune function, more research is required.

Keywords: Nutrition, Immunity, Infection, Inflammation, Selenium, Zinc, Vitamin D, Physical Activity, MUFA, PUFA

Key takeaway: Increasing dietary fats (fish and olive oils) and specific micronutrients may positively impact systemic immune function in healthy adults. Evidence suggests that these nutrients promote immunomodulatory properties useful in resolving acute infection.

Brief introduction to immune system functions

The immune system is a complex multi-cellular system that works in collaboration with the nervous, endocrine, cardiovascular and muscular systems to resolve dysfunction and restore homeostasis (Steinman, 2004). The human immune system operates through innate and adaptive arms. The innate arm provides the first "line of defence" through mechanical (skin and mucous membrane), chemical (temperature, pH and other mediators), phagocytic and inflammatory barriers (complement and coagulation systems). Innate immune cells, including granulocytes (neutrophils, eosinophils and basophils), macrophages, dendritic cells and natural killer (NK) cells as well as epithelial cells and endothelial cells, operate through pattern recognition receptors to fight pathogens (Marshall et al., 2018). These receptors recognize pathogen-associated molecular patterns, like lipopolysaccharides (LPS) from gram-negative bacteria. Innate immune cells are 'broadly-specific' against pathogens and are quick to respond, typically starting in hours and lasting for days. These processes are orchestrated through small signalling proteins called cytokines, involved in cell-to-cell communication (figure 1). Adaptive immunity includes T cells and B cells, which are more effective against antigens (a specific foreign molecular structure) and possess long-term memory through recognition specificity. The B and T cells detect specific-antigens via immunoglobulins (B-cell receptors) or T-cell receptors, respectively. The activation of T cells requires antigen-presentation by innate antigen presenting cells, such as dendritic cells and macrophages (Guerriero, 2019). The immune system consists of primary (bone marrow and thymus) and secondary (lymph nodes and spleen) immune organs, and the immune structures present in the gastrointestinal tract (GIT) form the gut-associated lymphoid tissue (GALT). The GALT tolerates commensal microbiota of the gut and surveils the

massive antigen load in the form of food. Nutrients and gut microbiota interact to regulate GALT immune responses, which in turn affects systemic immune responses.

Inflammation is characterized byswelling, redness, and edema of the infected region and systemic fever. This typically involves the production of markers of systemic inflammation, including acute phase C-reactive protein (CRP), prostaglandins, and cytokines. The immune system requires dietary nutrients for optimal function, and an insufficient supply of these nutrients may prevent optimal immune responses. During acute inflammation or infection, requirements for specific nutrients may increase, making the current dietary recommendations potentially inadequate. For instance, viral infections such as COVID-19 cause increases in virus-specific immunity and enhances the production of inflammatory biomarkers (Mortaz et al., 2020). During certain illnesses, nutritional interventions may improve nutrient status and clinical outcomes (Isfahani et al., 2021). Since sedentary lifestyles have been linked to inflammatory conditions, regular physical activity (PA) may also reduce the risk of infections(Shao et al., 2021).

This review focuses on (1) the impact of dietary fatty acids (FAs), that can modulate cell-membrane FA compositions, on systemic immune function, (2) the role of gut microbiota modulating nutrients in GALT function, and (3) key micronutrients that may help resolve acute infections, including COVID-19, through their immunomodulatory properties. We also comment on the synergistic role of PA during nutritional interventions on immune function. This review focuses on recently conducted clinical trials on predominantly healthy populations where significant immune parameters are reported. Finally, we develope a summary of these findings that can be used by researchers, students, and other professionals.

The role of dietary fats in systemic immune function through the modulation of cell membrane FA composition

Dietary fats dictate the FA profile of an individual and can modulate their cellular and systemic immunity (summarized in figure 2). The competitive incorporation of dietary saturated fatty acids (SFAs) and unsaturated (monounsaturated, and polyunsaturated fatty acids (MUFAs and PUFAs respectively)) into cellular membranes can modulate the differentiation of immune cells into either pro-inflammatory or anti-inflammatory dominant (Christ et al., 2019). For instance, the consumption of a high MUFA diet increases MUFA status by replacing SFA and PUFA, which results in dominant anti-inflammatory properties (Radzikowska et al., 2019). The total FA composition of dietary fats is described by other studies (Giakoumis, 2018; Orsavova et al., 2015). The current review only focuses on specific fatty acids or their family.

N-3 PUFA interventions

Several mechanisms related to the impact of PUFAs on immune function and inflammation have been explored (reviewed in (Philip C. Calder, 2013a)). In general, n-3 FAs like alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), exhibit anti-inflammatory effects, whereas n-6 FAs like includes linoleic acid (LA) and arachidonic acid (ARA) encourage pro-inflammatory properties. Moreover, the unique resolving role of n-3 long-chain PUFAs (LCPUFAs) during inflammation has also been described (Serhan et al., 2008). LCPUFAs (such as ARA, EPA and DHA) are present in seafood and animal sources, while ALA and LA are obtained from plant-based oils such as flaxseed, canola, soybean and corn oil (Saini & Keum, 2018).

In a randomized controlled trial (RCT), Rees et al. (2006) studied the impact of different doses of EPA (low 1.4 g, moderate 2.7 g or high 4 g from EPA-rich oil; daily for 12 weeks) compared to a placebo (corn oil), on innate immune cell functions. The dose-dependent EPA incorporation in peripheral blood mononuclear cells (PBMCs) led to the reduction in prostaglandin E2 (PGE2, an inflammatory lipid mediator derived from ARA). However, TNF-α, IL-1β, and IL-6 production by neutrophils or monocytes after *ex-vivo* stimulation did not change with any dose of EPA. Next, Yang et al. (2012) conducted a meta-analysis that evaluated the effects of n-3 PUFA supplementation (between 0.27 to 6.6 g/day) on plasma concentrations of soluble intercellular adhesion molecule-1 (sICAM), a biomarker of inflammation released by activated innate immune cells and endothelial cells. The n-3 PUFA supplementation (predominantly EPA and DHA) reduced sICAM-1 (weighted mean difference of -8.8; 95%CI of -15.20 to -2.53). Further, older adults (>55 years of age) showed higher sICAM-1 than younger adults, suggesting higher n-3 requirements in older adults.

To measure functional changes, J. F. Ferguson et al. (2014) evaluated the effect of n-3 LCPUFA supplementation (1:1 EPA+DHA from fish oil at 3.6 g/day or 0.9 g/day for 7 weeks) on acute inflammation response (towards lipopolysaccharide (LPS), bacterial challenge). In comparison to the placebo (corn oil), an increase in red blood cell incorporation of EPA and DHA was reported but only EPA showed dose-response. Although there were no significant changes in plasma levels of inflammatory markers, including (CRP, IL-1receptor agonist, IL-6, TNF-α and IL-10) pre- and post-bacterial challenge in any intervention group, there was a trend towards a greater reduction in TNF-α concentrations with higher dose. Further, those in the n-3 LCPUFA groups had less fever symptoms after the bacterial challenge compared to the placebo. Overall, a high

dose of n-3 LCPUFA (3.6 g/day) improved immune function and symptoms after a bacterial challenge.

In an RCT conducted with student participants, Kiecolt-Glaser et al. (2011) showed that a supplement of n-3 LCPUFA at 2.5 g/d (2.1 g EPA and 0.35 g DHA) for 12 weeks decreased IL-6 and TNF-α production (~14%) by PBMC following LPS stimulationcompared to the placebo group. Akin to Jane F. Ferguson et al. (2014), this study observed no difference in plasma levels of inflammatory IL-6 and TNF-α production without stimulation. However, there was a alleviation of self-reported stress in students that received the LCPUFA supplement, suggesting a possible inverse association between stress and n-3 LCPUFA. Kirkhus et al. (2012) used different sources of food to supplement n-3 LCPUFA (1.1g/day EPA+DHA for 7 weeks) and the results showed that there was no change in plasma CRP , IL-6, TNF-α or iCAM-1 concentration, but significantly improved EPA and DHA status at the cost of ARA plasma levels. Furthermore, EPA+DHA uptake was independent of n-3 sources (fish oil, fish pate and fruit juice).

Other evidence suggests that shark liver oil (1.5 g/day containing mostly EPA+DHA for 6 weeks,) resulted in the reduction of plasma CRP (1.51±2.67 vs. 1.78±3.03) (Zakrzewska et al., 2021). Further, shark liver oil also increased EPA, DHA and ARA in red blood cell at follow-up compared to baseline. However, this study lacked a placebo control group and only included female participants, which challenges the generalizability of the findings. Similarly, another RCT evaluated the effect of n-3 PUFA enriched eggs (~1.1 g/day for 3 weeks; 0.7 g ALA, 0.05 g EPA and 0.3 g DHA) on *ex-vivo* immune function (Kolobaric et al., 2021). The authors concluded that the anti-inflammatory effects of n-3 PUFA resulted in significantly higher TGF-β1 and resolvin mediator (RvE1) and lower prostaglandin-E2 (PGE2)/PGE3 ratio and IL-6 production by PBMC after PMAi stimulation. These differences between pre- and post-intervention associated with n-

3 PUFA were absent in placebo control (egg with low n-3 content). The study did not report data on FA profiles making it difficult to extrapolate their contribution to *ex-vivo* immune changes. Further, an RCT testing a lower dose of n-3 enriched egg (0.41 g/day for 3 weeks) reported a similar frequency of granulocytes and monocytes, but later showed that there was lower expression of CD11a+ in the supplemented group compared to the control (Mihalj et al., 2020). Despite the above findings, the use of eggs in supplementing n-3 may be confounded with other immunomodulating nutrients such as choline (Detopoulou et al., 2008).

N-3 supplementation provides substrate for the production of FA-derived mediators with immunomodulatory properties known as oxylipins. Ostermann et al. (2019) quantified plasma oxylipins in participants (aged 20-77 years, n=121) supplemented with low, moderate and high EPA+DHA doses (0.47 g/day, 0.93 g/day and 1.87 g/day, equivalent of 1, 2 and 4 servings of fatty fish, respectively, via fish oil capsules) for 12 weeks. The n-3 PUFA supplementation decreased plasma levels of n-6 hydroxy-PUFAs and n-6 epoxy-PUFAs, oxylipins involved in pro-inflammatory responses (Serhan, 2014), and increased n-3 epoxy-PUFAs, oxylipins involved in anti-inflammatory responses). Additionally, a linear dose response relationship was reported between the dietary supply of n-3 PUFAs and plasma oxylipin concentrations derived from those n-3 PUFAs. This study provides evidence of the potential regulation of lipid mediators involved in immune functions by n-3 PUFAs.

Several studies reporting immune function changes associated with n-3 PUFA supplementation do not report FA profile changes, which limit the interpretation of dose-related changes in such studies. Therefore, future studies should focus to improve 3 key parameters. First, the inclusion of both sexes and a sufficiently powered study sample size to capture in-vivo and ex-vivo immune outcomes. Second, utilize individual n-3 family FAs supplements with multiple doses to

isolate the specific immunomodulatory properties associated with each FAs. Lastly, report data on FA profile changes induced with supplementation to allow readers to understand the context in which immune outcomes may be modulated.

MUFA interventions

Oleic acid (C18:1-*cis*-n-9, OA) and olive oil

A systematic meta-analysis by Schwingshackl et al. (2015) showed that the Mediterranean diet, rich in oleic acid (OA), reduced CRP and IL-6 plasma concentrations in healthy populations. Olive oil and SFAs from foods representative of the western diet have been compared to determine their effects on plasma sICAM (Fuentes et al., 2008; Voon et al., 2011). These studies reported no significant changes in plasma CRP, IL-6 or TNF-α, however, a modest decrease in sICAM was seen with olive oil supplementation. Note, the use of olive oil to understand the roles of OA may be confounded with the anti-oxidative properties of polyphenols present in olive oil. Accordingly, Konstantinidou et al. (2010) isolated the effect of OA from olive oil, comparing virgin olive oil (rich in polyphenols and other bioactive components), washed-out olive oil (reduced polyphenols) and control supplemented groups. The results suggested an additive effect of OA and polyphenols in reducing plasma CRP levels *in-vivo*. Studies have shown a greater decrease in *ex-vivo* IL-6 response to LPS by PBMC when supplemented with fatty fish oil than olive oil, suggesting that they may have different anti-inflammatory mechanisms (Damsgaard et al., 2009). However, the lack of a robust placebo control group in several of these studies restricts our understanding of the qualitative differences in anti-inflammatory properties between MUFAs and PUFAs, complicating the interpretation of immunomodulatory effects of fats (Damsgaard et al., 2009; Maki et al., 2009; Singhal et al., 2013).

Palmitoleic acid (16:1-*cis*-n-7, POA)

Anti-inflammatory properties of palmitoleic acid (POA, an n-7 FA) have been shown in cell culture and pre-clinical models (Çimen et al., 2016). In models of obesity-inducing inflammation, POA suppresses pro-inflammatory genes, like NF-κB, IL-6, TNF-α, and IL-12, in macrophages and adipose tissues (Chan et al., 2015; Lima et al., 2014; Ravaut et al., 2020). Despite this, clinical trials that have examined immune function during POA supplementation are limited. Nonetheless, a recent RCT by Sasagawa et al. (2021) reported that a POA supplement (688 mg/day for 3 weeks), compared to a medium chain FA placebo, did not show significantly change plasma CRP, TNA-α or IL-6 concentrations. However, the absence of a washout period between interventions along with lack of treatment adherence and FA profile reporting are significant limitations (Sasagawa et al., 2021). Passos et al. (2016) compared the anti-inflammatory properties of POA and OA on human lymphocytes upon *ex-vivo* stimulation with a T cell mitogen Concanavalin-A, a plant-based lectin. POA (50 μM) decreased the production of inflammatory cytokines IL-2, IL-6, IFN-γ, TNF-α and IL-17 by activated lymphocytes compared to a control group. Whereas, OA (50 μM) only decreased IFN-γ production by activated lymphocytes. Lower OA concentrations (25 μM) promoted Th2 responses (IL-4 and IL-10), and IL-2 production, which may promote Th2 cell proliferation, that can suppress more inflammatory Th1 cells. More research is required to determine durations and doses of specific MUFAs that optimize immune function in healthy individuals.

Compounding effects of PA and dietary fats on systemic immune functions

Habitual PA promotes lasting beneficial immunological adaptations such as higher immunosurveillance (the interchange of immune cells between circulation and lymphoid tissues),

antibodies (vaccination response) and anti-inflammatory cytokine production. On the contrary, a single bout of high-intensity PA is considered a stressor which may promote acute and transient immunological responses (plasma levels of CRP and IL-6) (Nieman & Wentz, 2019). In this review, we investigated PA as bodily movement through exercise that result in energy expenditure, but we did not explore any particular type or intensity of exercise. There may be immunological differences amongst various types of exercises and intensities, however, this is not detailed in this review.

ALA supplementation (14g/day for 12 weeks) in adults (>60 years of age), during long-term resistance-training, was conducted to evaluate changes in inflammatory markers. In men, but not women, a significant reduction in plasma IL-6 was associated with ALA supplementation (Cornish & Chilibeck, 2009). An RCT of n-3 supplementation (1.8g/day for 4 weeks; 0.32g EPA and 0.24g DHA) significantly reduced plasma TNF-α, PGE2 and IL-6 concentrations measured at 24 and 48 hours after the exercise session, compared to placebo groups (Tartibian et al., 2011). Moreover, regular use of n-3 supplements (self-reported) by recreational athletes was associated with lower pre- and post-exercise plasma CRP levels compared to sporadic or non-users (Hansen et al., 2021). Similarly, in an RCT employed a higher dose of n-3 (3g/day for 4 weeks; 2.1g of EPA and 0.86g DHA),there was a reported alleviation of exercise-induced inflammation (EII, via plasma IL-6 and CRP) compared to the placebo (Kyriakidou et al., 2021). This study also reported improvement in perceived muscle soreness post-exercise with n-3 supplementation. On the contrary, Da Boit et al. (2015) reported Krill oil supplementation (0.36g/day for 6 weeks; 0.24g EPA and 0.12g DHA) increased plasma IL-6 post-exercise compared to placebo. Additionally, *ex-vivo* analysis in this study showed NK cell cytotoxicity and IL-2 production by concanavalin-A stimulated PBMC was higher post-exercise (3 hours) with supplementation.

Bloomer et al. (2009) evaluated 4.4 g/day n-3 LCPUFA supplementation (1:1 EPA and DHA for 6 weeks) on EII in exercise-trained men. They reported significantly lower plasma levels of CRP and TNF-α at both pre- and post-exercise (48 hours) in the n-3 supplemented group compared to placebo controls.

Overall, n-3 PUFA supplementation, especially EPA+DHA, may attenuate EII (Farley et al., 2021). The interaction between EII, n-3 PUFA supplementation and cardiovascular fitness requires more research as the current level of evidence is promising but preliminary. Future research should identify relevant dose-response and mechanisms involved in such interactions. This will help develop clinical and sub-clinical applications of PA and FA supplementation interventions.

The effect of food-derived SCFAs on GALT and systematic immune functions

Gut microbial communities are formed by trillions of live microorganisms including bacteria, viruses, fungi, archaea and protozoa (Li et al., 2019). Over the past few years, the modulatory effect of different nutrients or diets on the composition and diversity of gut microbiota has been well-established (Redondo-Useros et al., 2020; Vinelli et al., 2022; Y. Wu et al., 2019). The existing literature often summarize the effect of dietary consumptions on gut microbiota, but evidence regarding the immunomodulatory effects of the gut microbiota is limited. The intestine contains the GALT which exhibits immune defence against harmful pathogens and immunosuppression toward innocuous food antigens and microbiota (Armet et al., 2022; Eran Blacher et al., 2017). The gut microbiota and bioactive metabolites can work with the GALT and the systemic immune system to regulate local and systemic immunomodulatory responses (Armet et al., 2022; Qu et al., 2021). In this section, we aim to explore the diet-gut microbiota

axis further, with a focus on exploring the effect of diet or gut microbiota (that is affected by diet) on the local and systemic immune system and immune responses.

Relationships between the GALT and SCFAs associated with dietary fiber, prebiotics and probiotics

Short-chain FAs (SCFAs), such as acetate, butyrate and propionate, are produced by the gut microbiota in the colon through the fermentation of carbohydrates such as fiber, starch, simple sugar and oligosaccharides (Topping & Clifton, 2001). Diverse SCFAs can be produced by different gut microbes. Acetate is commonly biosynthesized by *Akkermansia muciniphila, Baterioide spp., Bifidobacterium spp., Prevotella spp.* And *Ruminococcus spp.* Through the pyruvate-acetyl-CoA pathway or the Wood-Ljungdahl pathway (Ratajczak et al., 2019; I. Rowland et al., 2018). Butyrate is often produced by *Clostridium symbiosum, Faecalibacterium prasnitzii* and *Bacteroidetes spp.* through the Butyryl-CoA pathway (Ratajczak et al., 2019; I. Rowland et al., 2018). *Akkermansia muciniphila, Bacteroidetes spp., Clostridium spp., Roseburia spp.* and *Ruminococcus spp.* are common producers of priopnate via acrylate, succinate and propanediol pathways (Ratajczak et al., 2019). More recently, SCFAs have been explored for their immunomodulatory functions. Acetate is the most abundant SCFA produced by gut microbes and it can inhibit the production of nitric oxide by macrophages after LPS stimulation, suppress the expression of pro-inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α and increase anti-inflammatory cytokine IL-10 (Nakkarach et al., 2021). SCFAs may also act as signalling molecules to regulate the function of various immune cells directly. For example, acetate, propionate and butyrate activate the free fatty acid receptor-2 and free fatty acid receptor-3 on immune cells while propionate and butyrate can activate the PPAR-γ receptor, therefore affecting the downstream immune response (Ratajczak et al., 2019). Butyrate has been

shown to decrease the expression of pro-inflammatory cytokines TNF-α, IL-6, and IL-12 and increase the expression of anti-inflammatory cytokine IL-10 in intestinal macrophages under inflammatory challenge (Parada Venegas et al., 2019). Figure 3 provides a summary of major connections between diet, gut microbiota and GALT functions.

Dietary fiber may elevate the abundance of SCFA-producing gut microbes and increase host SCFAs, which can regulate the GALT and systemic immune systems, please see the review by Ian Rowland et al. (2018) for an extensive list of SCFA producers in the gut. A high-fiber diet (39g/day for 5 days), rich in legumes, whole grains, fruits and vegetables significantly increased plasma acetate and propionate concentrations in healthy participants (Gill et al., 2020). In another study, a higher intake of dietary fiber from food in a healthy adult population was associated with increased relative abundances of *Eubacterium eligens* and *Faecalibacterium prausnitzii*,both SCFAs producers, as well as an improvement in their abilities to degrade oligosaccharides and produce butyrate (W. Ma et al., 2021). The authors reported that higher dietary fiber consumption wasrelated to lower plasma CRP concentrations. Similarly, a 3 months intervention of a prebiotic supplement containing 16g of inulin-type fructan,a type of dietary fiberand food rich in inulin-type fructan (such as asparagus, brussels sprouts, cauliflower) among obese participants led to increases in fecal SCFAs and decreases in calprotectin (a marker of gut inflammation) (Neyrinck et al., 2021). Interestingly, this study found that this prebiotic intervention did not affect the α-diversity of gut microbiota (i.e. species diversity within individuals) but affected the β-diversity of gut microbiota (i.e. species diversity between individuals) (Neyrinck et al., 2021). Together, these results indicate that the effects of increased SCFA production and decreased gut inflammation were caused by prebiotic-induced modification of gut microbiota composition instead of individual variance. Specifically, this

prebiotic intervention significantly decreased the Bacillota (formerly named Firmicutes) at the phylum level and increased Bifidobacterium at the genus level (Neyrinck et al., 2021). According to other research, Bifidobacterium is related to immune system maturation and a decline in Bifidobacterium is related to allergy as well as inflammation in infants while increased Bifidobacterium administration is related to reduced TNF-α production, increased IL-10 production and improved phagocytosis activity in healthy adults (Ruiz et al., 2017). These results further exemplify the immunoregulatory effects of gut microbiota and SCFAs.

According to the World Health Organization definition, probiotics are live microorganisms that have beneficial health effects if administered in a sufficient amount (Hill et al., 2014). When it comes to foods and diets, commercial supplements and some fermented foods such as yogurts, kefirs, pickles and kimchi are found to contain probiotics, implicating their potential health benefits for humans (Marco et al., 2017). Among a variety of probiotic strains, Bifidobacterium and Lactobacillus (lactic acid and SCFAs producers) are two of the most commonly studied probiotics in the literature. In healthy people, a daily supplement of multi-strain probiotics for 6 weeks (containing Bifidobacterium and Lactobacillus, 450 billion colony forming units (CFU) for people < 11 years old and 900 billion CFU daily for people >11 years old) did not affect the α-diversity or β-diversity of gut microbiota (Cabrera et al., 2022). However, this probiotic intervention increased the level of circulating anti-inflammatory SCFAs, reduced the production of proinflammatory cytokines (IL-12, IL-13, IL-15, IL-18) and lowered CD4+CD45RO+/ CD4+CD45RA+ T cell ratio (consistent with inhibition of systematic inflammation) (Cabrera et al., 2022). Besides the intestinal immune function, Bifidobacterium and Lactobacillus were also associated with preventing respiratory infections and alleviating symptoms (Hegazy et al., 2022; Williams, 2010). A systematic review and meta-analysis also found that prebiotics and probiotic

(various strains including Bifidobacterium and Lactobacillus) supplements improve influenza vaccination efficacy, significantly higher influenza haemagglutination inhibition antibody titers after vaccination (Yeh et al., 2018). Nonetheless, the safety of probiotics should be prioritized to determine if they increase the risk of infection and inflammation in immunocompromised people (Sanders et al., 2010).The efficacy of these interventions should be evaluated using long-term studies, since systemic immune changes associated with GALT-specific immune outcomes may be difficult to capture.

Immunomodulatory effects of other gut microbiota produced metabolites

More recently, there is interest in other nutrients that may be capable of interacting with the gut microbiota and changing the SCFA levels, to modulate immune functions. In a RCT, middle-aged healthy participants with 8 weeks of n-3 PUFA supplementation (4g of EPA and DHA mixture) showed an increased abundance of Bifidobacterium (SCFAs producer), Roseburia (Bacillota phylum) and Lactobacillus (Bacillota phylum), which of them are known for their butyrate-producing and immunoregulatory properties (Watson et al., 2018). The ratio of Bacillota/Bacteroidota is commonly used as a biomarker of intestinal homeostasis, where an imbalanced ratio is related to gut dysbiosis. Certain food patterns may also affect the gut microbiota (Stojanov et al., 2020). The CARDIVEG Study targeting clinically healthy people found that a 3-month intervention of a Mediterranean diet significantly decreased the abundance of Parabacteroides (Bacteroidota phylum), and showed a negative correlation between fecal SCFAs such as propionate, acetate, and butyrate, isovalerate and isobutyrate and some proinflammatory cytokines including VEGF, MCP-1, I1-17, IP-10 and IL-12 (Pagliai et al., 2020). This study also explored the effect of a 3-month vegetarian diet and reported significant decreases in Clostridium (Bacillota phylum, indicator of gut dysbiosis in adults) as well as a

negative correlations between fecal SCFAs isobutyrate and isovalerate and proinflammatory cytokines MCP-1 and IL-12 (Pagliai et al., 2020).

Dietary amino acid, tryptophan that escapes host digestion and absorption can be metabolized by different colonic bacterial species including *Bacteroides* spp. and *Clostridium* spp into various indole and indole derivatives which have been implicated in GALT function (Liu et al., 2021). Tryptophan metabolites have been reported to activate aryl hydrocarbon receptors, increase the expression of genes related to gut integrity and mucin production, inhibit the activation of NF-κB signalling, prevent transcription of pro-inflammatory cytokine IL-8 and activate the production of anti-inflammatory cytokine IL-10 in human cell lines (Bansal et al., 2010; Ehrlich et al., 2020; Meng et al., 2020). However, there is still limited evidence from human RCTs exploring the effect of tryptophan metabolites on gut microbiota and local or systematic immune functions, indicating the necessity for further research related to this area. More recently, compounds produced by gut microbe metabolism of bile acid have been shown to have immunoregulatory properties in animal models. These bile acid metabolites may maintain intestinal integrity, promote immune cell activation, downregulate inflammatory signalling pathways (NF-κB), suppress proinflammatory TNF-α, IL-12, IFN-γ, IL-6 and enhance anti-inflammatory IL-10 production (E. Blacher et al., 2017; Fiorucci et al., 2018; Vavassori et al., 2009; Wang et al., 1999). However, deoxycholic acid, a bile acid metabolite, supplements led to an increased concentration of fecal secondary bile acids, gut dysbiosis, intestinal inflammatory marker infiltration and increased expression of pro-inflammatory cytokines IL-1β and TNF-α (Xu et al., 2021). Considering the mixed findings, the impact of bile acid metabolites on humans is still under exploration.

Compounding effect of PA and SCFA or other gut-derived nutrients on GALT

The synergistic effects of diet and PA could alter the gut microbiome, leading to the modulation of the GIT inflammatory status (Bermon et al., 2015; Pedersini et al., 2020; Strasser et al., 2021; Tzemah Shahar et al., 2020). Despite a positive association between PA and fecal SCFAs, only a few RCTs have been conducted (Ortiz-Alvarez et al., 2020). Cronin et al. (2018) evaluated the effect of protein supplementation (24 g/day of whey protein) and/or a PA training routine (3 times/week for 8 weeks) in sedentary participants. Modest alterations in gut microbial characteristics and significant changes in body composition were identified in individuals undergoing PA, with or without protein supplementation, while significant changes in the gut virome were found in those taking the protein supplement (with or without PA). Although GALT functions were not specifically tested, an analysis of systemic inflammatory parameters was conducted with no clinically relevant changes observed amongst between the interventions (Cronin et al., 2018). Conversely, physically active individuals taking a synbiotic (prebiotic+probiotic for 3 weeks), showed a reduced increase in pro-inflammatory IL-16 concentrations when compared to individuals taking prebiotics only (West et al., 2012). Nonetheless, the difference reported between the group was small and its clinical significance needs to be interpreted with caution since there was no observed effect on mucosal immunity and GIT permeability in either intervention group.

A summary of diet-induced GALT functions is described in figure 3. Gut microbiota research in the field of nutrition has garnered interdisciplinary attention, particularly concerning the gut-brain axis, but more reproducible studies using pre-clinical models are required before clinical translation (Chakrabarti et al., 2022; Mayer et al., 2022; Murray et al., 2022; Peterson, 2020). Furthermore, the efficacy of these interventions should also be evaluated using long-term studies,

since systemic immune changes associated with GALT-specific immune outcomes may be difficult to capture.

Micronutrients involved in immunoregulation and the resolution of acute infections

Micronutrients form integral parts of immune cells to optimize their function and reduce the risk of infections and excessive inflammation (Elmadfa & Meyer, 2019; Dayong Wu et al., 2019). The proposed actions of micronutrients have been previously described in human health and disease, especially the antioxidative properties of Vitamin A, B complex, C and E and iron (reviewed by Gombart et al. (2020). This section will describe evidence related to the role of selenium (Se), zinc (Zn) and vitamin D (VitD) in the prevention and resolution of infections through the modulation of immune cell functions.

Overview of key micronutrients

Selenium is an important regulator of inflammation and T cell activation and the RDA for men and women is 55µg/day (figure 4)(Huang et al., 2012; Institute of Medicine, 2006). Despite this, clinical trials have sparingly studied Se supplementation in the modulation of the immune system (Avery & Hoffmann, 2018; Wu et al., 2009). Brown et al. (2000), showed that Se supplementation (50 µg/day for 4 weeks) increased the action of selenoproteins in lymphocytes and improved the virus-specific lytic function of CD8+ T cells (Roy et al., 1994). Ravn-Haren et al. (2008) demonstrated that in comparison to Se-enriched yeast (300 µg/day) and milk (480 µg/day), the supplementation of selenate (300 µg/day) for 1-week improved the function of platelet selenoproteins function, although the overall systemic immune function was unaffected. Similarly, Wu et al. (2009) determined the impact of high doses of Se (75 µg/day, 150 µg/day and 225 µg/day for 8 weeks) in healthy men. Findings indicated no apparent improvement in

immune markers within this Se-replete population, but Se-related toxicity was not reported in those that consumed the high doses.

Zinc modulates pro-inflammatory responses as well as the development of T cells and the RDA for this nutrient in men and women is 11 and 8 mg/day, respectively (figure 4) (Institute of Medicine, 2006; Prasad, 2014). Among healthy participants, Zn supplementation (45 mg/day for 8 weeks) lowered NF-κB related oxidative stress in PBMCs, which is known to promote pro-inflammatory cytokines (Prasad et al., 2004). Furthermore, Zn supplementation also reduced PBMCs IL-1β and TNF-α production after LPS stimulation. To demonstrate the effect of mild Zn deficiency on immune responses, Pinna et al. (2002) used a Zn-restricted diet (4.6mg/day for 10 weeks) and observed saw reductions in PBMC proliferation after phytohemagglutinin stimulation. Another clinical study induced a mild Zn deficiency (2-3.5mg/day for 20-24 weeks) followed by a repletion phase (25-55mg/day for 8-12 weeks) and significant changes in IFN-y, TNF-a, CD4+/CD8+ ratio, and CD8+ T cell subpopulations were identified. (Beck et al., 1997).

The regulation of immune function by VitD (RDA of 15ug/day, figure 4 (Institute of Medicine, 2011)) has been described, but there has recently been a resurgence of interest in its role in the prevention and treatment of SARS-CoV-2 symptoms (Zhang et al., 2020). In previous investigations, VitD supplementation among healthy participants [~4700 IU/day for 12 weeks (Bock et al., 2011) and 5000-10000 IU/day for 15 weeks (Allen et al., 2012)], was shown to increase PBMC proportions of T regulatory cells and decreased Th17 cells, respectively. The development of immunological tolerance depends on T regulatory cells (Faria & Weiner, 2005). VitD3 supplementation may play a vital role in white blood cell gene expression. Indeed, in a double-blinded RCT, VitD3 supplementation (400 and 2,000 IU/day for 8 weeks) was associated with multiple alterations in gene expression including reduced expression of TNF-α inducible

genes, which are implicated in NF-κB related pro-inflammatory responses in autoimmune diseases (Hossein-Nezhad et al., 2013). Maboshe et al. (2021) showed a similar reduction in IFN-γ production by stimulated T cells with 400 IU of VitD3/day for 43 weeks. Together, these findings suggest a potential role for VitD in modulating immune responses in healthy adults.

Micronutrients and acute infections

There is some evidence to support to use of micronutrient supplements in people with respiratory infections. Lenhart et al. (2020) explored a micronutrient supplement (containing 11 nutrients including VitD, Se, and Zn, at levels close to RDA; figure 4) and the incidence of common cold infections over two years in healthy participants. The supplementation appeared to reduce both the rate of infection and the duration of runny nose and cough symptoms among participants in the treatment group compared to the placebo group (Lenhart et al., 2020). Another RCT explored the impact of supplementation with several micronutrients, including Se (100 μg/day; a dose nearly twice the current RDA), among individuals with pulmonary tuberculosis (Villamor et al., 2008). Migration of the pathogen outside of the respiratory tract was decreased and the count of CD3+ and CD4+ T cells was elevated among participants receiving the supplement compared to the placebo. A meta-analysis of studies with broader inclusion criteria has suggested that the consumption of several micronutrients, including VitD and Zn), are correlated with a reduced risk of acute respiratory infections and shorter durations of infection symptoms (Abioye et al., 2021). However, findings were more limited in populations that were less likely to have nutrient deficiencies (Wang et al., 2019; M. X. Wang et al., 2020). A lower VitD status was associated with longer periods of acute respiratory symptoms, but not with illness prevention or severity in a series of studies with healthy participants (Wang et al., 2019). Other studies found that Zn, but

not VitD, status positively correlated with a faster resolution of common cold symptoms (M. X. Wang et al., 2020).

Certain micronutrients may have a role in the modulation of systemic illnesses, but there is limited supporting evidence. In a group of patients with systemic inflammatory response syndrome (similar to sepsis), plasma concentrations of Se were inversely associated with CRP concentrations and sequential organ failure assessment scores at baseline (Valenta et al., 2011). When these patients were given high Se doses (1000 μg on the first day then 500 μg/day for 2 weeks), compared to those that received a standard-of-care dose (Se <75 μg/day), selenoprotein functions significantly improved across the 2 weeks of hospitalization (Valenta et al., 2011). Accordingly, Se doses beyond the current RDA (Figure 4) may be beneficial in patients with excess inflammation during severe systemic infections. Furthermore, supplementation with 4000 IU/day of VitD3 for 10 days decreased TNF-α and increased IL-10 and IL-8 production by monocytes-derived macrophages that were infected with a dengue virus strain (Giraldo et al., 2018). These changes were not observed when a lower dose (1000 IU/day) was tested, suggesting that higher VitD3 doses may be beneficial against dengue virus challenge.

Despite some promising findings, there is an urgent need to identify the type, dose and duration of micronutrient supplements that may promote quicker immune responses and restorations to homeostasis. The study populations, criteria used to define who is 'healthy' and biomarker thresholds used to define deficiencies of micronutrients, especially VitD, were different across investigations. Baseline nutrient statuses are often not measured or disclosed, making it difficult to determine whether findings resulted from the correction of a deficiency or if additional supplements, beyond current DRIs (see figure 4), yields additional immune-related benefits.

The role of nutrients in the resolution of COVID-19-related inflammatory responses.

EPA, DHA and other n-3 PUFAs

The prevalence of COVID-19 could be higher in patients with n-3 PUFAs deficiencies, amplifying the production of cytokines, like IL-6 and TNF-α, and limiting inflammation resolution and recovery (Mazidimoradi et al., 2022). This could support why n-3 PUFA supplements have yielded encouraging findings for the management of cytokine storms associated with SARS-CoV-2 infections (Mazidimoradi et al., 2022; Taha et al., 2022). Doaei et al. (2021) reported increased blood lymphocyte counts and higher 1-month survival rates in critically ill COVID-19 patients after n-3 (400mg EPA and 200mg DHA per day) supplementation. Similarly, EPA+DHA (2g/day for 2 weeks) reduced serum CRP levels in addition to improving clinical symptoms, such as pain and fatigue reduction and appetite improvements (Sedighiyan et al., 2021). Kosmopoulos et al. (2021) tested an EPA metabolite, icosapent ethyl, at 8g for 3 days followed by 4g/day for 11 days in patients with COVID-19 and observed decreased CRP concentrations and the attenuation of adverse symptoms. Pimentel et al. (2022) also reported a larger decline in CRP during n-3 PUFA supplementation in severely ill patients with SARS-CoV-2 infection. Overall, current evidence suggests the benefit of n-3 PUFAs (ALA, EPA and/or DHA) supplementation as it appears to suppress the pro-inflammatory cytokine storms observed during SARS-CoV-2 infection. Future studies should focus on the independent effects of ALA, EPA, or DHA.

Micronutrients

Relationships have been examined between VitD status and COVID-19 outcomes (Gebremichael et al., 2022; Mohamed Hussein et al., 2022; Stroehlein et al., 2021). Higher concentrations of 25-

hydroxyvitamin D, a precursor of hormonally active VitD, were related to lower mortality rates following SARS-CoV-2 infection among populations in African countries (Gebremichael et al., 2022). On the contrary, among COVID-19 survivors, there was no difference in SARS-CoV-2-related symptoms among different categories of 25-hydroxyvitamin D status (Mohamed Hussein et al., 2022). Indeed, a systemic review by Stroehlein et al. (2021) reported inconsistent evidence and emphasized the need for future RCTs that explore whether the supplementation of VitD mediates COVID-19 outcomes.

Other studies related SARS-CoV-2 infection to the status of multiple micronutrients (Galmés et al., 2022; Notz et al., 2021; Vogel-González et al., 2021; Min Xian Wang et al., 2021). In a cross-sectional cohort of individuals from Spain, a higher rate of infection was related to insufficient dietary intakes of several micronutrients including VitD, VitA and Zn (Galmés et al., 2022). In Notz et al. (2021), median concentrations of Se and Zn biomarkers were either at or below normal cut-offs in severely ill COVID-19 patients that were admitted to the ICU. Pro-inflammatory cytokines, including CRP, IL-1β and IL-6, were negatively associated with selenoproteins concentrations, which were also correlated with other biomarkers of Se and Zn status in the same study (Notz et al., 2021). However, as these patients were hospitalized, the findings may not be reproducible in populations with milder cases. Similar inverse relationships were observed between systemic Zn status and both CRP and Il-6 concentrations among patients with COVID-19 (Vogel-González et al., 2021). Finally, a meta-analysis by Min Xian Wang et al. (2021) determined that the risk of initial infection and hospitalization was associated with micronutrient deficiencies, notably of VitD and Zn.

Overall, the improvement of Se, Zn or VitD status may reduce SARS-CoV-2 infection or related outcomes (Galmés et al., 2022; Notz et al., 2021; Min Xian Wang et al., 2021). However, more

mechanistic and clinical studies are required before evidence-based nutritional strategies can be employed in the prevention and treatment of COVID-19. Identifying the SARS-CoV-2 virus strain is also important as the micronutrient efficacy could change depending on the pathogenicity of different variants, which should be further explored.

Physical activity

Physical Activity has been observed to have positive immune-related impacts on COVID-19 outcomes. A multicenter observational study found that those who were physically active had a lower likelihood of COVID-19-like symptoms compared to physically inactive individuals(Nguyen et al., 2021). Another study reported that lower PA increased the risk of COVID-19 mortality, independent of BMI, and for individuals with obesity and low PA, it was suggested that higher pre-pandemic monocyte, neutrophil and leukocyte counts could explain the elevated risk (Hamrouni et al., 2021). The suspension of regular PA could increase proinflammatory biomarkers akin to declines in muscle strength and cardiovascular fitness (Heo et al., 2022). Accordingly, these populations could have an elevated risk of infection and symptom severity for COVID-19. A 2-weekintervention of aerobic exercise (40 minutes/session, 3 times/week at moderate intensity) resulted in significant reductions of COVID-19 symptoms and severity as well as increases in salivary immunoglobin A, peripheral blood lymphocytes and leukocytes compared to control groups (Mohamed & Alawna, 2021). A systematic review of exercise interventions assessing immune biomarkers in healthy adults suggested that moderate-intensity aerobic exercise (2-3 times/week for 20-60 minutes/session) appeared to be ideal for improving immune function and therefore a PA target for mitigating the COVID-19 severity (Alawna et al., 2020). These findings align with the current Canadian PA guidelines for adults recommending at least 150 minutes of moderate to vigorous activity per week, lasting at least 10

minutes or more in duration, as well as 2 days per week for bone and muscle strengthening (figure 4) (Tremblay et al., 2011). Individuals who were physically active with medium to high healthy eating scores had a lower likelihood of COVID-19-like symptoms compared to those that were physically inactive with low healthy eating scores (Nguyen et al., 2021), suggesting that PA in combination with better nutrition could provide synergistic benefits against COVID-19.

Conclusion

Dietary fatty acids, gut microbiota-GALT interactions and micronutrients appear to have impacts on immune outcomes which could be augmented by PA. Immunomodulatory effects of dietary MUFAs and PUFAs have also been observed. N-3 PUFAs have supported anti-inflammatory effects and MUFAs (like OA and POA) may also provide similar effects, but this remains to be clarified. The bidirectional connections between the GALT and gut microbiota via bioactive compounds likely plays an important role in systemic immune function. Dietary fiber provides the compounds need for the microbial generation SCFAs which have been shown to impact immune status and host physiology. Dietary patterns, like Mediterranean or vegetarian diets, may influence the relationship between the GALT and microbiota, but the effects of probiotics, and prebiotics are limited. Further pre-clinical research should aim to understand the mechanisms behind the modulation of neuroinflammation and GALT function associated with nutrient supplementation. Micronutrients, including Se, Zn and VitD may support the immune system in fighting foreign antigens and restoring homeostasis. Determination of the optional micronutrient doses, durations, and baseline status is needed for a better understanding of their impact on the risk, prevention and severity of acute infections or systemic illnesses. Recent COVID-19 studies provide promising evidence to support n-3 PUFA supplementation and beneficial effects on inflammatory status, where independent FA effects (like ALA, EPA and DHA) should be

considered. The use of Se, Zn or VitD may also have benefits for COVID-19 infection and severity however further clinical trials are needed to identify nutritional approaches. Lastly, synergistic effects between PA and nutrients could positively impact gut microbiota, GALT and infection risk (like COVID-19), however, the breadth of immune factors analyzed remains limited.

Figure 1: Description of cytokines and the source of immune cells involved in the pro-inflammatory versus anti-inflammatory responses.

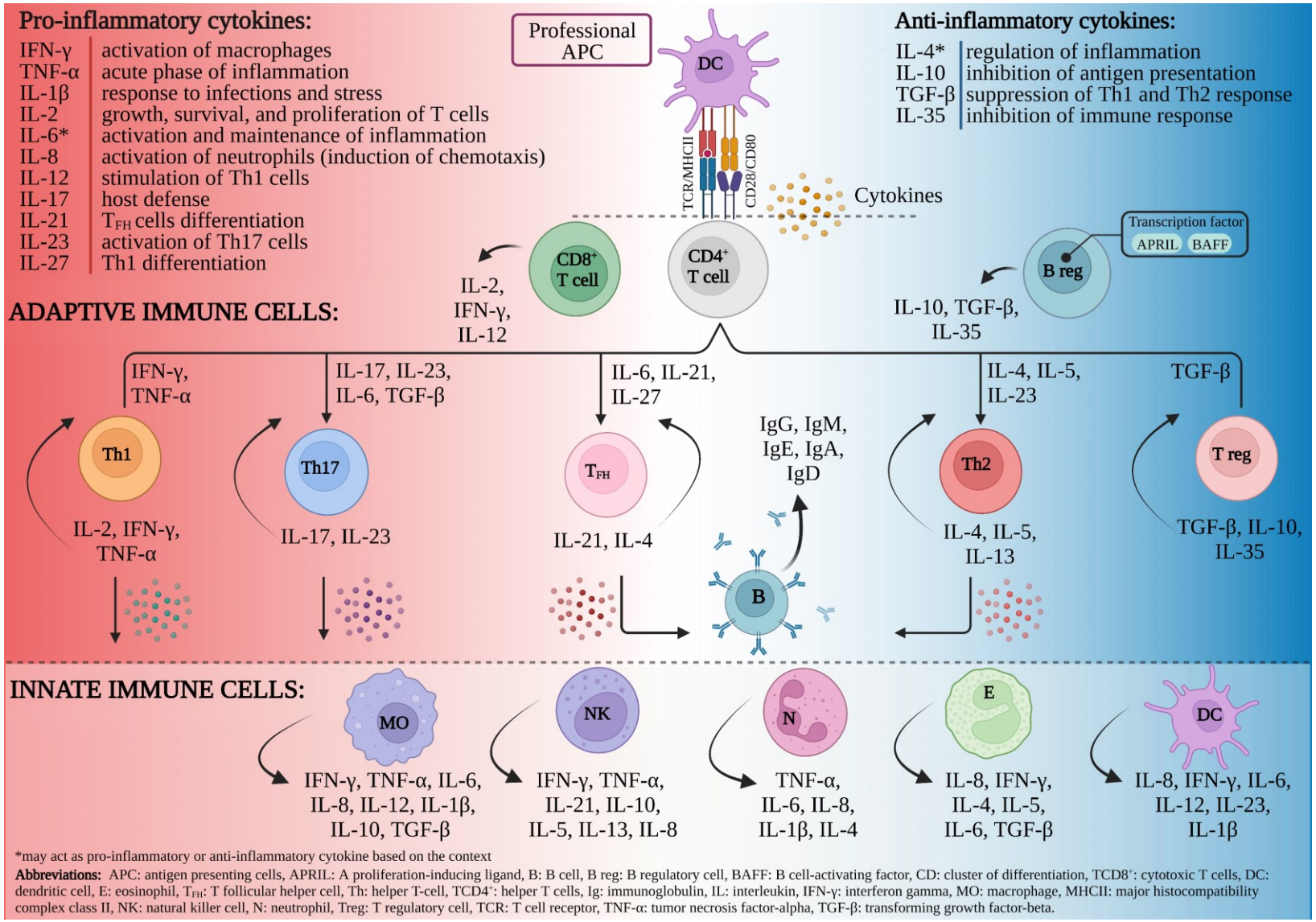


Figure 2: Summary of dietary fat's impact on immune outcomes in the context of pro-inflammatory versus anti-inflammatory response paradigms.

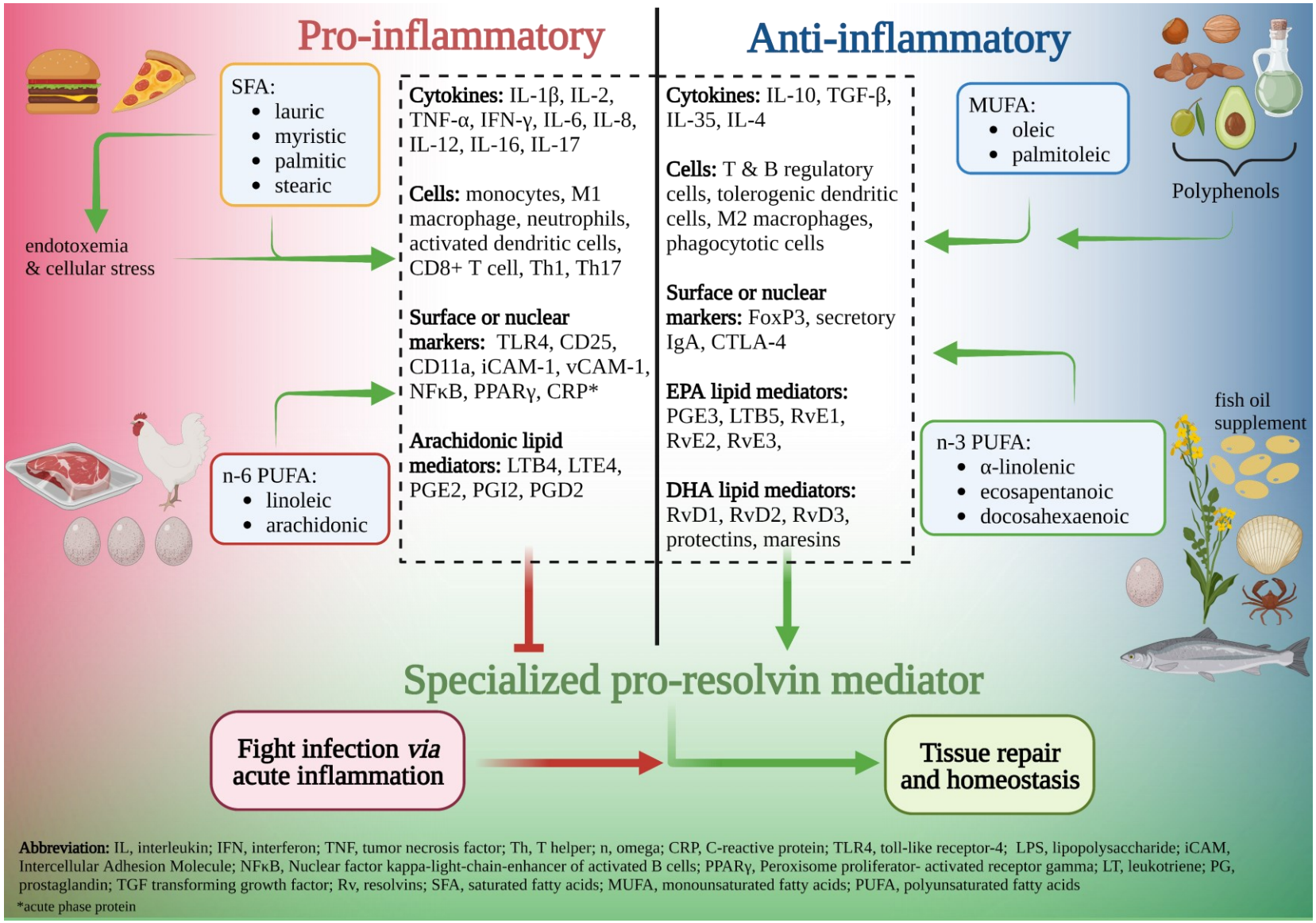
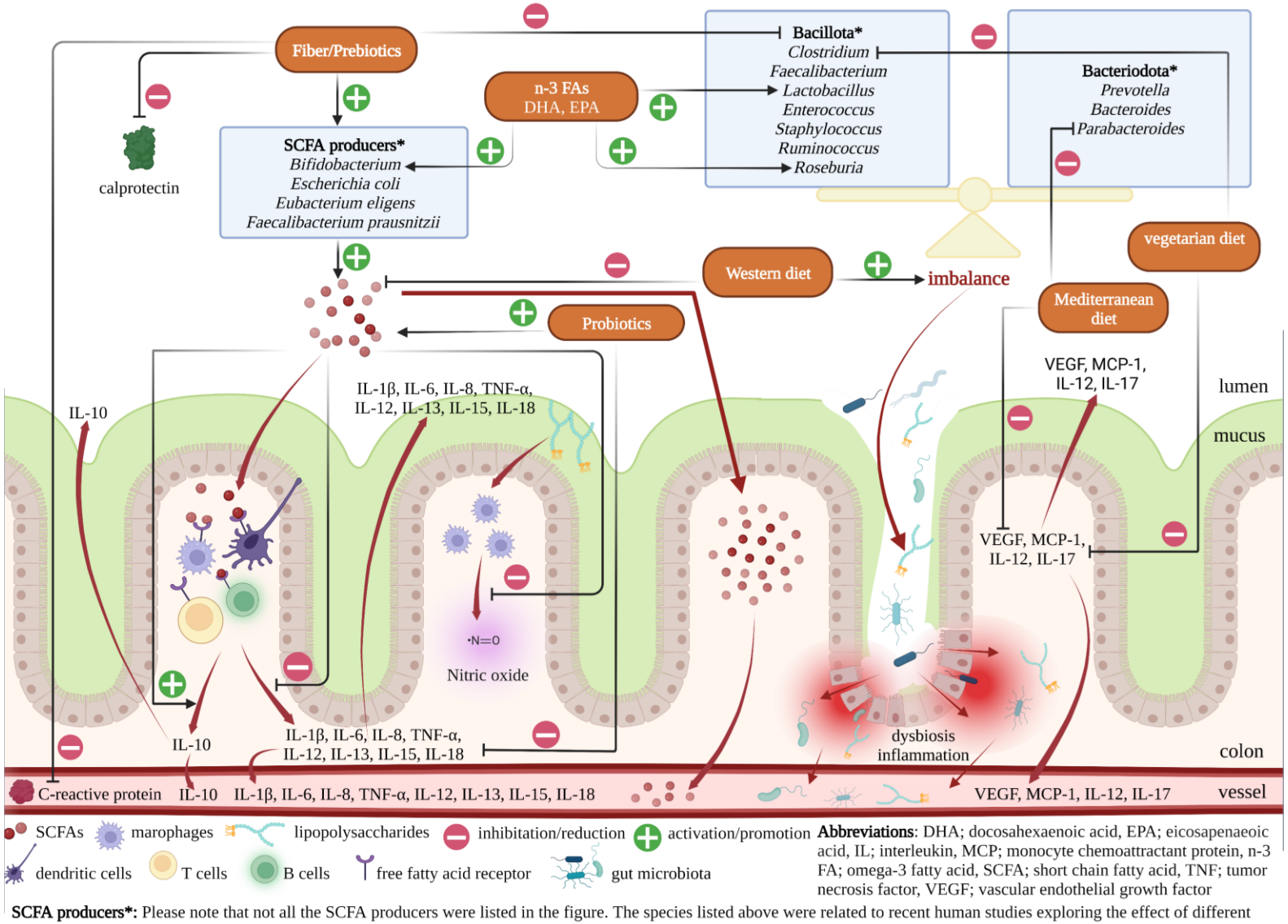


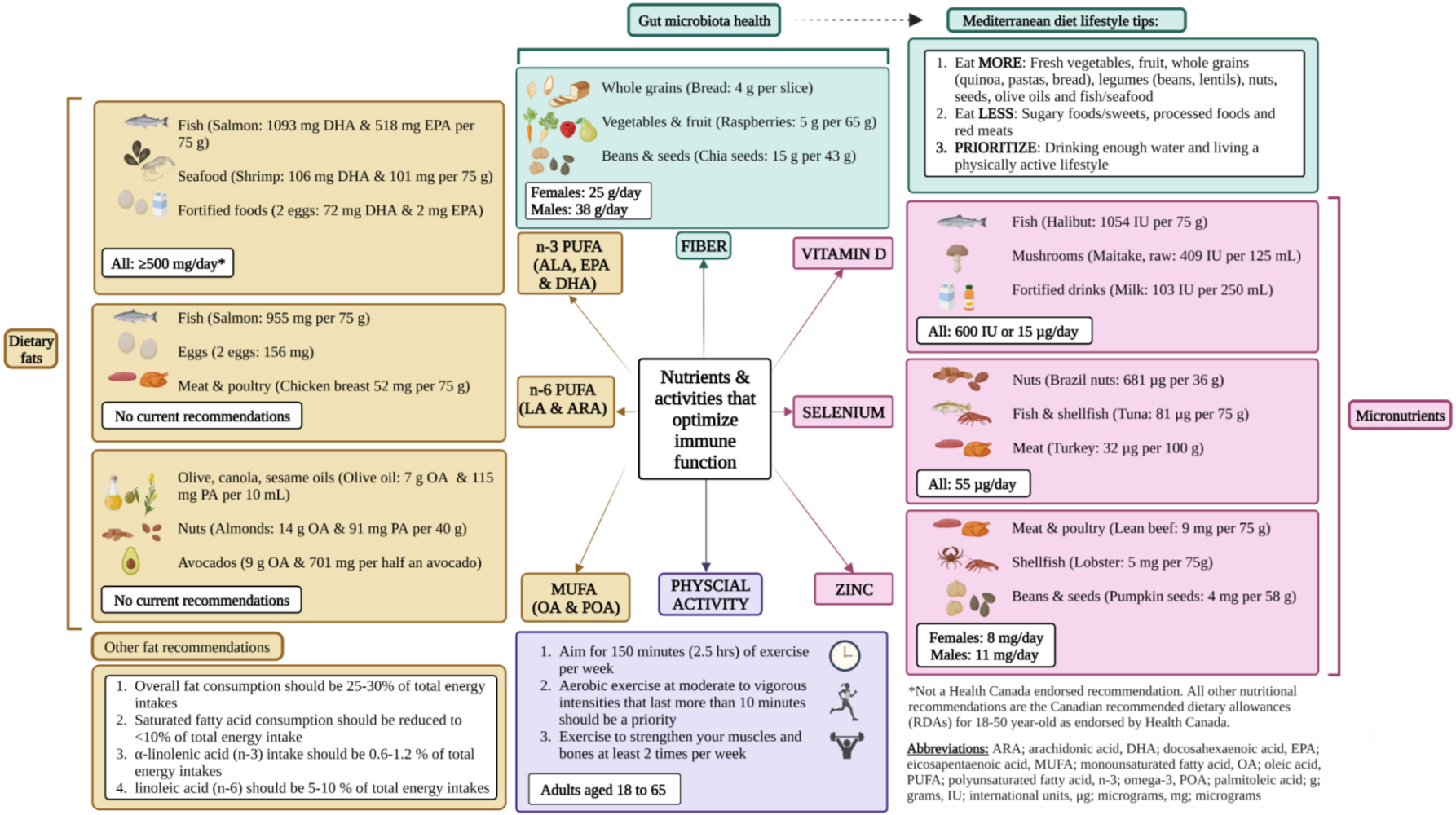
Figure 3: Summary of connection between diet- derived and microbial-derived metabolites and GALT functions.



Abbreviations: DHA; docosahexaenoic acid, EPA; eicosapenaeoic acid, IL; interleukin, MCP; monocyte chemoattractant protein, n-3 FA; omega-3 fatty acid, SCFA; short chain fatty acid, TNF; tumor necrosis factor, VEGF; vascular endothelial growth factor

SCFA producers*: Please note that not all the SCFA producers were listed in the figure. The species listed above were related to recent human studies exploring the effect of different nutrients or diets on intestinal/systematic immune system and immune function. For more details regarding SCFA producers, please refer to other review papers (Rowland et al. 2018). **Bacillota*** and **Bacteriodota*** were formerly named Firmicutes and Bacteroidetes respectively.

Figure 4: Sources and recommendations for nutrients and physical activities that may promote optimal immune function in healthy adults.



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Appendix B

Are There Benefits to Growth with Supplementing Long-Chain PUFAs to Toddlers Born Prematurely?[1]

In this issue of the Journal, Ingol et al. (2019) report no benefits on the growth of supplementing toddlers who were born <35 weeks of gestation for six months with long-chain PUFAs (LCPUFAs). Toddlers (aged 10–16 months) enrolled in the Omega Tots randomized placebo-controlled trial (NCT02199808) were provided with a daily LCPUFA supplement containing 200 mg DHA and 200 mg arachidonic acid (AA). Although this finding may be disappointing to many who believe there are insufficient LCPUFAs in the diet of infants, the results are not unexpected. The primary and several secondary outcomes of the Omega Tots trial have been reported by Keim et al. (2018). They found LCPUFA supplementation did not significantly improve cognitive development [on the Bayley Scales of Infant and Toddler Development (Bayley-III) Cognitive Composite] (Keim et al., 2018).

Despite supporting the null hypothesis, the current study will make some important contributions to understanding growth during the second year of life of infants who were born prematurely. Growth is an important measure to monitor and report in any dietary intervention, particularly in children who would have started life well below the 50th percentile. Currently, growth charts do not address toddlers born prematurely, and there are challenges using conventional growth charts

[1] A version of this is published as a commentary; Patel, D., Orsso, C. E., Haqq, A. M., & Field, C. J. (2019). Are There Benefits to Growth with Supplementing Long-Chain PUFAs to Toddlers Born Prematurely? *The Journal of Nutrition*. *149*(12), 2075-2076.

(Silveira & Procianoy, 2019). The mean and SD (adjusted for prematurity according to WHO growth standards) of key anthropometric measures (height, length, and head circumference) of 377 male and female toddlers from diverse socioeconomic backgrounds will now be available for inclusion in future growth charts. Growth velocity is also a useful clinical measure and would have been useful to include, but one assumes the authors would be willing to provide it if requested. Although the FFQ used to estimate the dietary intake of DHA and AA may not have been specific for this age group, the estimated intakes are consistent with those reported by others using more precise methods (Keim & Branum, 2015; Lien & Clandinin, 2009). Thus, the supplement provided was considerably more than the usual intake of these LCPUFAs. How much pre-formed DHA and AA are required to support growth in healthy toddlers is not known; however, the hypothesis that more is needed than currently consumed was not unreasonable.

There are, however, a number of limitations that need to be raised to determine if the investigators were able to test if there was an effect of LCPUFA supplementation on growth and adiposity. First, the study was powered for the primary endpoint. Growth and adiposity were not the primary outcomes nor even secondary outcomes of the Omega Tots trial (Keim et al., 2018). The authors justify their sample size by comparison with other studies, but it is quite clear that they did not have sufficient power with the 77% recruitment success of the trial. Second, the articles cited to support the statement of poor growth in this population were from a study of extremely low-birth-weight infants (Ehrenkranz et al., 2006) and another in which the children were born small for gestational age (<10th percentile) (Fewtrell et al., 2001). Based on the (gestation-adjusted) weight, length, head circumferences, and z scores, the children in the current

study were in the reference range. Thus, there is a less convincing hypothesis that an LCPUFA supplement would improve growth.

Despite the amount of training needed and effort to collect skinfolds, there are no data to support that they are a sensitive measure of adiposity in young children. With the number of staff required for this study, the CV for interobserver measures should have been included because the research staff were trained and recertified annually. Interobserver CVs of 10.0% and 8.6% for triceps and subscapular skinfolds, respectively, were previously reported in a study of infants aged 9–24 months (Johnston & Mack, 1985). This is in part due to the low subcutaneous fat in toddlers, behavioural compliance, and the difficulty in identifying the bony landmarks to standardize measures (Demerath & Fields, 2014). The potential variation in the measure is greater than the decrease observed in triceps (3–7%) and subscapular (2–3%) skinfolds from study entry to completion; thus, measurement error could have masked the study outcomes. Despite this, the rate of change in skinfold thickness is very low until four years of age (Addo & Himes, 2010), so it is unlikely that any dietary supplement could alter the thickness of these specific anatomical sites in healthy children. Fat percentage was estimated using anthropometric measures, but to our knowledge, there are no validated equations for toddlers born prematurely. The correlation studies that are cited in the article comparing skinfold estimates of body fat percentage using DXA do not necessarily support the precision of the measure to detect change.

Finally, and perhaps the major limitation of the study, it is not known if the number of LCPUFAs provided was sufficient to alter DHA and/or AA status in toddlers. The assumption was that based on the estimated intake at study entry and the amount in the supplement, the status of both

DHA and AA changed in the treatment group. However, examining the data on status (available for 85 and 88 toddlers in the treatment and control group, respectively) in the first article of this study, one is not confident that this was achieved in all children. The team reports the median increase in RBC DHA and AA content in the treatment was 1.1 mol% (36% increase) and 2.0 mol% (35% increase), respectively (Keim et al., 2018). That would represent a significant change in status if it occurred in most children, but the IQRs suggest that there was a range of responses, and a portion of the children (~25% of those measured) did not change their status of DHA and/or AA. This could be due to compliance or metabolic variability but would require a sub-analysis of responders/compliers compared with non-responders/noncompliers to be performed. Unfortunately, this was not possible because insufficient numbers of blood samples were collected in the treatment group at baseline (n = 99, 52%) and at the study end (n = 85, 47%). Compliance was based on the caregivers' daily records and returned packets, and this could have been validated against RBC content or concentration of DHA and AA in the treatment group.

Little is known regarding the optimal intake of DHA and AA required to support growth during toddlerhood, and unfortunately, this remains a question. The authors discuss their results throughout much of the study in relation to DHA. It is not clear why 200 mg AA was provided; the evidence that toddlers need a preformed source of AA beyond the first year is not strong. The major message from this trial is that this level of supplementation of LCPUFAs is safe in toddlers, but the study was not sufficiently powered or designed to specifically address growth, nor did it utilize a sufficiently sensitive measure or optimal time period to assess potential effects

on adiposity. Further research is needed before we can conclude that “DHA supplementation to toddlers has no effect on short-term growth or adiposity.” Long-term growth is another question and, as the authors state, more relevant. It is hoped that the researchers have the ability to continue to follow these toddlers, and we eagerly await those findings.

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