Dietary Polyunsaturated Fatty Acids Modulate Immunity to Influenza Virus Infection

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ABSTRACT

Nicole Marie Juliette Schwerbrock: Dietary Polyunsaturated Fatty Acids Modulate
Immunity to Influenza Virus Infection
(Under the direction of Melinda A. Beck, PhD.)

Dietary fish oils, rich in n-3 polyunsaturated fatty acids (PUFA), including

eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are known to have

anti-inflammatory properties. While the immunosuppressive effects of n-3 PUFA may

be beneficial for some chronic inflammatory disorders, these same anti-inflammatory

properties may be detrimental for a response to an infection when a functional

immune system is needed to eradicate an invading pathogen.

Despite the availability of vaccines and antiviral agents, influenza virus

continues to be a major cause of morbidity and mortality worldwide. Given that n-3

PUFA-rich fish oil supplementation by human populations is on the rise, and with the

increasing threat of an influenza pandemic, we tested the impact of fish oil feeding

on the immune response to influenza virus infection.

Using *in vivo* and *in vitro* models, we found n-3 PUFA, supplied at

physiologically relevant levels, suppressed immune responses following influenza

infection. Although the anti-inflammatory properties of fish oil resulted in decreased

lung inflammation in influenza infected mice, it also led to increased mortality and

increased viral titers in surviving mice post infection. Impairment of host resistance

was likely due to reduced inflammatory cell trafficking into the lungs in conjunction

with lower cytokine production during infection. Influenza virus infection also resulted in a significant increase in EPA content in the infected lung, while infection had little effect on DHA in fish oil fed mice. Although changes in fatty acid membrane profiles can result in functional changes, dendritic cells and T cells from infected fish oil fed mice were able to respond to viral antigen equivalent to control fed mice.

In response to viral infection, airway epithelial cells induce pro-inflammatory and antiviral mediators. We found that specific DHA to EPA ratios suppressed these mediators of infected human primary bronchial epithelial cells. Together, data from our studies indicate that n-3 PUFA treatment can have a profound effect in the host response to influenza virus infection.

Given that fish oil supplementation is used by both adults and children, there is a significant public health benefit in further investigating how PUFAs alter immune function.

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AA   arachidonic acid

ALA   alpha-linolenic acid

APC   antigen presenting cell

BALF   bronchoaveolar lavage fluid

CD   cluster of differentiation

CFU   colony forming units

COX   cyclooxygenase

d   day

DC   dendritic cell

DHA   docosahexaenoic acid

EPA   eicosapentaenoic acid

G3PDH   glyceraldehyde-3-phosphate dehydrogenase

HA   hemagglutinin

ICAM   intercellular adhesion molecule

IFN   interferon

IL   interleukin

LN   lymph node

LOX   lipoxygenase

LPS   lipopolysaccarides

LT   leukotrienes

MCP	monocyte chemotactic protein
MDCK	Madine Darby canine kidney
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
NA	neuraminidase
NK	natural killer
p.i.	post infection
PBMC	peripheral blood mononuclear cells
PG	prostaglandins
PLA_2	phospholipase A2
PR8	influenza A/Puerto Rico/8/34
PUFA	polyunsaturated fatty acids
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
RANTES	regulated upon activation, normal T cell expressed, and secreted
RDA	recommended daily allowance
TCID50	50% tissue culture infective dose
TCR	T cell receptor
Th1	T helper cell 1
Th2	T helper cell 2
TNF-α	tumor necrosis factor-α
USDA	united states department of agriculture
VCAM-1	vascular cell adhesion molecule-1

CHAPTER I
BACKGROUND AND SIGNIFICANCE

Polyunsaturated fatty acids (PUFAs) may have important roles in the prevention of chronic diseases because they are capable of reducing excessive inflammation (1). Suppression of the immune system, however, may be deleterious during influenza virus infection and others, when inflammation is essential for eliminating the invading pathogen (2). This introductory chapter reviews the structure and the uses of PUFAs and sets the stage for the presentation of new research on the relationship between the immune system and PUFAs, following infection with influenza virus *in vivo* and *in vivo.*

1.1 Polyunsaturated fatty acids: Their significance in public health

Over the years, researchers have become increasingly aware of the role diet plays in immune function. Studies have shown that dietary PUFAs derived from fish oil have beneficial effects on inflammatory and autoimmune disorders, suggesting that fish oil has both anti-inflammatory and immunomodulatory effects (3-5). Although the immunosuppressive properties of PUFAs may be beneficial during an inflammatory illness, their anti-inflammatory properties may be detrimental when the body is combating an infection, such as influenza virus (2, 6).

1.1.1 Dietary PUFA: Source and chemistry

PUFAs are unique fatty acids with 18 or more carbons, containing two or more double bonds in cis configuration. PUFAs are grouped into two series based on the origin of the position of the terminal double bond, located either on carbon 3 or carbon 6 from the methyl end (omega carbon) of the fatty acid chain (7, 8). Depending on these positions, PUFAs are classified as omega-3 (n-3) or omega-6 (n-6) fatty acids (8).

One of the subgroups of n-3 fatty acids, alpha-linolenic acid (ALA, 18:3n-3), is derived from plant oils. The other subgroup of n-3 PUFAs is derived from cold water fish; the major marine n-3 PUFAs are eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3). Two major kinds of n-6 PUFAs are linoleic acid (LA; 18:2n-6) and arachidonic acid (AA;20:4n-6), which are derived mostly from vegetable oils and red meats, respectively (7). Figure 1.2.1 shows the structures of the most prominent omega-3 fatty acids, eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), and the most prominent omega-6 fatty acid, arachidonic acid (AA; 20:4n-6).

Figure 1.2.1 Structures of polyunsaturated fatty acids (PUFAs).
The nomenclature of PUFAs indicates the number of carbon atoms in the chain, the number of
double bonds and the position of the first double bond from the methyl terminus of the chain,
indicated by the letter n (9).

PUFA Structures

Methyl end of the
fatty acid chain

EPA 20:5(n-3)

AA 20:4(n-6)

DHA 22:6(n-3)
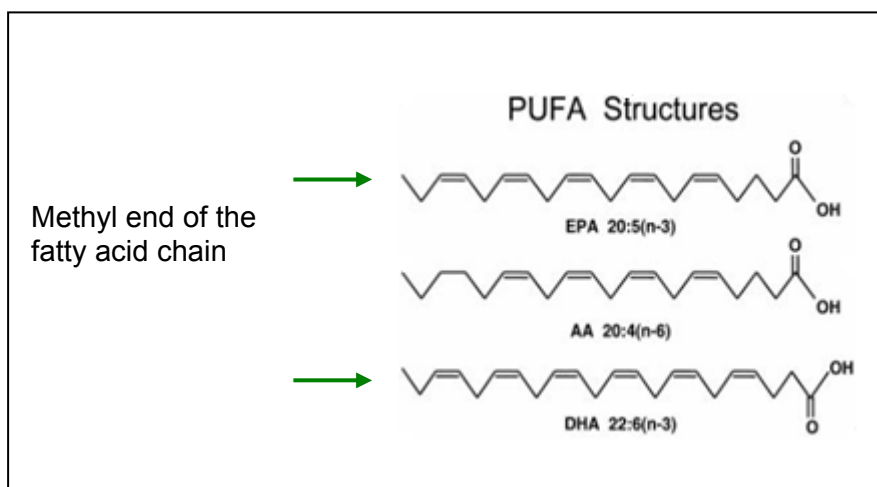
N-3 PUFAs are considered essential for the human diet. Humans and other
mammals cannot create double bonds before the 9th carbon because they lack the
enzymes Δ12-desaturase and Δ15-desaturase. Δ12-desaturase creates a double
bond at the 6th carbon, whereas Δ15-desaturase creates a double bond at the 3rd
carbon. Both of the desaturase enzymes, however, exist in plants and fish (8), the
major dietary source of n-3 PUFAs.

Following the absorption of PUFAs from foods, they are distributed by the
blood to all cells in the body. They typically incorporate into the sn-2 position of
phospholipids in the lipid bilayer of cellular membranes (Figure 1.2.2a) (4, 10).
Because of the multiple double bonds within the fatty acid chain, PUFAs can adapt
different confirmations within the cell membranes (Figure 1.2.2b)

Figure 1.2.2 A schematic representation of a phospholipid molecule.
1.2.2 b. EPA and DHA crustal structure. Modified from Eckhardt (11). Green arrows point to predominant incorporation of n-3 PUFAs.
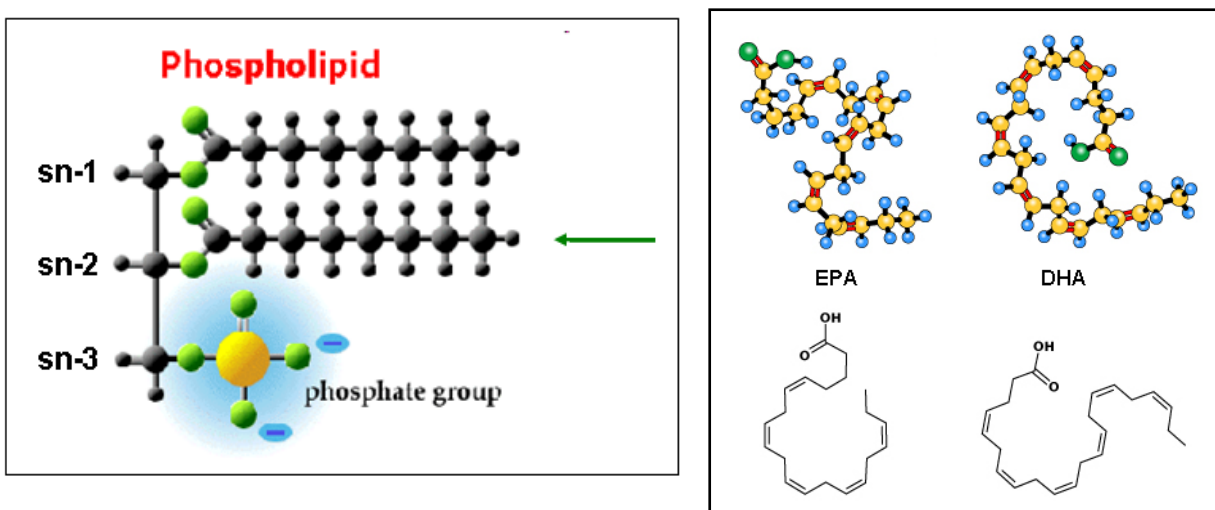
N-3 PUFAs are generally considered positive for human health, whereas n-6 PUFAs can be converted to pro-inflammatory agents and are therefore thought to be less beneficial when consumed in excess (3, 12). This notion stems from the fact that n-6 AA is the main precursor for prostaglandins, which have pro-inflammatory properties (discussed below in section 1.4.1) (8, 13). Maintaining a healthy n-6/n-3 balance is therefore important (discussed below in section 1.2.1)

Although most studies demonstrate a beneficial effect of n-3 PUFAs on inflammatory and autoimmune disorders, some concern remains that the anti-inflammatory properties of n-3 PUFAs may be detrimental during acute infections (2, 14). However, there has not been sufficient investigation to determine whether the intake of n-3 PUFAs affects the immune response to infectious disease.

1.1.2 Evolutionary aspect of diet: How has the American diet changed?

The human diet has undergone striking changes in evolution and agricultural development. The n-3 PUFA content of today's diet is noticeably lower than during earlier periods in human history because of changes in dietary habits resulting from the development of agricultural techniques and the consumption of processed foods (15, 16). Foods of the hunter and scavenger period (2.5 million years ago) were considered to be richer in n-3 PUFA consumption and a healthier balance between n-6 and n-3 PUFAs existed (i.e., n-6/n-3 ratio ≈ 1:1). Deviation from this balance began about 10,000 years ago with the adoption of agriculture and animal husbandry (16).

Industrial revolution over the last 300 years or so increased meat consumption and decreased consumption of plant foods and fish in western society. This increase has led to an imbalance in intakes of the two food types of PUFAs, resulting in an increased n-6/n-3 fatty acid ratio, as high as 15 –20:1 (5, 16, 17). In the last century, total fat intake increased and both absolute and relative changes of n-6/n-3 ratio in the diet of Western societies has occurred (Figure 1.2.3) (16).

Figure 1.2.3 Scheme of total fat and fatty acid intakes throughout evolution.
Data were extrapolated from cross-sectional analyses of contemporary hunter-gatherer populations and from longitudinal observations and their putative change during the preceding 100 years. Modified from Simopoulos (18).
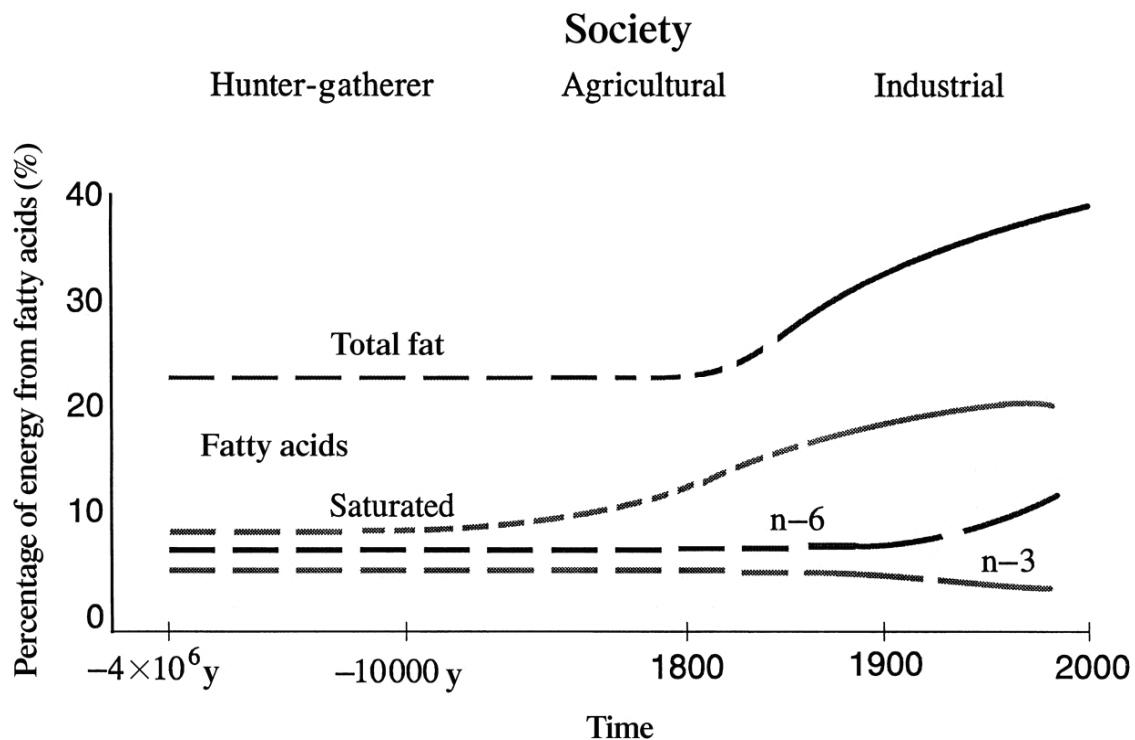
A high n-6/n-3 ratio has been suggested to have adverse effects on human health, which has in turn led to a recommended increase in the intake of n-3 PUFAs and a decrease the consumption of n-6 fatty acids (16, 17). To achieve these goals, the American Heart Association recommends 1g/day of n-3 PUFAs for the prevention of cardiovascular disease (19). In addition, the Center for Nutrition Policy and Promotion, a division of the USDA, recommends eating specific types of fish, such as salmon, trout, and herring, for their high content in EPA and DHA (20). The "Dietary Guidelines for Americans 2005" published jointly by DHHS and USDA, suggests that total intake of PUFAs should remain at 7% of energy intake and not

exceed 10% of energy intake (21, 22), but with no recommendation of a healthy ratio of the two types of PUFAs.

Although U.S. food disappearance data from 1985 to 1994 shows a decreased ratio of n-6/n-3 PUFA from 13:1 to 11:1, an additional 4-fold increase in fish consumption would still be necessary to attain the AHA recommended Adequate Intake (AI) of 0.65 g per day in EPA and DHA, resulting in a suggested ratio of 4:1 or lower (19, 23).

Currently no Recommended Dietary Allowances (RDA) have been established for n-3 or n-6 PUFAs. The health benefits of n-3 PUFAs are considered to outweigh the health risks (24), but quantitative requirements have not been adequately investigated. The promotion of an increase in n-3 PUFA intake has resulted in an industry initiative to promote supplementation with fish oil capsules and fortification of n-3 PUFAs in processed foods (25). A survey conducted among health care professionals in 2006 found that fish oil capsules were consumed by 24% of those surveyed (26). A follow-up study, conducted by the same group, reported an increase in fish oil supplementation from 24% to 30% in 2007 (27).

1.2 Specific objectives

The goal of this dissertation is to investigate if fish oil feeding can influence host resistance to influenza infection. We characterized phenotypes, numbers and functionality of cells infiltrating the infected lung using an in vivo model that closely resembles human conditions in dietary fish oil intakes and pathogen. Additionally, we used in vitro models to illustrate effects of PUFAs treatment on influenza virus

growth and related our cell culture experiments to explain mechanisms occurring in fish oil fed mice.

1.3 Influenza virus infection and its widespread influence

Influenza A virus is a RNA virus that can cause severe illness in humans and animals. Influenza A virus can infect cells throughout the respiratory tract and cause acute and diffuse inflammation of the bronchoaveolar tract (28, 29). Infection with influenza is characterized by a rapid infiltration of immune cells, such as neutrophils, monocytes, macrophages and natural killer cells, in the infected lungs. These cells are necessary for viral clearance but also contribute to lung pathology (30).

Influenza virus continues to be a major worldwide cause of morbidity and mortality despite the availability of vaccines and antiviral agents. Infection with influenza virus was reported to be responsible for greater than 200,000 hospitalizations and more than 30,000 deaths in the US annually, and these numbers are increasing among groups at high risk, such as the very young, elderly, and immuno-compromised individuals (31-33). During a periodic pandemic, 20-40% of the world's population might be infected (34), and the increasing threat of an influenza pandemic underscores the importance of knowing the linkage between an n-3 PUFA-enriched diet and the body's immune response to this virus.

1.3.1 Effects of dietary PUFAs on infectious disease

Epidemiological, clinical, and animal studies have demonstrated that PUFAs attenuate immune-mediated inflammatory diseases (1, 5). However, for example, epidemiologists have noted that populations that consume large quantities of oily

fish, such as Greenland Eskimos, suffered low death rates from ischemic heart disease, but higher incidence rates of tuberculosis (35). But, in addition, northern Canadian Indian and Inuit children suffered from recurrent and persisting upper respiratory tract and chest infections associated with weakened immunity (36).

In animal models, fish oil diets have been shown to delay and weaken the immune response to infection, resulting in reduced clearance of some bacteria and viruses (2, 37). For example, diets supplemented with fish oils have been shown to lower host resistance to Mycobacterium tuberculosis (14), to reduce the survival of mice infected with Listeria monocytogenes (38, 39), and to decrease natural resistance of mice infected with Salmonella typhimurium (40). Similarly, fish oil feeding may diminish host defense against influenza virus due to delays in influenza virus clearance (37). These studies support the conclusion that the immunosuppressive effects of n-3 PUFAs are associated with a decline in cytokine production (41, 42), natural killer (NK) cell activity (43), antigen presentation (44, 45), lymphocyte function, and T-cell responses (46, 47). Each of these will be discussed in section 1.6.

1.4   The synthesis of polyunsaturated fatty acids

As stated earlier, mammals lack the enzymes Δ12-desaturase and Δ15-desaturase, and therefore cannot convert oleic acid (18:1) into linoleic acid (18:2 n-6) or into α-linolenic acid (18:3 n-3). Consequently, these fatty acids must be obtained through the diet. Dietary sources rich in linoleic acid (18:2 n-6) include corn, sunflower, safflower, and soybean oils, and dietary sources rich in α-linolenic acid (18:3 n-3) include rapeseed and flax seed oil. Once consumed, linoleic acid can

be converted via intermediates γ-linolenic acid (18:3n-6) and dihomo-γ-linolenic acid

(20:3n-6) to arachidonic acid (AA; 20:4n-6) (Figure 1.2.4). Using the same pathway,

dietary α-linolenic acid can be converted to eicosapentaenoic acid (EPA; 20:5n-3),

docosapentaenoic acid (22:5n-3) and docosahexaenoic acid (DHA; 22:6n-3). This

conversion however is very inefficient and only accounts for ~10% of the

endogenous synthesis (7). n-6 and n-3 PUFAs compete for the same metabolic

enzymes, thus causing the n-3 PUFA synthesis to occur at the expense of n-6 PUFA

synthesis, and vice versa (13). As a result, the dietary intake of either n-6 or n-3

PUFAs determines the amounts of the products synthesized.

Figure 1.2.4 Fatty acid biosynthetic pathways.
Adapted from (48).
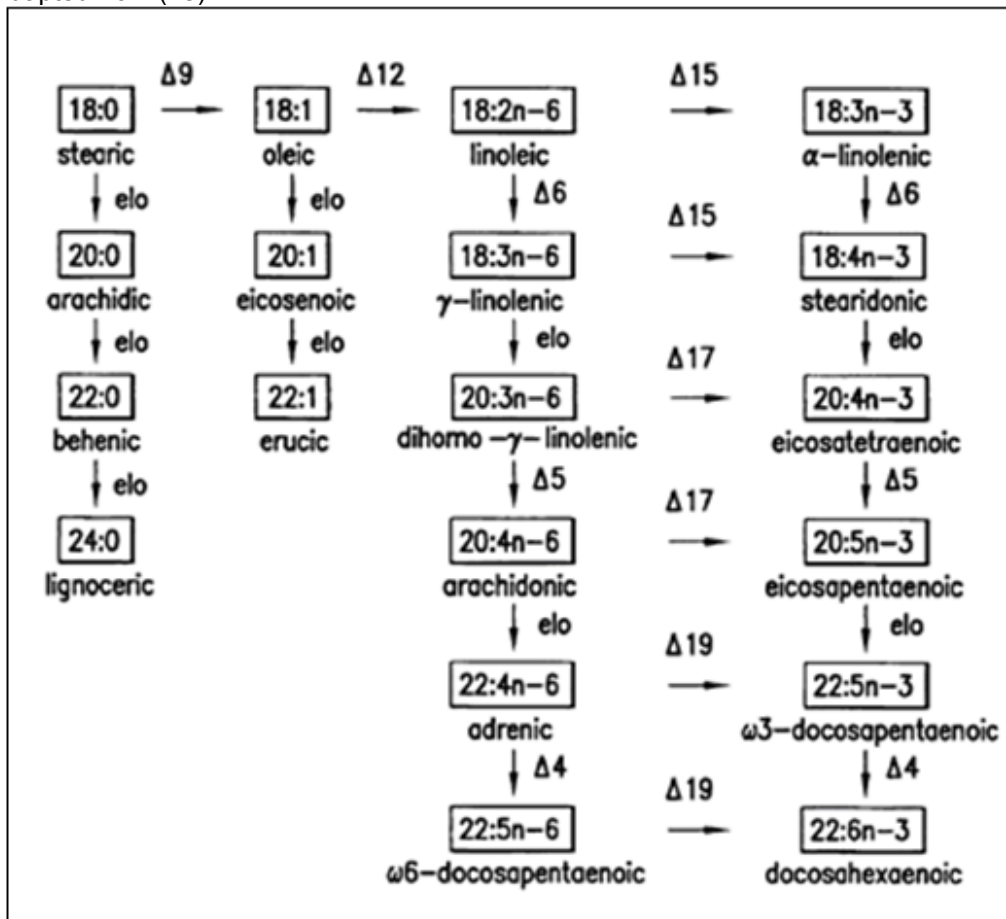
1.4.1 PUFA content in cells membranes of the immune system

Baseline fatty acid profiles are similar in rodent splenocytes and human peripheral blood mononuclear cells (PBMC) (49). The phospholipids of PBMC contain about 6-10% linoleic acid, 1-2% dihomo-γ-linolenic and 15-25% archidonic acid (50, 51). The proportions of n-3 PUFAs are low, with EPA and DHA compromising only 0.1-0.8% and 2-4%, respectively (52). Feeding studies in humans and rodents have shown a dose-dependent relationship with PUFA intake correlating to a higher proportion of PUFAs in immune cells (53). In general, the increase in content of n-3 PUFAs occurs at the expense of n-6 PUFAs and vice versa (54-56). In rodents that are maintained on chow diets, 18:3n-3 (α-ALA) and EPA are minor PUFAs in the phospholipid fraction. However, supplementing diets with fish oil significantly increases tissue levels of EPA and DHA; these changes occur at the expense of AA (4, 7). Time-course studies have shown that, depending on diet, the incorporation of EPA and DHA into immune cells reaches its peak within 2-4 weeks of commencing increased intakes (49). Thus, the feeding of diets high in n-3 PUFAs is reflected in the membrane content of immune cells.

1.5 Mechanisms by which PUFAs modulate the immune system

Fatty acids fulfill a variety of roles within immune cells, including 1) fuels for generation of energy, 2) components of cell membrane phospholipids contributing to the physical and functional properties of those membranes, 3) covalent modifiers of protein structure influencing the cellular location and function of proteins, 4) regulators of gene expression either through effects on receptor activity, on

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intracellular signaling processes, or on transcription factor activation, 5) precursors for synthesis of bioactive lipid mediators like prostaglandins (PGs), leukotrienes (LTs), lipoxins and resolvins (1).

The next two sub-chapters will focus on PUFAs effect on eicosanoid metabolism and membrane structure.

1.5.1 PUFAs effects on eicosanoid metabolism

Eicosanoids are synthesized from 20-carbon PUFAs that are esterified with the hydroxyl group at the sn-2 position on the glycerol backbone of polar phospholipids (Figure 1.2) (7). Normally, membranes of immune cells contain AA at the sn-2 position, causing AA to be the predominant precursor for eicosanoid biosynthesis (1, 7). AA is hydrolyzed from the sn-2 position by the enzyme phospholipase A2 (PLA_2). Subsequently, non-esterified, free AA becomes a substrate for cyclooxygenase1 (COX1), a constitutive enzyme, or COX 2, an inducible enzyme. Cyclooxygenase products of AA lead to the synthesis of prostaglandins (PG). Alternatively, AA can act as a substrate for one of the lipoxygenase (LOX) enzymes, forming 4-series leukotrienes (LT) (Figure 1.2.5).

With an increased consumption of fish oil, a decrease in the amount of AA in immune cells and therefore less AA substrate becomes available for synthesis of 2 series PG (Figure 4). Peterson et al. and Yaqoob et al. demonstrated a direct relationship between AA content of immune cell phospholipids and the ability of those cells to produce PGE_2, such that PGE_2 production is increased by AA feeding and decreased by EPA or DHA feeding (53, 57).

PGE$_2$ generated from AA has both pro- and anti-inflammatory effects, thus making it a potent regulator of immune function. Pro-inflammatory effects include inducing fever, increasing the vascular permeability, increasing vasodilation and enhancing pain (52). Cell culture studies have demonstrated that PGE$_2$ induces the production of the inflammatory cytokine IL-6 by macrophages (58).

Alternatively, PGE$_2$ synthesis can inhibit lymphocyte proliferation and NK cell activity by reducing the production of cytokines IL-2 and IFN-γ. In lipopolysaccharide (LPS) stimulated monocytes, the production of PGE$_2$ inhibits the induction of cytokines TNF-α and IL-1β (59, 60), and PGE receptor knockout mice (EP2$^{-/-}$ and EP4$^{-/-}$) have been shown to experience reduced T cell function (61).

The synthesis of LTB$_4$, on the other hand, has pro-inflammatory effects. LTB$_4$ synthesized from AA induces the production of pro-inflammatory cytokines TNF-α, IL-1, IL-6, IL-2 and IFN-γ from inflammatory cells, enhances lymphocyte proliferation and promotes NK cell activity (52).

By adding fish oils to the diet or media to cell culture, EPA acts as a substrate for both COX and LOX, giving rise to derivatives which have a different structure from those produced from AA (i.e. 3-series PG and 5-series LT). When EPA is used instead of AA, series-3, rather than series-2 prostaglandins and leukotrienes are synthesized (Figure 1.2.5). With fish oil supplementation in humans, n-3 fatty acids derived from LTB$_5$ block the biosynthesis of pro-inflammatory AA derived LTB$_4$ and accelerate the production of LTB$_5$ in inflammatory cells (13, 62). Because the series-3 molecules derived from EPA have a slightly different structure, they are less potent inflammatory mediators than those formed from AA. Thus, the n-6/n-3 ratio in the

diet strongly affects eicosanoid production. The increased consumption of n-3

PUFAs leads to reduced synthesis of pro-inflammatory eicosanoids from AA, and

thus to a decrease in inflammation (7, 63, 64).

Figure 1.2.5 Effects of PUFA on eicosanoid metabolism.
Modified from Calder (64).
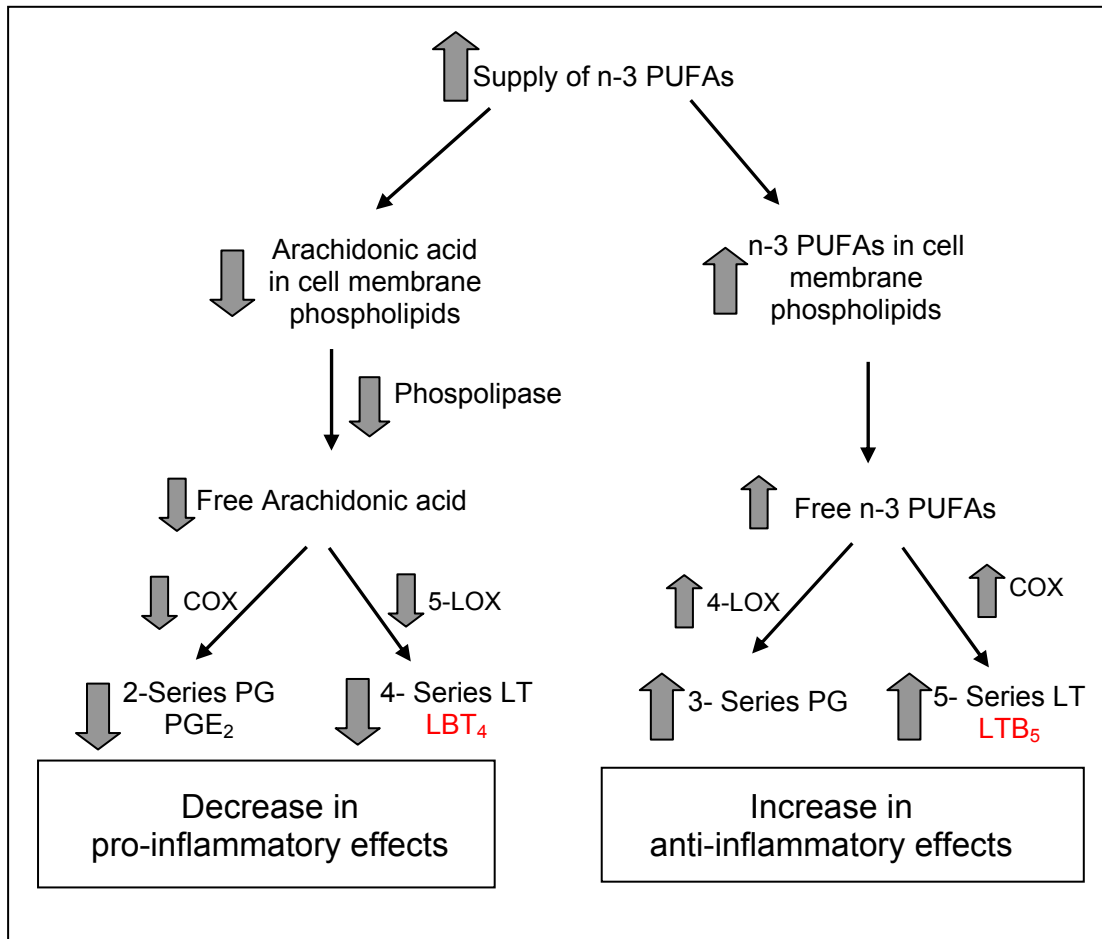
1.5.2 PUFAs effects on membrane structure.

Within the lipid bilayer of cell membranes there are lipid domains with a

distinct, structural composition that act as platforms for proteins involved in

intracellular signaling pathways. Membrane lipid domains are classified as

detergent-resistant membranes when they are insoluble in cold, nonionic detergents (65).

Cholesterol, phospholipids and glycosylphosphatidylinositolanchored proteins concentrate within these lipid domains (66). The acyl side chains of cholesterol and phospholipids are highly enriched in saturated fatty acids compared to the surrounding regions of the membrane (non membrane lipid micro-domains). The enrichment of phospholipids and cholesterol with saturated fatty acids enables the lipid membranes to pack closer together. This placement results in lipid-micro-domains or regions which are structurally more ordered and they contain less fluid than the surrounding membrane (67).

The role of lipid domains in immune system activation was observed in T cell signal transduction (68). When T cells interact with antigen presenting cells (APC), the T cell receptor clusters within lipid domains to form an immunological synapse, which initiates intracellular signaling (69). Stulnig et al. demonstrated in human Jurkat T cell lines that n-3 PUFA treatment inhibits T cell activation and immunological synapse formation by the displacement from the membrane of the linker for activation (LAT) of T cells (70), a protein that plays a role in T cell activation, from lipid micro-domains (71).

Fish oil enrichment of cell culture media or the diet primarily affects the composition of phosholipids within the lipid micro-domains of immune cell membranes. An increase in unsaturated fatty acids results in increased fluidity of cell membranes (72, 73), and PUFA treatment increases the size and distribution of lipid domains in HeLa cells (74) by changing the basic properties of cell membranes.

Furthermore, treatment of cells with n-3 PUFAs has an effect on acylated proteins that are anchored to the inner lipid leaflet by replacing saturated fatty acids with unsaturated fatty acids. This effect was demonstrated by the displacement of signal molecules expressed on n-3 supplemented Jurkat T cells from the cytoplasmic layer of the lipid-micro-domain (46). Moreover, dietary fish oil, as well as pure DHA treatment, has been postulated to reduce the susceptibility to autoimmune disorders and chronic inflammatory diseases by affecting CD4$^+$ T cell plasma membrane fatty acid composition. Nevertheless, in this study fish oil feeding specifically changed the phosphatidylserine and phosphatidylethanolamine fractions within CD4$^+$ T cells membranes, while the TCR/CD3 complex was not affected (75). These data suggest that the anti-inflammatory properties of n-3 PUFAs may result from altered T cell function via the alteration of lipid membrane domain structure.

Figure 1.2.6 Schematic representation of a lipid micro-domain and T-cell activation.
Modified from Hughes DA (76, 77). Green arrows point to potential impact position of feeding n-3
PUFAs.
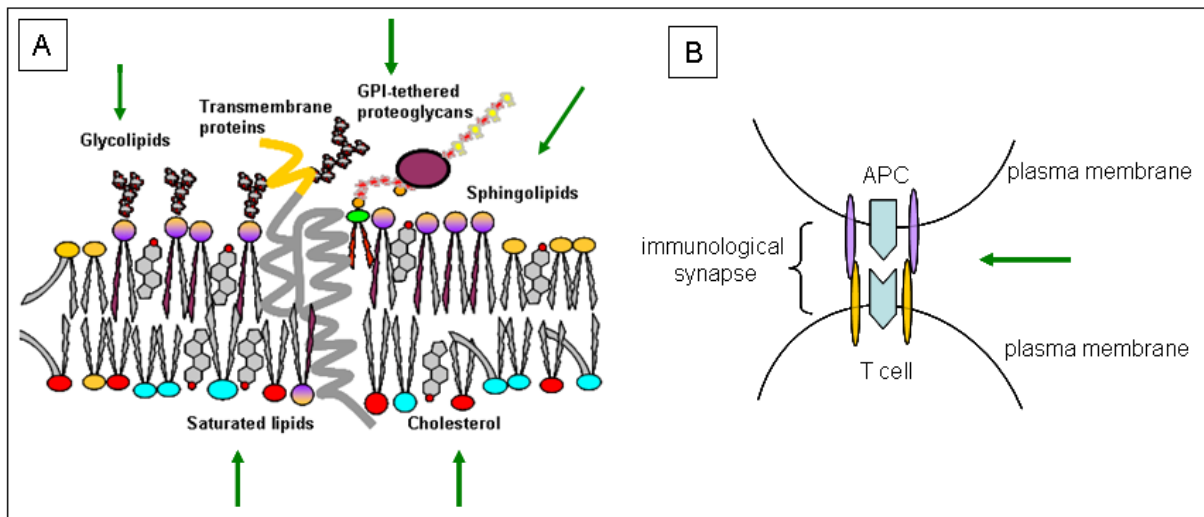
1.6   Influenza A virus

Influenza viruses are membrane enveloped, negative-strand RNA viruses in

the Orthomyxoviridae family. Influenza viruses are classified as influenza A, B or C,

based on the antigenic differences of their core nucleoproteins and matrix proteins

(Figure 1.2.7) (78). The influenza A virus infects host respiratory epithelial cells, is

highly contagious, and is the most pathogenic of the influenza strain types (79).

The mouse provides a well characterized model for influenza virus infection

(80, 81). Following intranasal infection, mouse-adapted strains of influenza virus can

cause pneumonia, tracheitis, and flu-like symptoms similar to those found in human

infections (29, 82-84).

1.6.1 Structure of Influenza A virus

Influenza A virus RNA is composed of eight RNA segments which encode for ten different proteins: envelope glycoproteins hemagglutinin (HA) and neuraminidase (NA), matrix protein (M1), nucleoprotein (NP), three polymerases (PB1, PB2 and PA), ion channel protein M2, and nonstructural proteins NS1 and NS2 (79). Influenza A viruses are classified according to their hemagglutinin (H1–H15) and neuraminidase (N1–N9) genetic and antigenic types (85). The HA forms a spike-like structure on the surface of the virus and binds to the host cell receptor. Binding of the virus to the host cell occurs when HA influenza virus particles attached to cell surface sialic acid, present on glycoproteins or glycolipids on the surface of host cells (86, 87). The specificity of the sialic acid (α-2,6-linked or α-2,3-linked) determine species-specific restriction of influenza viruses (88). The NA is also expressed on the viral surface and functions to remove sialic acid on host cells which then allows for the release from the cell and spread of virus particles throughout the body via the blood circulation (87). The viral membrane envelope surrounding the viral RNA (vRNP) consists of a lipid bilayer containing spikes composed of viral glycoproteins (HA and NA) on the outer side and M1 on the inner side (Figure 1.2.6). Viral lipids, derived from the host plasma membrane, are selectively enriched in cholesterol and glycosphingolipids that are derived from the host plasma membrane (Figure 1.2.7) (87).

Figure 1.2.7 Structure of influenza A virus.
Showing 3 structural proteins: Haemagglutinin (HA), neuraminidase (NA) and matrix protein (M2). The ratio of HA to NA molecules in the viral envelope usually ranges from 4:1 to 5:1. The influenza virus matrix protein M1 associates inside the viral membrane. The viral genome consists of eight negative-strand RNA segments and is packaged into the particle as a ribonucleo-protein with nucleocapsid protein (NP) and the viral polymerases PA, PB1 and PB2. Adapted from Karlsson (89).
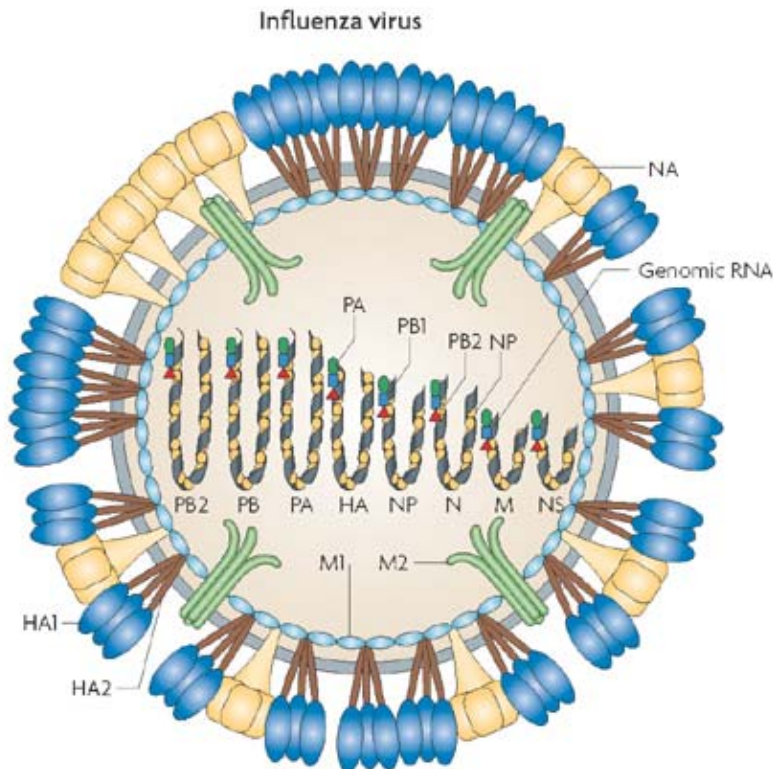
1.6.2 The life cycle of Influenza A

Influenza A viruses preferentially replicate in host respiratory epithelial cells, but macrophages and other leukocytes may also become infected (90). The first stage in influenza virus entry to a host cell is the recognition of a terminal sialic acid on target cell surface molecules by the viral HA protein (91). Host cell-bound viruses are internalized by receptor-mediated endocytosis of cholesterol and

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glycosphingolipid enriched membrane micro-domains via clathrin-coated pits or clathrin-independent endocytosis (92, 93). Following endocytosis, the virus is transported to endosomes, where the low pH induces membrane fusion between the viral and endosomal membranes. Viral replication is a two-step process that begins with a full-length positive-sense copy of the vRNA being transcribed to complementary (c) RNA. cRNA is then used as a template to produce additional vRNA (Figure 1.2.8) (94, 95). Viral replication and mRNA synthesis depends on host cell nuclear function.

Figure 1.2.8 The life cycle of influenza A virus.
Modified from Bao Y (96).
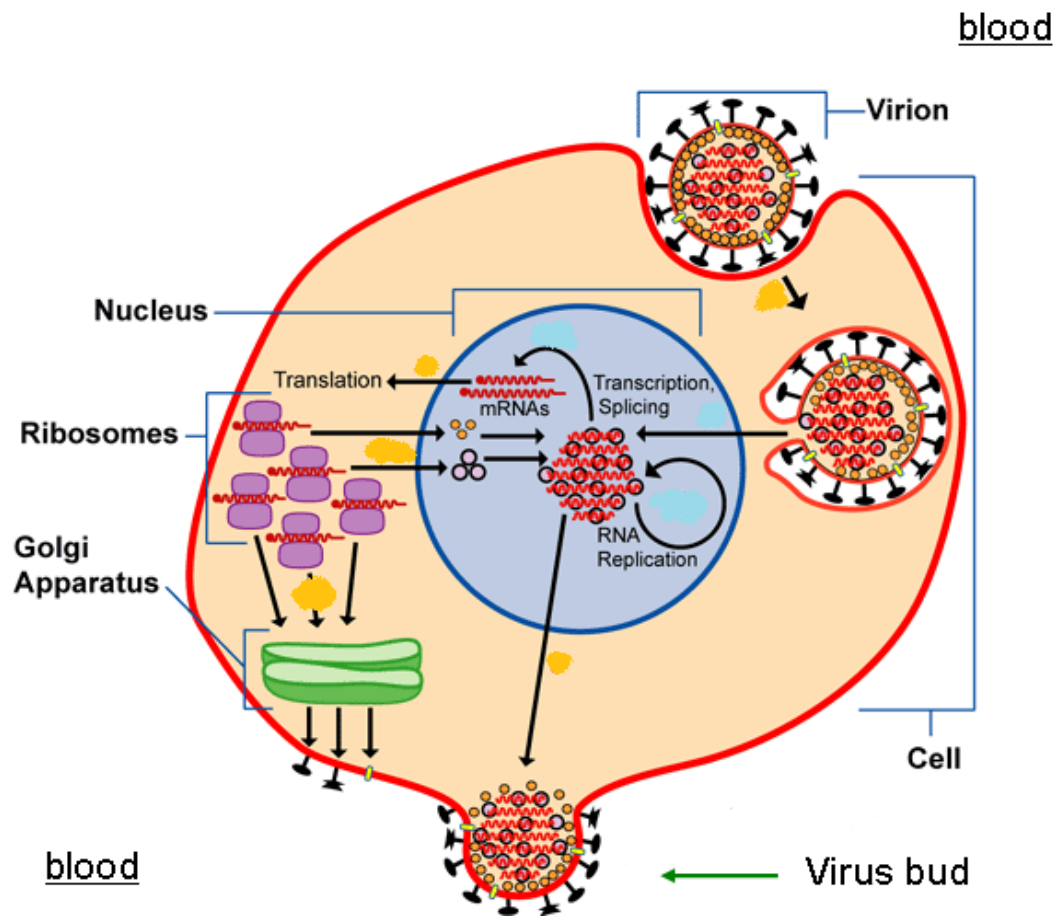
1.6.3 PUFA role in interfering with virus budding

In polarized epithelial cells, influenza A viruses assemble and bud at the apical

surface of the cells using cholesterol and glycospingolipid enriched membrane

domains (97, 98). Buds are released by release of the apical membrane and fission

of the bud from the cell membrane (87). Viruses that bud from the host cell consist of

18-37% lipids by weight. The composition of viral lipids and host cell membranes are

similar (99).

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The composition of the host lipid bilayer affects properties of the virus membrane, including virulence, lipid heterogeneity and lipid–protein interaction (92, 100). Given that Influenza A virus budding relies on the existence of cholesterol and glycospingolipid-enriched membrane domains (101, 102), n-3 PUFA enrichment of cells has been suggested to interfere with virus-budding and fission processes (Figures 1.2.6 and 1.2.9). Unpublished data from our laboratory (Dr. Beck) have suggested that the influenza virus becomes "trapped" in PUFA-treated cells when compared to non-PUFA treated infected cells.

Figure 1.2.9 A schematic representation of virus budding.
Modified from Nayak (87). Green arrows point to potential impact position of feeding n-3 PUFAs
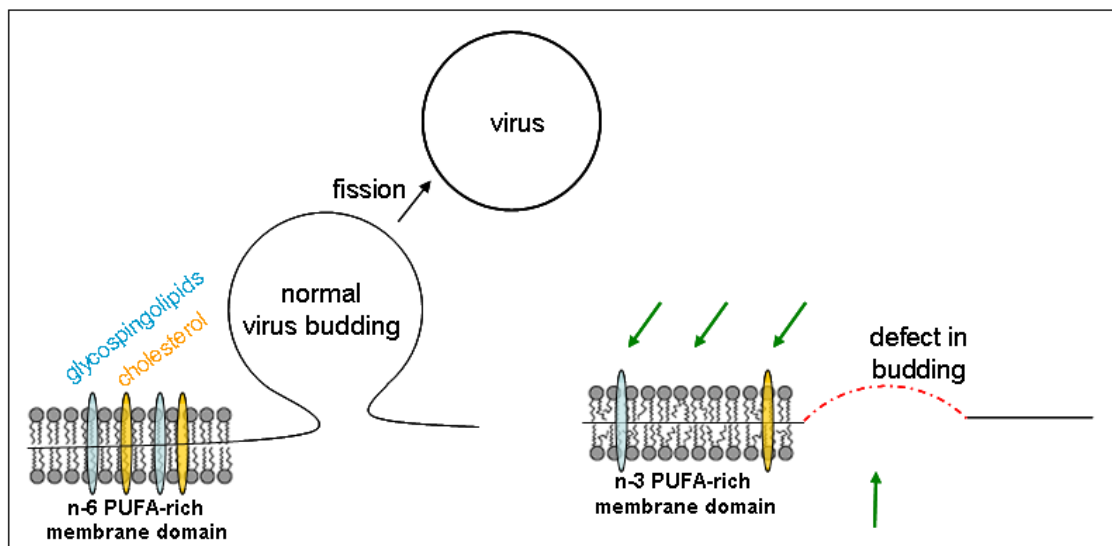
1.7   Roles of PUFAs in modulating cell responses to influenza infection

Immune responses to influenza virus infection contain virus spread and reduce virus infectivity. To do so, the host requires an effective and coordinated activation of the innate (early) and adaptive (delayed) immune response.

Innate immunity is the first line of defense that restricts influenza viral spread by the activation of non-specific immune responses (103). The first step in the innate immune response is the release of cytokines and chemokines from virus-infected cells (29). The expression and release of these inflammatory mediators induces a rapid infiltration of neutrophils, which is followed by infiltration of other inflammatory cells such as monocytes, macrophages and natural killer (NK) cells. For example, during influenza infection, the influx of immune cells into the lung alveolar space is recognized as a key characteristic of the host defense mechanism against viral infection (104). Further elimination of virus-infected cells requires cell-mediated mechanisms that form the adaptive immune response. In particular, virus-specific T cells are essential in this process. Several days after the initial infection are required before adaptive immunity becomes effective, as antigen specific T cells have to be expanded de novo during a primary infection (29). During the transition time from an innate to an adaptive immune response, neutrophils, macrophages, dendritic cells (DCs), and NK cells help control viral spread (104).

Studies of mice fed fish oil enriched diets illustrate, that in response to immune system stimulation, the anti-inflammatory properties of PUFAs reduced natural killer (NK) cell activity (105), decreased lymphocyte proliferation (55), and diminishing antigen presentation by monocytes and dentritic cells (75, 76, 106, 107). In addition, decreases in ex vivo production of TNF-α (108), interleukin (IL)-1, IL-2, IL-6 and interferon (IFN)-γ, have been reported in cells of mice fed fish oil enriched diets (Figure 1.2.10) (42, 109, 110).

Figure 1.2.10 A schematic representation of the temporal nature of the host immune response to invasion by a pathogenic microorganism
Modified from Burleson et al (111). Green arrows point to potential impact position of feeding n-3 PUFAs.
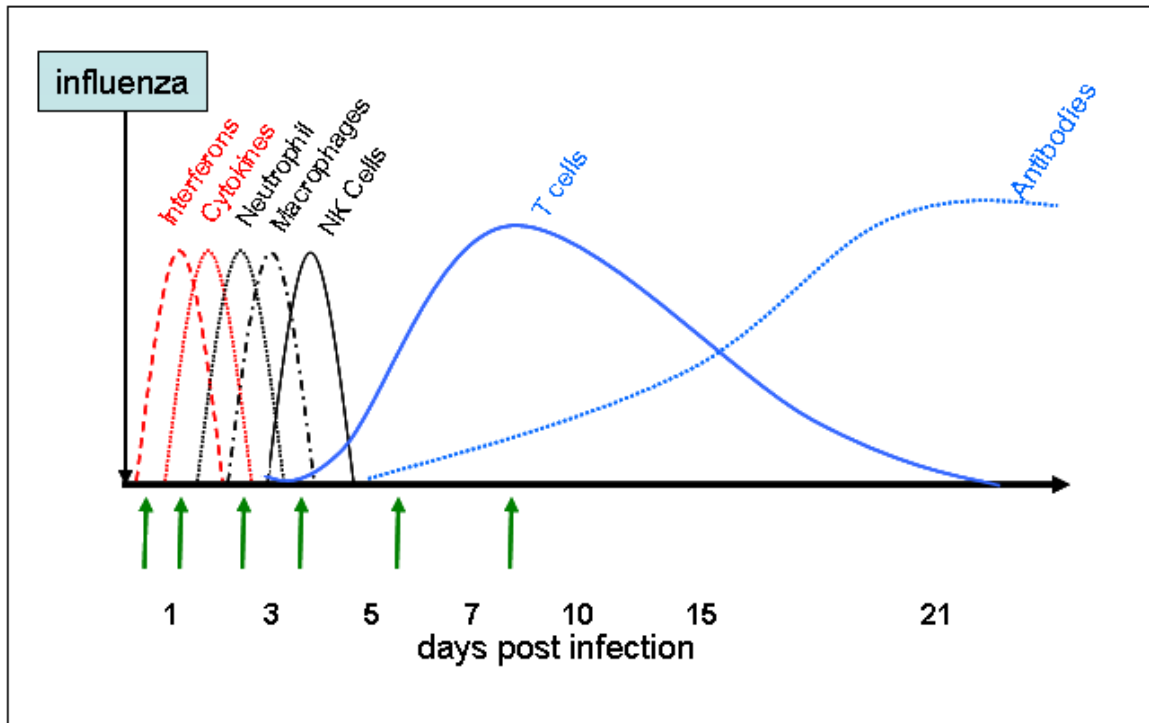
1.7.1 Virus infection-induced cytokine responses

The **interferon (IFN) isoforms α and β (IFN α/β)**, which are primarily secreted from virally infected epithelial cells, are the cytokines initially produced in response to infections. IFN α/β enhance immune function by up-regulating cytokine receptors (112, 113), activating NK cells (114), promoting the differentiation of monocytes to DCs (115) and controlling the gene expression of the major histocompatibility complex (MHC) (116). The few studies that have investigated the effect of n-3 PUFA treatment on IFN α/β secretion found no association (37, 117).

Interferon-γ (IFN-γ) is synthesized by activated T cells and NK cells in response to infection. IFN-γ is associated with upregulation of MHC II expression

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and is responsible for the activation of macrophages to produce cytokines and enhance macrophage-mediated killing of intracellular pathogens (118, 119). With influenza infection, IFN-γ production provides a positive feedback loop among macrophages, NK cells, and T cells.

The impact of fish oil feeding on IFN-γ secretion during infection has been analyzed. Byleveld et al. showed that influenza virus-infected fish oil fed mice had increased virus titers with decreased IFN-γ levels in lungs compared to beef tallow fed mice. The authors suggested that an impaired production of IFN-γ may have been responsible for delayed influenza virus clearing in lungs of mice (37). Additionally, Fritsche et al. showed that feeding mice a diet rich in n-3 PUFAs from fish oil significantly lowered the production of IFN-γ, while bacterial Listeria monocytogenes load increased (120). These studies suggested that n-3 PUFA impairment of IFN-γ secretion plays a significant role in the clearance of an infectious pathogen.

Influenza virus-infected **monocytes** contribute to the host response through the production of cytokines tumor necrosis factor (TNF)-α, interleukins (IL)-1β, IL-6 and IFN-α/β (121, 122). TNF-α acts on blood vessels to increase vascular permeability to fluids, proteins, and cells and to increase endothelial adhesiveness to leukocytes and platelets (123). During viral infection, TNF-α is also produced by activated T and NK cells (29, 124).

The production of **IL-1α and IL-1β** enhances the immune response by increasing the expression of adhesion factors on endothelial cells (125), the activation of T cells (126) and the gene expression of cyclooxygenase and

phospholipase A$_2$ enzymes (127). IL-1β does not influence the killing of virus-infected cells directly, but enhances secondary antibody responses and recruits T cells to the site of infection (128). Induction of **TNF-α** and IL-1β results in the synthesis of **IL-6** from infected monocytes, macrophages and endothelial cells (29). IL-6 production during influenza infection has pleiotropic effects, including the activation of NK cells and macrophages in addition to stimulation of T-cell differentiation (129). IL-6 acts as both a pro-inflammatory and anti-inflammatory cytokine and plays a role in haematopoiesis and acute-phase immune responses of the host (130). Cell culture studies suggest that n-3 PUFAs play a central role in altering cytokine induction. EPA and DHA enrichment of culture medium was shown to inhibit the production of endotoxin-induced IL-1β and TNF-α by monocytes (131). Additionally, pretreatment with EPA and DHA significantly decreased in vitro TNF-α, IL-1β and IL-6 production by lipopolysaccharides (LPS)-stimulated macrophages compared to LPS-stimulated macrophages that were not fatty acid pre-treated (132). Fish oil feeding in mice reduced LPS-induced production of TNF-α, IL-1β and IL-6 by macrophages and decreased TNF-α, IL-1β and IL-6 concentrations in the circulation (133, 134). Thus, diets high in n-3 PUFAs, including EPA and DHA may decrease inflammatory responses to infection by decreasing the cytokine production of mononuclear phagocytic cells, such as circulating monocytes and tissue macrophages.

In addition to the previously mentioned cytokines, influenza virus-infected macrophages also secrete chemokines. **Chemokines** act as chemoattractants that enhance the trafficking of immune cells to the site of infection. During viral infection,

chemokines additionally stimulate the secretion of cytokines from infected monocytes. The chemokines secreted by virus-stimulated macrophages include: regulated upon activation normal T cell expressed and secreted (RANTES), monocyte chemotactic protein (MCP-1) (135, 136) and macrophage inflammatory protein 1α (MIP-1α) (137). Pretreatment with EPA was shown attenuat the up-regulation of MCP-1 and adhesion molecules in umbilical vein endothelial cells after stimulation with TNF-α (138). In other studies, however, the incorporation of n-3 PUFA into human alveolar epithelial cells had no measurable effect on the production of chemokines (i.e. RANTES) in response to respiratory syncytial virus infection (139).

1.7.2 Roles of PUFAs in virus infection-induced cell-mediated responses

T cell activation requires recognition of antigen by the T cell receptor. T cells recognize antigen in the context of major histocompatibility complex (MHC) glycoproteins presented on the surface. Antigen presenting cells (APCs) (such as macrophages and dendritic cells) express MHC class II molecules, whereas all nucleated cells express MHC class I molecules. MHC class I molecules present antigens to CD8$^+$ T cells and MHC class II molecules present antigen to CD4$^+$ T cells (140, 141).

Figure 1.2.11 The role of PUFA feeding in clonal expansion of naïve T cells.
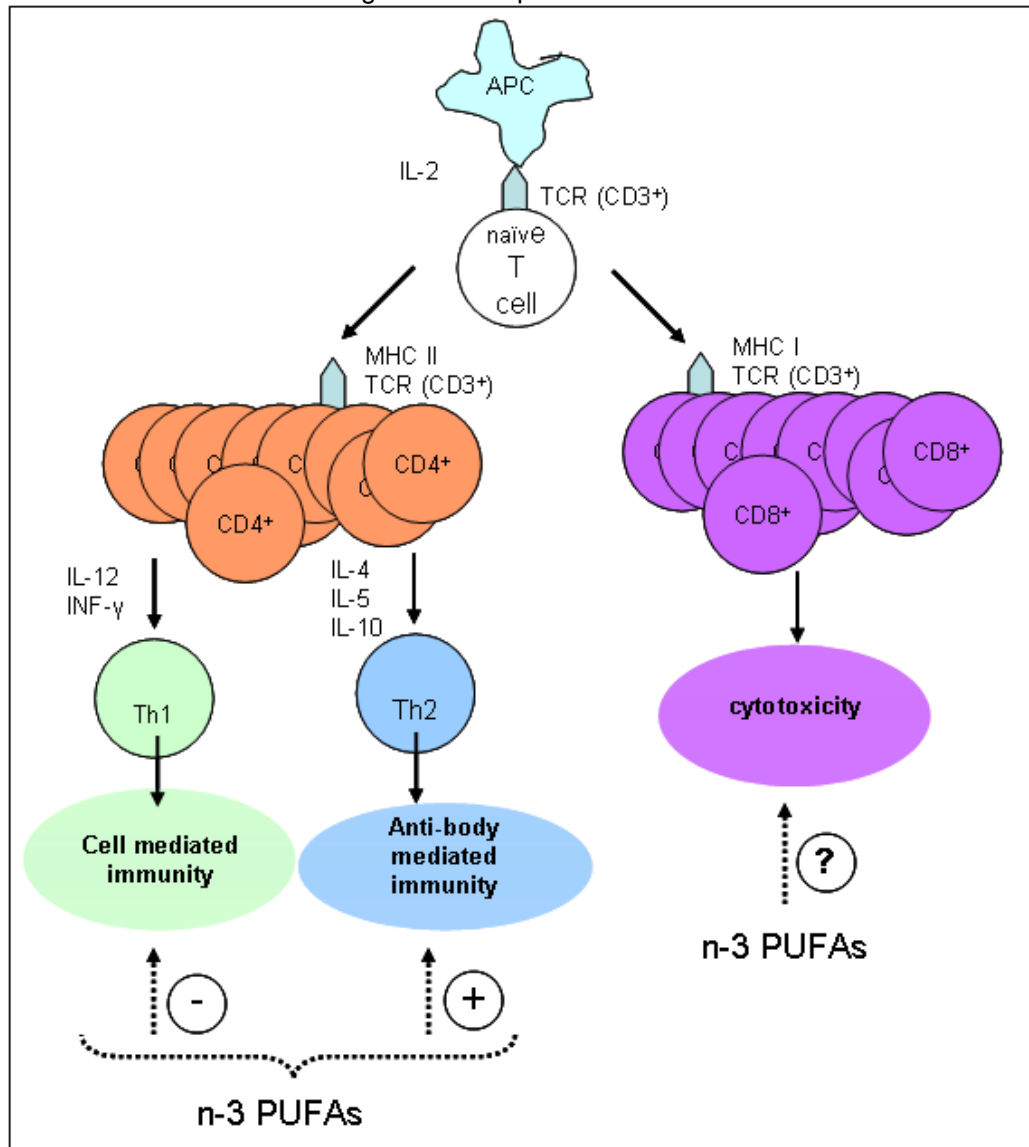
Most studies have tested the effects of n-3 PUFAs on MHC class II surface expression because MHC class II molecules are up-regulated in many autoimmune and inflammatory disorders. These studies show that n-3 PUFA enrichment reduces MHC class II surface expression (44, 45, 142). For example, treatment of stimulated monocytes with EPA and DHA decreased MHC class II expression and diminished the ability of APCs to present tetanus toxoid antigen to lymphocytes (76). In rats, fish

oil feeding diminished the expression level of MHC class II and therefore reduced the antigen presentation function of dendritic cells (45). Recently, Shaikh et al. demonstrated the effect of PUFAs on MHC class I expression and on MHC class I-mediated antigen presentation. Expression of MHC class I was decreased with in vitro treatment of B lymphoblasts (JY cells) with ararachidonic acid (AA) or DHA. This study showed that the effect was fatty acid concentration dependent, with AA being more effective than DHA. Functionally, the reduced MHC class I expression resulted in decreased T cell lysis of the PUFA treated JY cell (143). Thus, because fish oil feeding may reduce antigen presentation, adaptive immunity may be negatively affected when faced with infectious disease.

Natural Killer (NK) cells are non-specific cytotoxic lymphocytes activated by interferons or macrophage-derived cytokines in response to infection. NK cells help to control viral infections while the adaptive immune system generates a more specific antigen response to clear the infection. NK cells distinguish pathogen infected cells from uninfected cells based on recognition of 'activating' or 'inhibiting' receptors (114). Cytolysis of the target cell occurs following the secretion of cytotoxic perforin granzyme B protein that penetrates the targeted cell and leads to cell death (144). Studies of cytolysis have compared the effects of fish oil and corn oil feeding in mice on NK activity (37, 53, 105, 145-147). These studies demonstrated that mice fed fish oil diets exhibited decreased NK activity compared with mice on corn oil diets. Considering that NK cells kill virally infected cells and represent the first line of the innate immune responses during influenza infection

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(148), the attenuated inflammatory response due to fish oil has be hypothesized to impair viral clearance by NK cells.

Dendritic cells (DCs) and **macrophages** are considered antigen presenting cells (APCs) and each type forms a bridge between innate and adaptive immunity. DCs reside in peripheral tissues, where they survey for incoming pathogens. Invading viruses can directly infect DCs, or DCs can phagocytose virus-infected apoptotic or necrotic cells. After viral uptake, DCs migrate from the infected tissue to draining lymph nodes, where they present viral antigens to T cells. DCs modulate immune responses to infection by the secretion of IL-12, which acts on CD8^{+} T cells to augment cytolytic activity and IFN-γ production (149-151). Additionally, DCs produce IFN α/β, which, together with the production of IL-12, enhances NK cell expansion and activation (30). Sanderson et al. demonstrated that dietary fish oil diminished the antigen presentation of rat dendritic cells because fish oil feeding significantly diminished the levels of adhesion molecules and MHC class II expression on dendritic cells (45). The diminished APC function of DCs that accompanies fish oil feeding suggests that, upon infection, fish oil feeding would result in decreased elimination of virus-infected cells and/or an altered antigen presentation to T cells.

1.7.3 PUFAs role in clonal expansion of T cells

Clonal expansion of T cells is an essential process in cell-mediated immunity (152). T cells mediate regulatory and effector functions through the expansion and differentiation of T cells expressing surface protein CD4 or CD8. One of the

important immunomodulatory activities of n-3 PUFAs is their ability to diminish T cell differentiation and proliferation (60, 153).

As circulating T cells migrate from the thymus through peripheral lymphoid tissues, T cells sample MHC-peptide complexes on antigen presenting cells (Figure 10) (140, 141). If the T cell receptor (TCR) recognizes the antigen presented by the MHC, then clonal expansion, differentiation and activation is triggered. T cells recognize antigens by the TCR. When the TCR recognizes antigens, an activation signal is transmitted by the CD3 complex on the T cell (154). Upon the initial encounter with antigen, naïve T cells produce IL-2. The antigen recognition by TCR and the induction of IL-2 by naïve T cells leads to rapid clonal expansion and differentiation of T cells, which are unique to specific antigen presented to them.

CD4$^+$ T helper cells. T cells expressing the surface protein CD4 (CD4$^+$ T cell) can further be differentiated into a T helper cell (Th1) or Th2 phenotype. Th1 or Th2 subtypes are determined by the type of invading pathogen and the cytokines secreted during CD4$^+$ T cell activation. When naïve T cells recognize antigens, which are presented by APC's, then T cells are also exposed to IL-12. IL-12 promotes the differentiation of the T cell into the Th1 subset, which then produces IFN-γ, which primarily interferes with viral infection and stimulates phagocyte-mediated killing. If the invading pathogen does not elicit IL-12 production by APC's, then T cells themselves produce IL-4, which induces the differentiation of these cells towards the Th2 subset (155). Th2 cells produce cytokines IL-4 and IL-5, which stimulate phagocyte-independent mediated immunity. Some of the cytokines produced by Th2 cells, such as IL-4, IL-10, and IL-13, additionally inhibit macrophage activation and

suppress Th1 cell-mediated immunity (156). The Th1 cell responses help to activate CD8$^+$ cytotoxic T cells and are important for dealing with viral infection. Th2 responses provide help for B cells to enhance antibody production and activate eosinophils (140).

Since dietary n-3 PUFAs can alter cytokine induction, it has been suggested that the anti-inflammatory effects of fish oils may be due to their ability to modulate the Th1/Th2 balance (60, 106). For example, Fritsche et al. demonstrated that n-3 PUFA consumption was associated with a significant impairment of in vivo IL-12 production in mice. Fritsche et al. suggested that the attenuated inflammatory response seen with fish oil feeding may be explained, at least in part, by suppression of a Th1 response (120). Zhang et al. illustrated that dietary n-3 PUFAs inhibited the proliferation of both antigen-stimulated CD4$^+$ T cells and the IL-2 induced proliferation of antigen-specific Th1 cells. His group concluded that dietary n-3 PUFAs inhibited Th1 cell responses by suppressing the clonal expansion of Th1 cells (106, 157). Furthermore, during influenza virus infection, cytokine IL-12 and IFNγ production by CD4$^+$ T helper cells activate CD8$^+$T cells (141). Because n-3 PUFAs are able to suppress Th1 responses they also may impair CD8$^+$ T cell function.

CD8$^+$ T cytotoxic cells. Successful host defense against intracellular pathogens involves activation of phagocytes and the generation of cytotoxic CD8$^+$ T cells. Upon encountering an antigen, CD8$^+$ T cells produce IFN-γ, which inhibits viral replication by several mechanisms. The production of INF-γ increases expression of MHC class I on the cell surface, increases antigen presentation by macrophages,

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increases NK cytotoxic activity and enhances adhesion expression that are necessary for leukocyte migration, together these immune responses to INF-γ can lead to a suppression of Th2 cell activity (118). CD8$^+$ cells, additionally, eliminate virally infected cells through direct lysis of targeted cells (158). In mice that lack CD8$^+$ cells (CD8 $^{-/-}$), infection with influenza A/PR/8/34 (PR8) led to increased viral replication and mortality (159). In contrast, CD4 $^{-/-}$ mice effectively cleared PR8, implying that CD4$^+$ T cells are not required for elimination of influenza infection (160). Few studies have examined the effects of n-3 PUFAs on CD8$^+$ cytotoxicity and/or CD8$^+$ T cell proliferation and T cell trafficking (161, 162). Most of these studies investigating the antigen-driven expansion of CD8$^+$ T cells reported that antigen-specific CD8$^+$ T cells were unaffected by n-3 PUFAs (163).

In summary, immune responses to influenza virus infection aim to restrain virus spread and control virus infectivity. Because n-3 PUFAs have been shown to decrease inflammatory responses such as the production of cytokine (41, 42), natural killer cell activity (43), antigen presentation (44, 45), lymphocyte function, and T cell responses (46, 47) the inflammation due to infection, may not be induced. Thus, n-3 PUFA feeding may have profound effects when combating an infection in which an intact immune response is necessary to eliminate the pathogen.

1.8   Dietary n-3 PUFAs and reduced resistance to pathogens

Publications that have investigated the influence of n-3 PUFAs on infectious disease resistance in mice are summarized in Table 1.1. Most studies examined the effects of the immune response in mice to bacterial challenges and found host survival to be dependent on the type of pathogen. For example, PUFA-fed mice

challenged with Listeria monocytogenes and Pseudomonas aeruginosa had less survival than those fed other types of fat (38, 164). In contrast, other studies demonstrated that fish oil feeding did not affect resistance of mice to bacteria such as Salmonella typhimurium and viral challenges such as Cytomegalovirus and Influenza A/Queensland. Other studies have shown that dietary fish oil can enhance mouse survival to Klebsiella pneumoniae and mouse AIDS virus (Table 1.1). Only one of these studies examined the effects of PUFAs in mice infected with influenza virus.

Table 1.1 N-3 fatty acids and infectious disease resistance.
Modified from Anderson et al (2).

Mouse sex and strain	Pathogen and dose	Site of infection	Dietary treatment	Duration (165)	Survival of control vs. fish oil diet	Authors
Viruses						
Male BALB/c	Influenza A/Queensland/6/72 (H3N2) $\log_{10}$ 5 CFU	Intranasal	6% sunflower oil with 34% beef tallow or 34% fish oil	2	No change	Byleveld et al. (37)
Female C57BL/6	Mouse AIDS (MAIDS) LpBMS MuLV 5 X IO^5 PFU	Intra peritoneal (i.p)	5% corn oil (CO), 5% CO with 40% energy restriction or 20% Fish oil	4	Increased	Fernandes et al. (166)
Female NZBxW	Cytomegalovirus (CMV) 10^4-10^7 CFU	i.p.	46% beef tallow or 46% menhaden fish oil	4-5	No change	Rubin et al. (167)
Bacteria						
Female NZBxW	Pseudomonas aeruginosa 10^5-10^9 CFU	i.p.	46% beef tallow or 46% menhaden fish oil	4-5	No change	Rubin et al. (167)
Female CF1	Salmonella typhimurium 10^4 CFU	i.p.	40% coconut oil, safflower oil, MaxEPA mix	2-3	No change	Clouva-Molyvdas et al.(168)
Female C57Bl/6	Klebsiella pneumonia $0.5 \times 10^{4-5}$	Intra muscular (i.m.)	30% corn oil 30% plam oil 28% fish oil (+2% corn oil)	6	Increase	Blok et al. (169)
Female BALB/c	Pseudomonas aeruginosa ~10^5 CFU	Burn side	10 and 40% safflower oil or MaxEPA mix	2-3	Decrease	Peck et al.(164)
Female C3H/HeN	Listeria monocytogenes 10^5-10^7 CFU	i.p.	20% lard, soybean oil or 17% menhaden fish oil (+ 3% corn oil)	4-5	Decreased	Firtsche et al. (38)
Sex not reported BALB/c	Listeria monocytogenes 10^5 cfu	i.p.	Low fat as control, 20% coconut oil, olive oil, fish oil	4	Decreased	Puertollan oet al. (39)
Female swiss webster	Salmonella typhimurium 6 x 10^6 CFU	i.p.	20% menhanden fish oil	4	Decreased	Chang et al.(40)
Male C57BL/6	Pseudomonas aeruginosa 2 x 10^5 cfu	Trans-tracheal insertion	fatty acid mixtures EPA 10% DHA 5%	5	Increased	Pierre et al. (170)

With the anticipated increase of fish consumption and the increase in the usage of fish oil supplements per person, there is growing concern that the beneficial anti-inflammatory properties of n-3 PUFAs that prevent chronic inflammatory illnesses may have adverse effects when inflammation is necessary to combat infection (2, 4, 171).

To date, limited data have reported the effects of fish oil supplementation on the progression of virus infections (2, 37, 167, 172). More information is needed on how increased fish oil intake affects the immune response to infection. We chose the influenza virus as a model system to study the effects of PUFAs on infection because its infectivity is well characterized in animal models and, therefore this model has applications to human infectious with this virus.

1.9   Limitations of studying the immunomodulatory effects of PUFAs

The ability of PUFAs to influence the immune system has been established (117). Much of what is known about the immunosuppressive effects of PUFAs on inflammatory illnesses has come from feeding studies, but results have been highly variable. Human dietary studies rarely exceed 3 to 4% of n-3 PUFA total energy intake, whereas most animal studies used percentage of n-PUFAs that exceed what is possible in humans, even for those consuming n-3 PUFA supplements (Table 1.1) (49). Another recurring problem with these studies is the choice of appropriate control diets. When selecting a control diet for studying the influence of PUFA, its is important to consider equivalent amounts of total dietary calories obtained from protein, carbohydrates and fats in addition to the vitamin mix and mineral content used. Suitable control diets should include all components of the treatment diet in

the same amounts expect the dietary component in question. Therefore, only chemically defined diets are appropriate.

The advantage of using animal feeding studies is that they are of physiological relevance and can control for genetic variability. Compared to cell culture experiments, animal studies often avoid "cell culture artifacts" that can alter characteristics from the original cell population (6, 173). Animal experiments are preferred, but results may not be consistent. Factors which may influence experimental may include the 1) limited number of studies, 2) nature of the pathogen, 3) challenge dose administered, 4) route of administration, 5) genetic background and sex of the animal, 6) length of feeding before the immune challenge and, 7) specific n-3 PUFAs fed.

Because this study was designed to test if fish oil feeding can modulate the immune response to influenza virus infection, we chose an animal model to reflect as close as possible human conditions with a dietary intervention and use of infectious challenge. The treatment diet consisted of a natural fatty acid mixture that provided 4% menhaden fish oils (+ 1% corn oil) at physiologically achievable levels of n-3PUFAs, rather than pharmacological concentrations. A two week feeding period before the immune challenge was chosen based on prior feeding studies (2, 49, 74, 106, 157). The influenza virus was administered intra-nasally, which represents the most natural route of administration. Much of what is known about how PUFAs modulate the immune system stems from in vitro studies employing nonspecific polyclonal activators, and in vivo studies are often limited to bacterial infection (2). The strength of our in vivo study is that we analyzed a wide range of

immune cells involved in innate and adaptive immunity and also measured these variables during the recovery phase from virus infection.

Beneficial anti-inflammatory properties of fish oil feeding were found to protect mice from lung inflammation, although these same properties can adversely affect overall immune function when the body needs to fight an acute infection. Given that n-3 PUFA-rich fish oil capsules are readily available and the supplemental inclusion of n-3 PUFAs in prepared foods is on the rise, the understanding of how PUFAs can affect the immune response to infection becomes even more important. Better dietary recommendations for the public can be made only after detailed understandings of PUFAs in modulating innate and adaptive immunity are achieved.

The next two chapters represent manuscripts that will be submitted for publication or are already under review. The final chapter provides a general summary of the results of the experimental studies and suggestions for future investigation.

CHAPTER II

2 Impaired resistance to influenza infection in fish oil fed mice

Nicole MJ Schwerbrock, Erik A Karlsson, Qing Shi, Patricia A Sheridan, Melinda A Beck.

2.1 Abstract

Dietary fish oils, rich in n-3 polyunsaturated fatty acids (PUFA), including eicosapentaenoic acid and docosahexaenoic acid (DHA), have been shown to have anti-inflammatory properties. Although the anti-inflammatory properties of fish oil may be beneficial during a chronic inflammatory illness, the same anti-inflammatory properties can suppress the inflammatory responses necessary to combat acute viral infection. Given that n-3 PUFA-rich fish oil supplementation is on the rise and with the increasing threat of an influenza pandemic, we tested the impact of fish oil feeding for 2 weeks on the immune response to influenza virus infection. Male C57BL/6 mice fed either menhaden fish oil (4:1, w/w at 5g/100g) or a control corn oil diet were infected with influenza A/PuertoRico/8/34 and analyzed for lung pathology and immune function. Although fish oil fed mice had a reduction in lung inflammation compared to controls, fish oil feeding also resulted in a 4-fold increase in mortality, a 7-fold increase in lung viral load at d 7 post infection and a prolonged recovery period following infection. While splenic natural killer (NK) cell activity was

suppressed in fish oil fed mice, lung NK activity was not affected. Additionally, lungs of infected fish oil fed mice had significantly fewer CD8^{+} T cells and decreased mRNA expression of MIP-1α, TNF-α and IL-6. These results suggest that the anti-inflammatory properties of fish oil feeding can alter the immune response to influenza infection resulting in increased morbidity and mortality.

2.2 Introduction

Dietary long chain polyunsaturated fatty acids (PUFA) derived from fish oil have been shown to have beneficial effects on chronic inflammatory and autoimmune disorders, suggesting that fish oil has anti-inflammatory and immunomodulatory properties (4, 5). Abundant in marine fish oils, long chain PUFA of the omega-3 series such as eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), appear to be most beneficial (7, 174). A number of studies report that the immunosuppressive effects of PUFAs are a result of decreased cytokine production and from reductions in T cell proliferation, activation, and signaling (41, 47, 175). Studies of rodents fed with fish oil enriched diets have shown a reduction in natural killer (NK) cell activity (105), decreased lymphocyte proliferation (106, 109) and decreased antigen presentation functions (6, 106, 176, 177). In addition, decreases in ex vivo production of tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-2, IL-6 and interferon (IFN)-γ, have also been reported (109, 133, 134, 178, 179)

While the anti-inflammatory properties of PUFA may be beneficial for some chronic inflammatory illnesses, these same anti-inflammatory properties may be detrimental for response to an infection when an intact immune system is needed to

eradicate an invading pathogen. For example, diets supplemented with fish oils have been shown to lower host resistance to Mycobacterium tuberculosis (14), to reduce the survival of mice against infection with Listeria monocytogenes (38, 39) and to decrease resistance of mice infected with Salmonella typhimurium (40). Similarly, fish oil feeding can diminish host defense against influenza virus due to delays in viral clearance (37).

Despite the availability of vaccines and antiviral agents, influenza virus continues to be a major cause of morbidity and mortality worldwide (32, 34). Influenza virus infects cells of the respiratory system resulting in an acute and diffuse inflammation of the bronchoaveolar tract (29). Following infection, a coordinated immune response consisting of both innate and adaptive mechanisms results in an accumulation of immune cells and secretion of immunomodulatory proteins designed to limit viral spread. In the mouse model of influenza virus infection, NK cells, neutrophils, and T lymphocytes increase in the lung post infection and contribute to host protection (180). The secretion of both inflammatory and antiviral cytokines helps to eliminate the virus and reduce further spread. While this inflammatory response is necessary for viral clearance, it also contributes to lung pathology (180). Although there are many studies documenting the anti-inflammatory properties of fish oil, there are few studies that examine the effects of fish oil on viral infection (2).

Because of the known health benefits, fish oil supplementation is on the rise. A survey conducted among health care professionals reported an increase in fish oil supplementation from 24% to 30% in 2006 to 2007 (27). Additionally, in 2007, 37.4

% of the US population reported using fish oil or fish oil supplements for health reasons (181). With the anticipated increase of fish consumption and the increase in the usage of fish oil supplements per person, there is a growing concern that the beneficial anti-inflammatory properties of n-3 PUFAs may have adverse effects when inflammation is necessary to combat infection (2, 4, 171). This study was undertaken to investigate effects of fish oil feeding on the immune response to influenza virus infection in mice.

2.3   Materials and Methods

Animals and diet: Six-week-old male C57BL/6J mice were purchased from Jackson Laboratories. All mice were housed at the University of North Carolina Animal Facility, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. Animals were maintained under protocols approved by the Institutional Animal Care and Use Committee. All mice were housed under a 12 hour light/dark schedule with free access to food and water. Mice were fed ad libitum a control semipurified diet containing 5% corn oil (D07092502, Research Diets, Inc) or a fish oil-corn oil diet at physiologically levels (1% corn oil + 4% fish oil, D07092503, Research Diets, Inc) for 2 weeks (Table 2.1). Fish oil diet was supplemented with 0.405 g/kg Vitamin E to keep normal Vitamin E levels during infection (182, 183) .

Virus and infection: The mouse-adapted strain of influenza A/Puerto Rico/8/34 (A/PR8, American Type Culture Collection) was propagated in the allantoic fluid of 10-day-old fertilized hen's eggs, and the viral titer was determined by hemagglutination assay (184). Following 2 weeks dietary treatment, mice were anesthetized with an intraperitoneal injection of a ketamine (0.6 mg/kg)/xylazine

(0.35 mg/kg) solution and infected intranasally with 0.05 mL of 2 hemagglutinating units of A/PR8 virus diluted in PBS. Previous studies from our laboratory determined that this dose of virus is sufficient to effectively elicit an immune response with normal mortality in mice (184). Mice were weighed daily following infection and percent weight loss compared to starting weight at time of infection was calculated.

Pathology: The left lobe of the lung was removed at day 0 (uninfected mice) 3, 7, 10, 15 and 21 post infection (p.i) and perfused with 4% paraformaldehyde, paraffin embedded, cut in 6 μm sections, and stained with hemotoxylin and eosin. Pathology grading was performed semiquantitatively according to the relative degree of inflammatory infiltration as previously described (185).

Quantification of lung virus titer: As previously described, lung viral titers were determined by a modified tissue culture infection dose 50 ($TCID_{50}$) using hemagglutination as an endpoint (184) . Briefly, supernates from lung homogenates were serially diluted and used to infect Madin-Darby canine kidney cells. Virus titers were determined based on the presence or absence of hemagglutination of human O red blood cells and $TCID_{50}$ was determined by the method of Reed and Muench (186).

Enumeration of NK cell populations: Lungs and spleens were removed at d 0 and 3 p.i. Lungs were incubated in a collagenase solution (1500 units/ lung) for 1 h. Lung and spleen were processed into single cell suspensions using a stomacher (Seward, West Sussex, UK) and strained through a 40 μm nylon filter. Cell numbers between different samples were equalized to 5×10^5 cells/sample and stained with flourescein anti-DX5 isothicyanate (FITC) and anti-CD3 phycoerythrin (PE) (BD Pharmingen).

Fluorescence was measured using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 488 nm argon laser and a 647 nm diode laser. The lymphocyte population was gated and NK cells were identified as CD3$^-$ DX5+ within the gate.

Determination of natural killer cell cytotoxicity: Total lung and spleen cells were analyzed using a standard chromium-51 release assay in triplicate following a previously published method (184). Percent specific lysis was calculated by the following equation:

$$\% \text{ specific lysis} \quad \frac{\text{cpm [maximum release]} - \text{cpm [spontaneous release]}}{\text{cpm [sample]} - \text{cpm [spontaneous release]}}$$

Quantification of immune cells: To obtain bronchoaveolar lavage fluid (BALF) whole lungs were lavaged three times with PBS at d 3, 7, 10 and 15 p.i. Red blood cells were lysed using ACK lysis buffer (0.15mol/L NH_4Cl, 1 mmol/L $KHCO_3$, 0.1mmol/L Na_2EDTA in ddH_2O, pH 7.4). Samples were washed twice in PBS/2% bovine serum, and stained with fluorescent antibody for 30 min on ice, followed by three additional washes in PBS/2% bovine serum. At least 5x10^5 cell suspensions were stained with the following anti-mouse monoclonal antibodies: FITC anti-CD3, APC-anti-CD4 and PerCP anti-CD8, PE anti-CD11b and FITC anti-GR-1 (BD Biosciences). Cell populations were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). T cells were identified using the follow antibodies: FITC-anti-CD3, APC-anti-CD4 and PerCP-anti-CD8 and neutrophils: PE anti-CD11b and FITC anti-GR-1(187).

Quantitation of lung mRNA cytokine levels: The right lobe of the lung was removed at d 0 (uninfected), 3, 7 and 10 p.i. Total RNA was isolated using the TRIzol method and reverse transcription was carried out with Superscript II First Strand Synthesis kit (Invitrogen). Following previously described methods (184), mRNA levels were measured for tumor necrosis factor α (TNF-α), interleukin (IL)-2, IL-6, IL-12, interferon-α and IFN-β, regulated upon activation, normal T cell expressed and secreted (RANTES), macrophage inflammatory protein-1 alpha (MIP-1α) and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) using quantitative real time polymerase chain reaction. All data were expressed as fold change from uninfected mice of the matching diet group. There were no significant differences between control and fish oil fed mice with respect to G3PDH levels at any time point. All data were expressed as fold change from uninfected mice of the matching diet group.

Statistical analysis: Statistical analyses were performed using JMP 7 Statistical Software and SAS 9.1 software (SAS Institute). Normally distributed data were analyzed by 2-way ANOVA with diet and day post infection as main effects. Student's t test was used for post-hoc comparison between the dietary groups and Tukey's HSD was used for post-hoc comparisons among the days p.i. Nonparametric data were analyzed using the Kruskal Wallis test. Counted data were analyzed using logistic regression analysis $p < 0.05$ when Wald confidence interval for odds ratios did not include 1. Survival data were analyzed using the Kaplan-Meier survival estimates. Significance that compare the survival curve estimates of

the two diet groups were analyzed by the log Rank test. Differences were

considered significant at P < 0.05.

Table 2.1 Composition of experimental diets, including fatty acid composition.

Ingredient	Control[1]		Fish Oil[2]	
	g/kg	kcal%	g/kg	kcal%
Casein	200	800	200	800
DL-Methionine	3	12	3	12
Corn Starch	219	876	219	876
Sucrose	420	1680	420	1680
Cellulose, BW	60	0	60	0
Corn Oil	50	450	10	90
Fish Oil	0	0	40	360
t-BHQ	1.008	0	1	0
Mineral Mix S10001	35	0	35	0
Vitamin Mix V10001	10	40	10	40
Choline Bitartrate	2	0	2	0
Vitamin E, Acetate (500 IU/g)	0	0	0.405	0

Fatty acid	Control (g/100g)	Fish Oil
C18, Stearic	1.9	2.6
C18:1, Oleic	24.9	9.6
C18:2, Linoleic	60.1	1.9
C18:3, Linolenic	1.4	1.5
C18:4	0.0	3.1
C20, Arachidic	0.0	0.2
C20:1,	0.0	1.5
C20:2		0.2
C20:3,		0.4
C20:4, Arachidonic	0.0	2.1
C20:5,	0.0	14.2
C21:5		0.8
C22, Behenic	0.0	0.1
C22:1, Erucic	0.0	0.3
C22:2		1.5
C22:4, Clupanodonic	0.0	0.2
C22:5	0.0	2.8
C22:6,	0.0	12.2
C24, Lignoceric	0.0	0.6
C24:1		0.2

[1] Formulated and supplied from Research Diets, Inc. (D07092502) containing corn oil.

[2] Formulated and supplied from Research Diets, Inc. (D07092503) containing menhaden oil with

2.1% AA, 14.2% EPA and 12.2% DHA

2.4   Results

Fish oil fed mice recover more slowly from influenza virus infection. In mice,

weight loss is a marker for illness severity following influenza infection and

subsequent weight gain is an indicator for recovery. To determine if fish oil feeding

influences body weight following influenza virus infection, mice were weighed daily

post infection. Both control and fish oil fed mice lost significant amounts of weight

during the course of infection. Control mice began to regain weight after d 10 p.i.;

however, fish oil fed mice did not begin to regain weight until d 14 p.i. By d 20 p.i.,

the body weights were not different between groups (Fig. 2.1).

Figure 2.1 Percent body weight change following influenza infection.
Values are expressed as means ± SEM, n = 9-23 per group per time-point. *P < 0.05, control diet
versus fish oil diet.
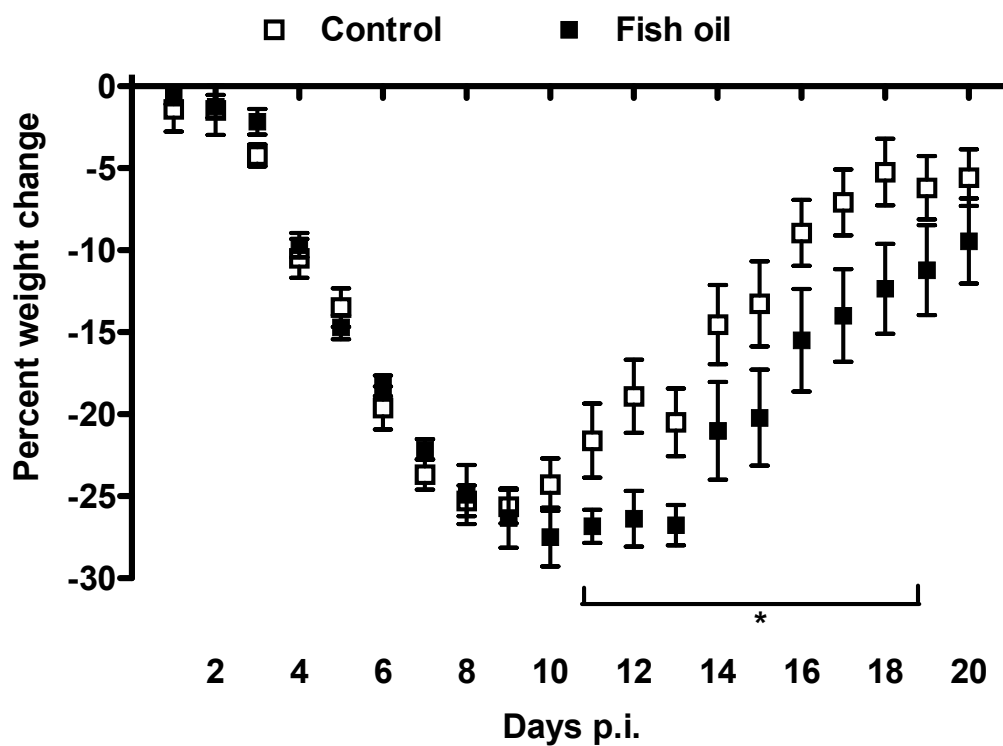
Fish oil feeding results in a higher mortality rate following influenza infection.

Increased severity of infection in the fish oil fed mice was also reflected in the

mortality rates. Beginning at d 11 p.i., fish oil fed mice had a significantly higher

mortality rate compared to control fed mice. At d 20 p.i., the fish oil fed mice had a

51% mortality rate compared to a 10% mortality rate in control diet fed mice (Fig.

2.2).

Figure 2.2 Percent survival following influenza infection.
n = 9-23, per group per time point.
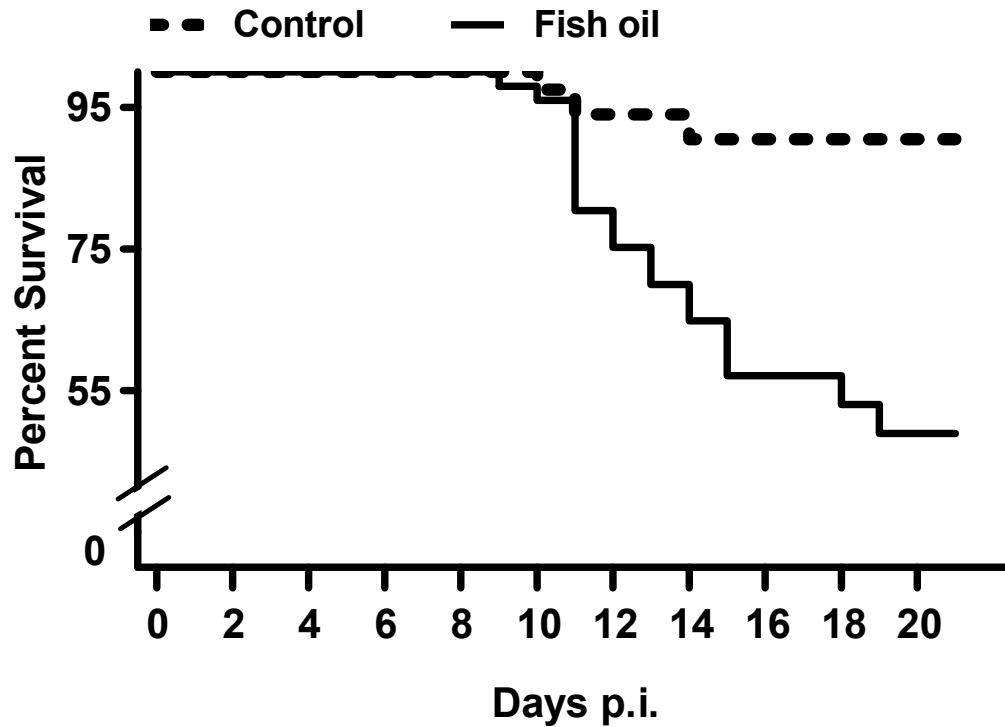
Fish oil feeding improves lung pathology post influenza virus infection.

Influenza virus infection results in a significant infiltrate of inflammatory cells in the

lung. To determine if the anti-inflammatory properties of a fish oil enriched diet could

decrease this inflammation, lung pathology was examined at various times p.i. As

shown in Fig. 2.3, lung pathology was significantly reduced in influenza-infected fish

oil fed mice at d 7, 10, 15 and 21 p.i. compared to infected control fed mice.

Figure 2.3 Degree of lung pathology following influenza infection.
Values are expressed as means + SEM, n = 4-10 per group per time-point. * P < 0.05, control diet versus fish oil diet.
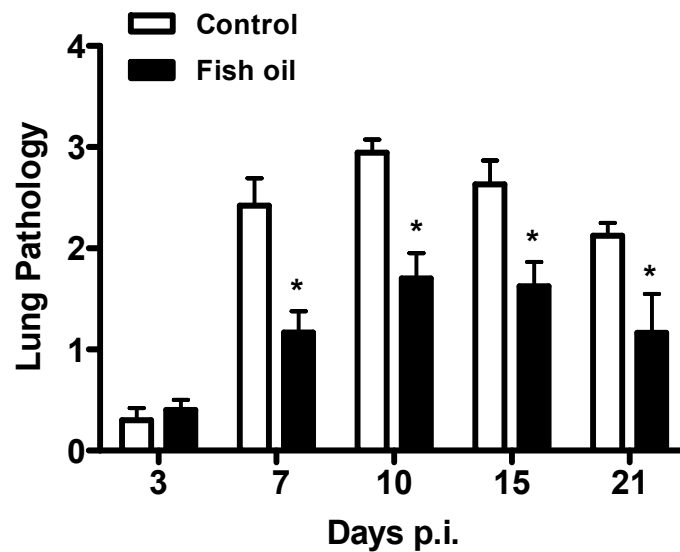
Fish oil feeding increases virus load in the lung post infection. Because the fish oil fed mice had reduced inflammation in the lungs and the inflammatory response is necessary for viral control, lung viral titers were measured in the mice. By d 7 p.i., fish oil fed mice had a 7.1- fold increase in viral load versus controls (Fig. 2.4).

Figure 2.4 Lung influenza viral titers in control and fish oil fed mice during influenza infection. Data are expressed as means + SEM, n = 5-10 per group per time point. *P < 0.05, control diet versus fish oil diet.
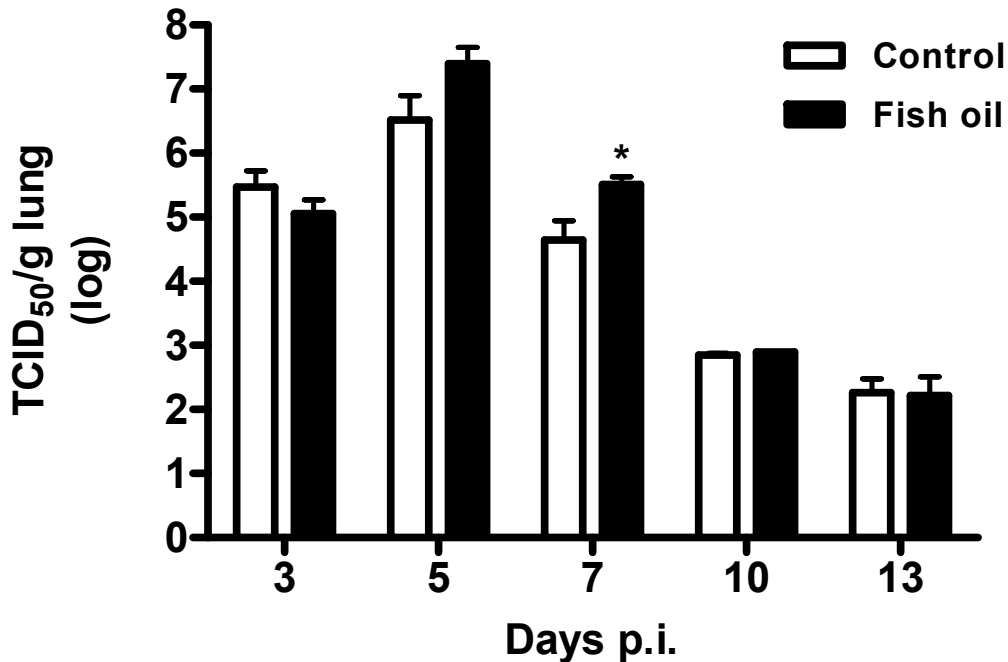
Fish oil feeding affects NK cell cellularity and cytotoxicity. NK cells represent the first line of defense post influenza infection (148). Previous studies have demonstrated reduced NK activity with fish oil feeding (105). At d 3 p.i., the splenic NK population was significantly reduced in the fish oil fed mice compared to controls (Fig. 2.5A). In addition, splenic NK activity was also reduced in the fish oil fed mice (Fig. 2.5B). In the lungs at d 3 p.i., the fish oil fed mice had less then half the total number of NK cells compared with control mice (Fig. 2.5C); however, lung NK activity did not differ between groups (Fig. 2.5D).

Figure 2.5 Effect of influenza infection and fish oil feeding on NK cells.
spleen NK cell number (A), spleen NK cell cytotoxicity (B), lung NK cell number (C), and lung NK cell cytotoxicity (D). Flow cytometry was used to identify NK cells. The lymphocyte population was gated and NK cells were identified as CD3⁻DX5⁺. NK cell activity was analyzed in a standard NK cell cytotoxicity assay. Values are means ± SEM, n = 6 per group. *P < 0.05, control diet versus fish oil diet.
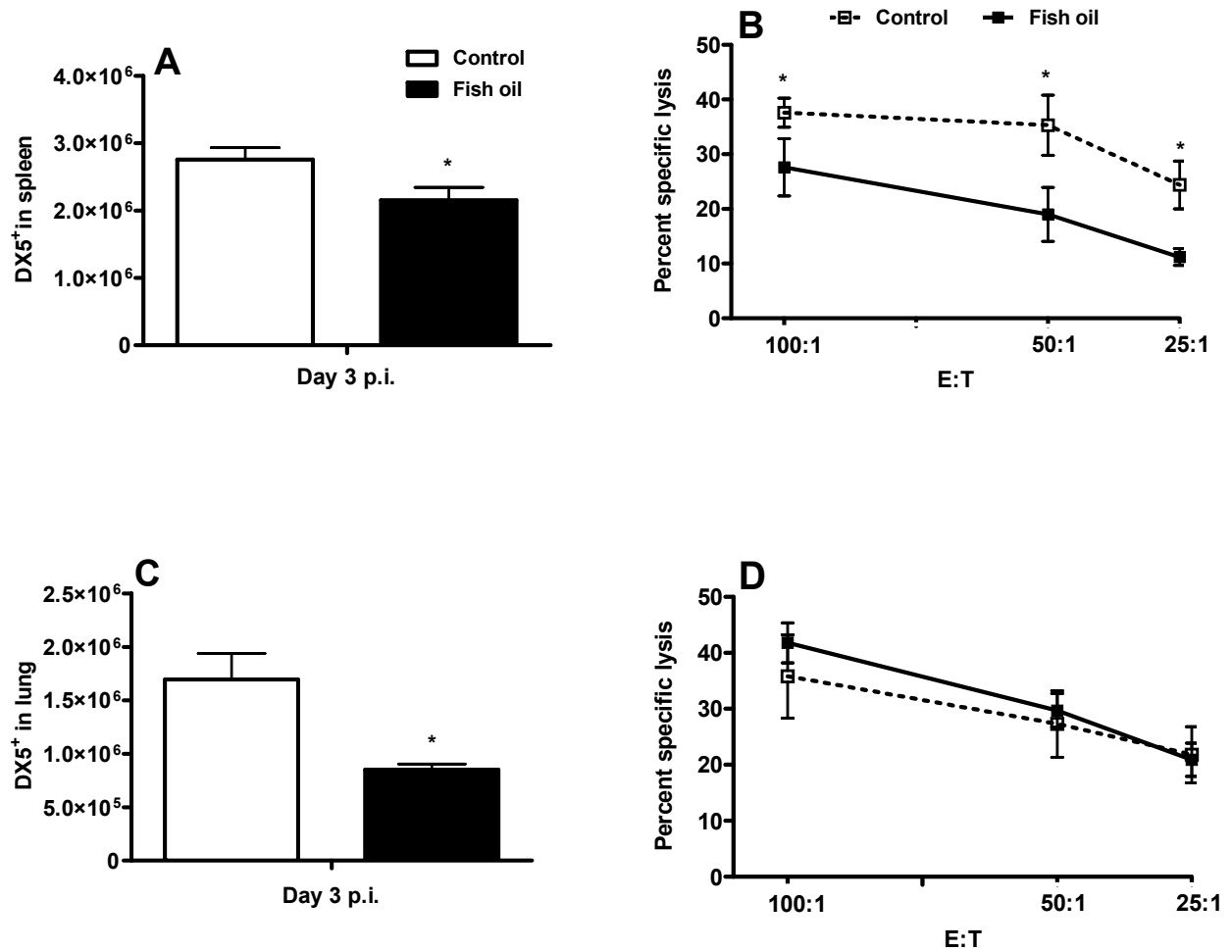
Fish oil feeding reduces neutrophils in lung of infected mice. Recruitment of

neutrophils to the site of infection is an essential early component of the immune

response to influenza infection (188). In the lungs of fish oil fed uninfected mice (d

0), there was a 71% reduction in total neutrophil number compared to control fed

mice (Fig. 2.6). Following infection, neutrophil numbers in the lung increased for both

control and fish oil fed mice; however, the total neutrophil number remained

significantly lower in the fish oil fed mice. Influenza infected control diet fed mice had

a significant 2.94-fold increase in neutrophils at d 3 p.i while fish oil fed mice had a

significant 3.7-fold increase in neutrophil post infection.

Figure 2.6 Effect of fish oil feeding on neutrophil infiltration of the lung.
Populations of neutrophils in lung were identified by flow cytometry based on CD11b$^+$ GR-1$^+$
expression. Values are means + SEM, n = 5-6. Means for a group without commune letter calculated
with logistic regression analysis, *P < 0.05.
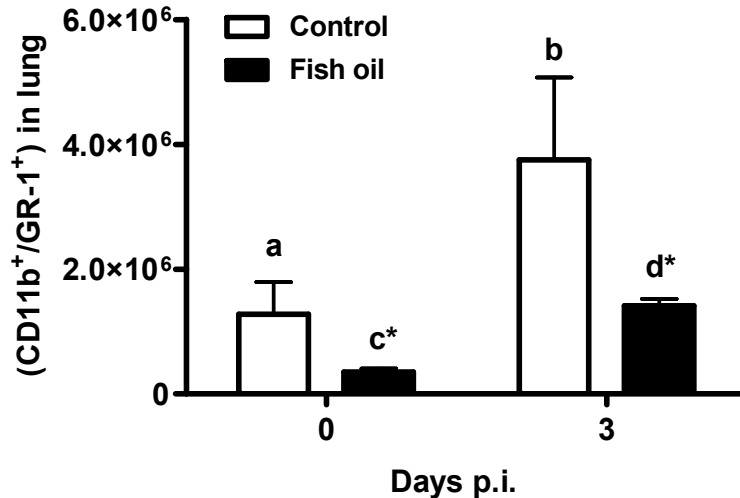
Fish oil feeding reduces CD8$^+$ trafficking to the infected lung. To determine if

fish oil feeding can decrease T cell trafficking to the lung, total numbers of T

lymphocytes (CD3$^+$) as well as CD4$^+$ and CD8$^+$ populations were identified in the

lung following influenza challenge. Although the CD3$^+$ T cell number peaked at d 10

p.i in both diet groups, fish oil fed mice had a significant decrease in CD3$^+$ T cells at

days 7 and 10 p.i. (Fig. 2.7A). This reduction in CD3$^+$ T lymphocytes was due to

decreased numbers of CD8$^+$ T cells (Fig. 2.7B), as the number of CD4$^+$ T cells was

53

not different at any time point (data not shown). By day 15 p.i., lymphocyte numbers

were not different between groups.

Figure 2.7 Effect of fish oil feeding on CD3^{+} and CD8^{+} T cell infiltration of the lung.
Lung cell population of CD3^{+} lymphocytes (A) and CD8^{+} cytotoxic T cells (B) in the lungs at days 7,
10 and 15p.i. Values are means + SEM, n = 5-6. *P < 0.05, control diet versus fish oil diet.
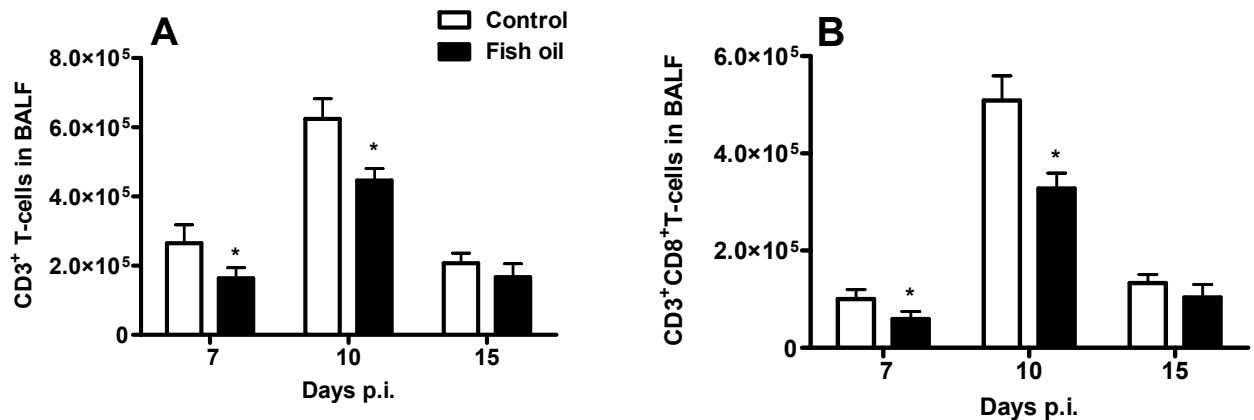
Fish oil feeding decreases cytokine and chemokine mRNA expression in lungs

of influenza infected mice. A coordinated production of cytokines and chemokines

is produced in the lungs during an influenza virus infection (180). In order to

determine if fish oil feeding could influence production of these inflammatory

mediators, mRNA levels were measured for various cytokines and chemokines. Fish

oil feeding resulted in significantly reduced mRNA expression of MIP-1α , TNF-α,

and IL-6 at d 7 p.i. compared to controls (Fig. 2.8 A,B,C). Levels of mRNA for IL-2,

IL-12, IFN-α, IFN-β and RANTES were not different between groups at any time

point post infection (data not presented).

Figure 2.8. Effect of fish oil feeding on mRNA levels of pro-inflammatory cytokines.
Lung mRNA levels for MIP-1α (A), TNF-α (B) and IL-6 (C). Values are normalized to G3PDH and are expressed as fold increase over uninfected mice of the matching diet group. Values are means + SEM, n = 4-6, per group per time-point. *P < 0.05, control diet versus fish oil diet.

Figure 8
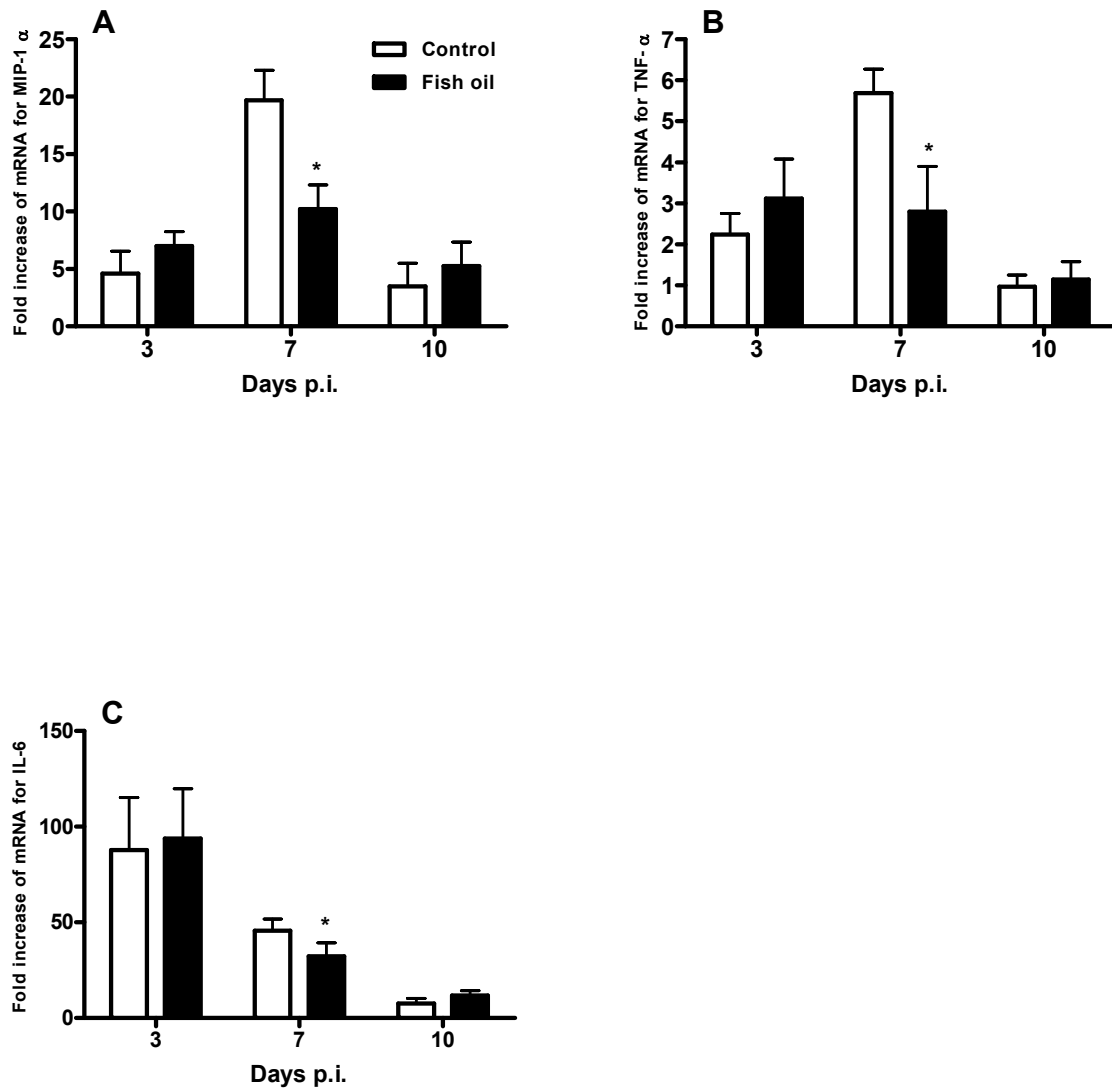
2.5 Discussion

The favorable effects of dietary fish oils stem from the potential of n-3 PUFA to reduce excessive inflammation (1, 174). Studies have shown beneficial anti-inflammatory properties of dietary fish oils on chronic diseases such as rheumatoid and osteoarthritis, inflammatory bowel disease, cardiovascular disease, type 2 diabetes and Alzheimer's disease (12). However, suppression of the immune system may be deleterious when inflammation is required to eliminate the invading pathogen, such as during influenza virus infection (2). Following infection with influenza, a controlled and coordinated immune response is essential for resolving the infection. The availability of n-3 PUFA-rich fish oil supplements and the increasing inclusion of n-3 PUFA in prepared foods (189) add to the importance of understanding how the immunosuppressive properties of fish oils may impact the host's ability to respond to influenza virus infection.

Mice fed a fish oil rich diet had decreased lung pathology following influenza infection compared to controls; however, the fish oil fed mice also had a prolonged recovery time, significantly increased viral titer and importantly, a significantly higher mortality rate. Thus, in the case of influenza virus infection, reduced lung inflammation was associated with a poor outcome post infection. In order to understand the direct impact of fish oil feeding on the immune response to influenza virus infection, numbers and phenotypes of cells infiltrating the lung tissue were measured. At d 3 p.i., mice had reduced numbers of NK cells infiltrating their lungs compared with control mice. NK cells provide an early defense against influenza virus infection and are important in reducing viral load prior to activation of the

adaptive immune response (148). NK cell trafficking to the site of infection is dependent on a variety of inflammatory mediators, including the expression of a cytokine/chemokine gradient, up-regulation of adhesion molecules and activation of G-protein-coupled receptors (190-192). n-3 PUFAs have been shown to decrease surface expression of vascular adhesion molecules and to alter G-protein-coupled membrane receptors (1, 193, 194) suggesting that fish oil feeding may have interfered with the signaling required for NK cell trafficking into the lungs.

Although the NK cell number was reduced in both the spleen and lungs of fish oil fed mice, impairment in NK activity was found only in splenic NK cells. The differences between spleen and lung NK activity may be due to exposure to cytokines. There are a number of cytokines that enhance NK cytotoxicity, including IL-12, IL-18 and IFN-α and β (148, 191), therefore NK activity in the lungs of fish oil fed mice may be increased due to exposure to the cytokine milieu at the site of infection. During influenza virus infection, production of inflammatory cytokines occurs at the site of infection and therefore the spleen is not exposed to this localized inflammatory response. Indeed, Yaqoob et al. demonstrated enhanced NK activity in PUFA treated NK cells following exposure to INF-γ (105).

 In addition to altered NK cell trafficking, fish oil feeding also affected trafficking of neutrophils to the lung following infection. During early virus induced inflammatory responses, neutrophils rapidly traffic to into infected airways where they play a critical role in limiting virus replication and activating innate immunity (188, 195). Neutrophil migration is controlled, in part, by the release of chemokines, cytokines and leukotrienes. MIP-1α has been shown to play a critical role in each

aspect of neutrophil trafficking, including rolling, stationary adhesion tissue recruitment in vivo (196, 197). In addition, studies by Ramos et al. demonstrated that ovalbumin (OVA) induced neutrophil migration in immunized mice was mediated by MIP-1α via the release of TNF-α and LTB$_4$ (198). Moreover, influenza infected MIP-1α knockout mice exhibited reduced lung inflammation and delayed viral clearance compared with infected wild type mice (199). In our study we show that fish oil fed mice lacked MIP-1α and TNF-α mRNA induction. Together, these data suggest that the failure to up-regulate MIP-1α and TNF-α mRNA in fish oil fed mice may have resulted in reduced neutrophil trafficking to the lungs following influenza infection.

On days 7 and 10 p.i., CD8^{+} T cells were decreased in the lung of influenza infected fish oil-fed mice. Influenza-specific CD8^{+} T cells kill infected target cells by direct lysis and also play an essential role in influenza virus clearance and controlling morbidity (200-202). In mice that lack CD8^{+} T cells, infection with influenza A/PR/8/34 led to increased viral replication and mortality (159). CD4^{+} T cells, on the other hand, help to resolve inflammation during influenza infection; however they are not essential for virus clearance (141, 203). Our data suggests that reduction in CD8^{+} T cell number post infection coupled with reduced neutrophil influx likely contributed to the increase in lung virus titer in the fish oil fed mice.

The recruitment of T cells is dependent on cytokine-induced expression of adhesion molecules. For example, TNF-α and IL-1β stimulate endothelial cells to increase expression of adhesion molecules, selectins and intergrins (204, 205). As expected with an influenza virus infection, cytokine mRNA expression of TNF-α peaked at d 7 in control fed mice; however, with fish oil feeding this induction did not

occur, suggesting that a lack TNF-α may have contributed to the decrease in CD8$^+$ T cell trafficking to the lung.

The migration of CD4$^+$ cells however, was not affected in fish oil fed influenza infected mice. Fish oil feeding may have altered pathways required for CD8$^+$ T cell and not CD4$^+$ T cell trafficking. For example, although both CD4$^+$ and CD8$^+$ T cells express CXCR3, administration of anti-CXCR3 antibody reduced CD4$^+$ T cell infiltrate in the brain, while CD8$^+$ trafficking was not affected (206). Similarly, CCR5 is also expressed on both CD4$^+$ and CD8$^+$ T cells, although only CD4$^+$ T cell trafficking was affected by lack of CCR5 expression in a mouse model of hepatitis virus (207). Interestingly, mRNA for MIP-1α, the ligand for CCR5, was underexpressed in the fish oil fed mice. While no studies have examined a direct effect of selective T cell recruitment with fish oil feeding during influenza infection, our results suggest that chemokines and perhaps their receptor expression may play a key role for the immunomodulatory effects of fish oil during influenza infection.

TNF-α is produced by infected lung epithelial cells, activated macrophages, dendritic cells, neutrophils, T cells (CD8$^+$ and CD4$^+$) and NK cells (29, 122). During viral infection, TNF-α exerts antiviral activity (124), enhances the recruitment of leukocytes to the site of infection and it activates innate immune responses (208, 209). IL-6 induction has pleiotropic effects, including the activation of NK cells and macrophages, and stimulation of T cell differentiation during influenza infection (129, 208). The potential for n-3 PUFA to reduce pro-inflammatory cytokines has been shown previously (210). For example, studies of fish oil fed mice, that have been infected with lipopolysaccharide had decreased the ex vivo production of TNF-α, IL-

1β and IL-6 by peritoneal macrophages and decreased TNF-α, IL-1β and IL-6 concentrations in circulation (133, 134, 211).

Together, these data suggest that the anti-inflammatory properties of fish oil that resulted in reduced neutrophils, NK cells and CD8$^+$ T cells in the lung and decreased expression of mRNA for pro-inflammatory cytokines, likely led to the increased viral titer and subsequent higher mortality rate in the fish oil fed mice. Further mechanistic studies are needed to determine how PUFAs can influence the immune response to influenza infection. For example, studies have shown that lipid raft disruptions by n-3 PUFAs can affect signaling pathways in T lymphocytes and disrupt immunological synapse formation needed to activate T cells (47, 212). However, potential adverse effects of these alterations in vivo during an influenza virus infection have not been investigated.

In this study, we utilized both a physiologically relevant concentration of dietary fish oil supplementation and a natural route of administration of a viral pathogen. Results from our study suggest that fish oil consumption has the potential to increase the severity of an influenza virus infection and perhaps other viral illnesses as well.

Chapter III

3   MODULATION OF THE INFLAMMATORY RESPONSE TO INFLUENZA VIRUS INFECTION BY N-3 POLYUNSATURATED FATTY ACIDS

Nicole MJ Schwerbrock, Whitney Franz, Vivian Ariail, Erik A Karlsson, Patricia A Sheridan, Qing Shi, Melinda A Beck

3.1   Abstract

Anti-inflammatory properties of n-3 polyunsaturated fatty acids (PUFA), including eicosapentaenoic acid (24) and docosahexaenoic acid (DHA), are well known, however less is understood about their role during viral infection. We have previously reported that feeding mice a 4% menhaden fish oil diet increases morbidity and mortality following influenza virus infection. To further characterize the effects of n-3 PUFAs on the response to viral infection, we utilized both a mouse model of influenza infection as well as primary human bronchioalveolar cells. We found that influenza virus infection induced a 6.3-fold increase in EPA content in the infected lung, whereas infection had little effect on DHA content. Although changes in fatty acid membrane composition can result in functional changes, the effectiveness of DCs from fish oil fed mice to stimulate T cells, or of pulmonary CD8^{+} T cells from fish oil fed mice to be activated by dendritic cells was not impaired. In response to viral infection, PUFA treated airway epithelial cells treated with DHA to EPA ratios of 25µM/25µM and 80µM/20µM significantly suppressed mRNA for type

1 interferons, TLR-3, IRF-3/7 and RIG-1 in addition to mRNA levels for pro-inflammatory cytokines IP-10 and IL-6. A DHA to EPA ratio of 20μM/80μM did not have this suppressive effect. Thus, n3-PUFA can substantially alter the response of primary epithelial cells towards a viral infection, with DHA being more effective than EPA.

3.2 Introduction

Polyunsaturated fatty acids (PUFA), particularly the n-3 PUFAs eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), are abundant in fish oils, and exert anti-inflammatory functions in cell culture and *in vivo* (1, 134, 178, 213). A number of studies have demonstrated the anti-inflammatory properties of fish oils, including reduced natural killer function, reduced production of pro-inflammatory cytokines and decreased ability of macrophages and dendritic cells to present antigen to T cells (6, 143, 176, 177).

Although the anti-inflammatory properties of PUFA have been shown to be beneficial for chronic inflammatory illnesses (12), these same anti-inflammatory properties can be detrimental for the response to acute infections when a pro-inflammatory state is necessary for pathogen eradication (2, 214, 215).

In our previous study, we demonstrated that feeding fish oil to mice increased their morbidity and mortality following infection with influenza virus (11). We found reduced NK and neutrophil infiltrate in the lungs of the infected fish oil fed mice, as well as a significant reduction in CD8+ T cells, which are required for influenza virus elimination.

Influenza viruses primarily target bronchial airway epithelial cells, in which they replicate and initiate innate immune responses to help limit the spread of the virus. Following infection, viral RNA is recognized by the pattern recognition toll like receptor (TLR)-3 and cytoplasmic retinoic acid-inducible gene (RIG)-1. Once activated, these host-virus interactions signal to trigger transcription factors needed to induce the production of type I interferon (IFNα/β) genes (216-218). Following infection, bronchial epithelial cells also produce various mediators that signal an inflammatory response including IL-6 (219), and interferon-induced protein-10 (IP-10) (220).

In addition to the innate response initiated by infection of the bronchoalveolar cells, the adaptive immune response begins within 7-10 days following infection. Dendritic cells take up and process viral antigen into peptide fragments which are presented on major histocompatibility I molecules to activate CD8+ T cells (149). PUFA treatment has been reported to interfere with the antigen presentation capability of dendritic cells (6)**.**

In order to further understand how fish oils can modulate the immune response to influenza virus infection, we utilized both an animal model as well as an *in vitro* primary cell culture model to determine the effects of PUFA on infected epithelial cells as well as investigating the ability of dendritic cells from PUFA fed mice to present influenza antigen to T cells.

3.3   Material and Methods

Animals studies

Diet: Six week old male C57BL/6J mice were obtained from Jackson Laboratories. All mice were housed at the University of North Carolina Animal Facility, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. Animals were maintained under protocols approved by the Institutional Animal Use and Care Committee. Mice were housed 4/cage with free access to food and water. Mice were fed a control semipurified diet containing 5% corn oil (D0792502, Research diets INC) or a fish oil-corn oil diet at physiologically achievable levels (1% CO + 4% FO, D0792503, Research diets INC) for 2 weeks.

Virus and infection: The mouse adapted strain of influenza A/Puerto Rico/8/34 (A/PR8) (American Type Culture Collection) was propagated in the allantoic fluid of 10 day old fertilized chicken eggs, and the viral titer was determined by hemagglutination assay (221). After 2 weeks on the diets, mice were anesthetized with an intraperitoneal injection of a ketamine (0.6 mg/kg)/xylazine (0.35 mg/kg) solution and infected intranasally with 0.05 mL of 2 hemagglutinating units (HAU) of A/PR8 virus diluted in PBS. Previous studies from our laboratory determined that this dose of virus is sufficient to effectively elicit an immune response while causing little mortality in infected control mice (222).

Extraction and measurement of fatty acids. Lipids were extracted from the lungs of non-infected and infected (day 7 post infection) mice using the method of Bligh and Dyer (223). An antioxidant (0.01%, w/v, BHT) was added to all solvents. Residual lipids were saponified (224). Resulting fatty acid methyl esters were analyzed using capillary gas chromatography (Perkin Elmer AutoSystem XL Gas Chromatograph, Shelton, CT). Heptadecanoic acid (17:0) was used as an internal

standard. Individual fatty acids were identified by comparing their retention against

authentic standards (Nu Chek Prep, Elysian, MN). Data were analyzed with the

Perkin Elmer Totalchrom Chromatography Software, version 6.2. The obtained

results were provided as % FA of total tissue sample.

Antigen presentation assay. Dendritic cells (DC) were isolated from spleens of

control diet and fish oil diet uninfected mice (n=10 per group) and processed into

single-cell suspensions by mechanical agitation as described previously (225). Cells

were counted and 1×10^8 cells were used to isolate DCs using the DC enrichment

kit from Dynal Biotech (Carlsbad, CA). This population was shown to be greater than

92% pure by FACS analysis. T cells were isolated from spleens of control diet fed

mice (n=8) that had been infected with influenza 7 days previously. Cells were

counted and 1×10^8 cells were used to isolate T cells using the T cell enrichment kit

from Dynal Biotech (Carlsbad, CA). DCs were incubated at a multiplicity of infection

(MOI) of 2 with 56°C heat-inactivated influenza A/PR8 for 2 hr followed by extensive

washing to remove any excess virus.

DCs were incubated with T cells in an 1:2 ratio with 1×10^5 DC in a 96-well round-

bottom microplate. Samples were incubated for 2 hr at 37°, followed by the addition

of Golgi Plug (BD Biosciences) and incubation for an additional 4 hr. Prior to surface

staining, Fc receptors were blocked with anti-FcγII/III. Cells were stained with DC

marker: FITC-anti-CD11b, PE-anti-MHCII, APC-anti-CD11c, PeCy5- anti- CD86 or

FITC-anti-CD80, PE-anti-11b, APC-anti-CD11c, PeCy7- anti- CD45R.

T cell function. DC from control fed mice were processed as described above. T

cells from fish oil fed mice infected with influenza 7 days earlier were isolated as

described and cultured with DC at a DC/T cell ratio of 1:5. After 2 hours of incubation, cultures were processed as described above and stained with: FITC-anti-CD3, PE-anti-INF-γ, APC-anti-CD4, PeCP - anti- CY5.5 Cy5, PE- anti-gramzyme B (BD Biosciences), followed by fixation and permeabilization for subsequent intracellular staining with PE-anti-IFN-γ. An irrelevant PE-IgG (Sigma) was used as a staining control.

Preparation of PUFA media for cell culture: DHA and EPA were supplied in ethanol solution (Cayman Chemical) and dried under N_2 gas to minimize oxidation. All fatty acids were prepared as 50mM stock solutions in 35% BSA and incubated for 1 h at at 37°C. Stock BSA-fatty acid complexes were added to growth media at final DHA to EPA growth media concentrations of 25μM/25μM; 20μM/80μM and 80μM /20μM. Media were prepared freshly for each experiment.

Primary human bronchial epithelial cells were obtained from healthy nonsmoking adult volunteers by cytologic brushing at bronchoscopy and plated as described previously (226). Upon confluence, PUFA were added to the bronchial epithelial growth media (Cambrex Bioscience Walkersville, Inc. Walkersville, MD) under air liquid interface (ALI) culture conditions (removal of the apical medium).

Cell culture infection with influenza. Influenza A/Bangkok/1/79 (H3N2 serotype) was propagated in 10-day-old embryonated hen's eggs. The virus was collected in the allantoic fluid and titered by 50% tissue culture infectious dose in Madin-Darby canine kidney cells and hemagglutination as described before (227). For infection of differentiated bronchial epithelial cells (ALI) approximately 3×10^5 cells were incubated with 320 HAU of influenza A Bangkok 1/79 on the apical side for 1 h, after

which the remaining unattached virus was removed. This infection protocol results in a mild infection in which about 10–20% of the cells become infected with influenza. Following infection, apical cell surface was washed with DMEM/2% fetal calf serum and control or PUFA bronchial epithelial growth media was added. Samples were harvested at 8 hours and stored at - 70 °C for further analysis.

Determination of mRNA levels. At 8 hours post infection, total RNA was isolated from the ALI cells using TRIzol and reverse transcription reaction was carried out with the RNeasy Mini Kit (Qiagen). Using quantitative real time polymerase chain reaction (RT-PCR), mRNA levels were measured for human β-actin, interferon-inducible protein 10 (IP-10), interleukin 6 (IL-6), interferon regulatory factor (IRF)-3 and IRF-7, toll like receptor-3 (TLR-3), and retinoic acid-inducible gene I (RIG-I). All measured gene expression was normalized with respect to the reference gene of β-actin, which is ubiquitously expressed in eukaryotic non-muscle cells. Resulting data were expressed as percent reduction compared with infected non-PUFA treated cells.

Statistical analysis. Statistical analyses were performed using JMP Statistical Software (SAS Insititute). Student's t test was used for post-hoc comparison between the dietary groups. Nonparametric data were analyzed using Kruskal Wallis test.

3.4   Results

Infection of fish oil fed mice with influenza virus alters lung fatty acid composition. The lung is the site of infection for influenza virus. We tested whether infection itself could alter the fatty acid profile of lung cells. As shown in figure 1, as

expected, fish oil feeding alone increased both DHA and EPA percentages in lung

tissue of uninfected mice. Interestingly, infection with influenza could also modify

PUFA incorporation into lipid membranes. As shown in Figure 3.1A, infection with

influenza caused a 6.3-fold increase in EPA concentration. DHA concentrations did

not change with infection (Fig 3.1B). In control fed mice, EPA and DHA levels did not

change with infection. Arachidonic acid (AA) concentration increased 42 % with

infection in the PUFA fed mice, although this percentage was still below the level

seen in control fed.

Figure 3.1 Effects of influenza infection and fish oil feeding on percent fatty acids in the lung. Lipids were extracted using the method of Bligh and Dyer (223) and resulting fatty acid methyl esters were analyzed using capillary gas chromatography. Percent total (A) EPA levels, (B) DHA and (C) AA. Values are expressed as means ± SD, n = 4-6 per group per time-point. *$P < 0.05$.
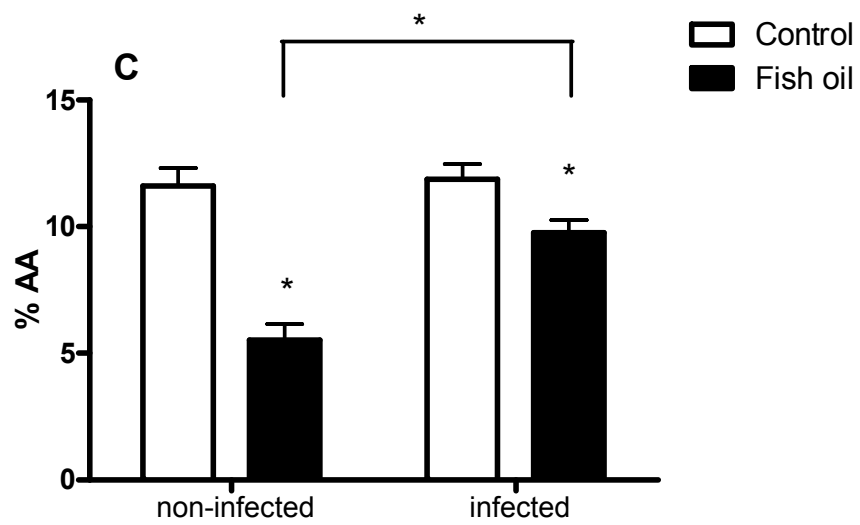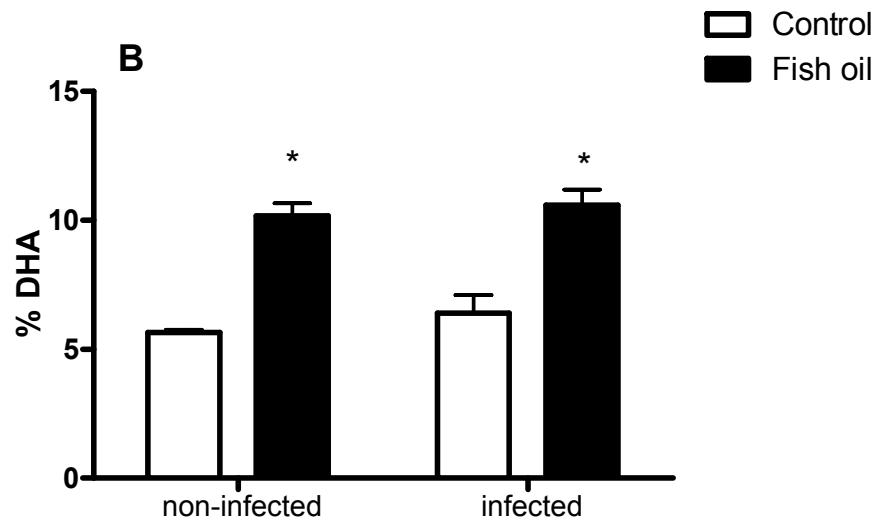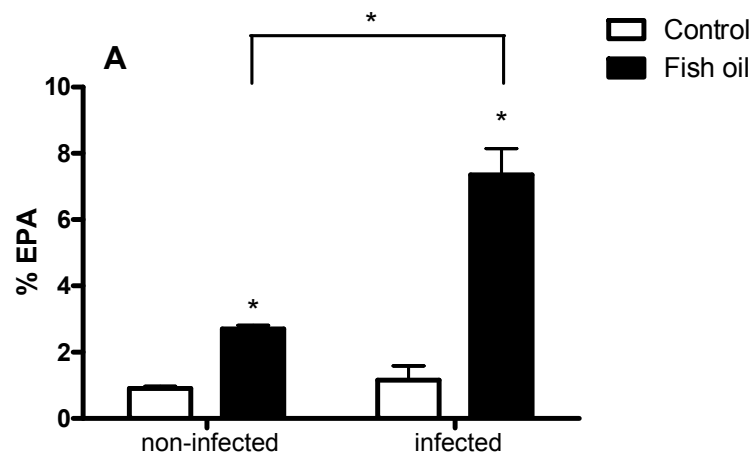
Fish oil feeding does not alter antigen presentation. Previous studies have demonstrated that fish oil feeding can alter the ability of dendritic cells to function properly (45, 228). To examine this possibility in our influenza-infected fish oil fed mice, we tested the ability of dendritic cells from PUFA fed mice to activate CD8+ and CD4+ T cells to produce IFN-γ. We found that influenza-pulsed DCs from spleens of control and fish oil fed mice effectively induced CD8+ (Fig 3. 2A) and CD4+ (Fig 3. 2B) T cells from control fed mice to produce IFN-γ. In addition, we tested the ability of dendritic cells from control fed mice to activate CD8+ T cells from PUFA fed and control fed mice. Similar to our results above, we found no differences in the ability of CD8+ T cells from PUFA fed mice compared to control mice to produce IFN-γ following dendritic cell stimulation (data not shown).

Figure 3.2 Fish oil feeding does not alter the ability of dendritic cells from PUFA fed mice to activate (A) CD8+ and (B) CD4+ T cells.
An antigen presentation assay was performed using T cells isolated from spleens of influenza-infected control diet fed mice at day 7 p.i. The T cells were incubated with influenza-pulsed DCs isolated from spleens of uninfected control and fish oil fed mice for 4hr. The percentage of interferon (IFN)-γ^+CD8$^+$ and (IFN)-γ^+CD4$^+$ T cells in the CD3$^+$ lymphocyte population was determined by intracellular cytokine staining and flow cytometry. Data are expressed as the mean ±SEM and are representative of two separate experiments (n= 8 pooled mice per group per experiment). Values presented have been corrected for the per cent non-specific immunoglobulin G (IgG) staining
*P $\leq$ 0·05.

A

B
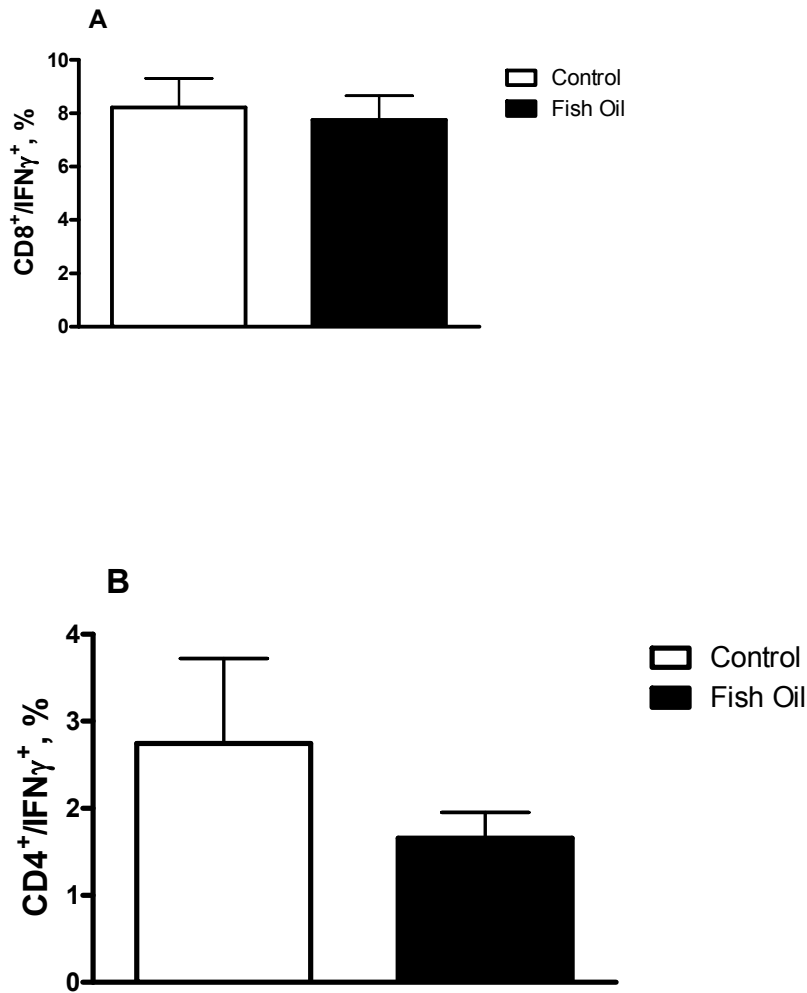
n-3 PUFA treatment of human bronchioalveolar cells decreases antiviral
responses to influenza virus. To test the ability of PUFA treatment to modulate
bronchial epithelial cell response to influenza infection, we treated primary
bronchoalveolar air liquid interphase (ALI) cells with 3 different ratios of DHA to EPA:
25µM/25µM; 20µM/80µM and 80µM /20µM. As shown in Figure 3. 3, mRNA levels
for TLR3 (Fig 3. 3A), RIG1(Fig 3. 3B), IRF-3(Fig 3. 3C), IRF-7 (Fig 3. 3D), IP-10 (Fig
3. 3E), and IL-6 (Fig 3. 3F), 8 hours post infection were all significantly reduced in

the 25μM/25μM and 80μM /20μM ratios compared with infected cells not treated with

PUFAs. Interestingly, the 20μM/80μM ratio had no effect on the mRNA levels.

Figure 3.3 Influenza infected human bronchoalveolar cells exposed to varying concentrations of DHA and EPA have altered mRNA expression.
Cells were treated for 48 hours prior to influenza virus infection. mRNA levels were determined 8 hours post infection for (A) TLR-3, (B) RIG-1 (C) IRF-7, (D) IRF-3 (E) IP-10 (F) IL-6 are shown. Uninfected n-3 PUFA treated cells and non PUFA treated control cells did not express mRNA levels for any antiviral and pro-inflammatory cytokine measured. Values are means + SD, $n = 2$.

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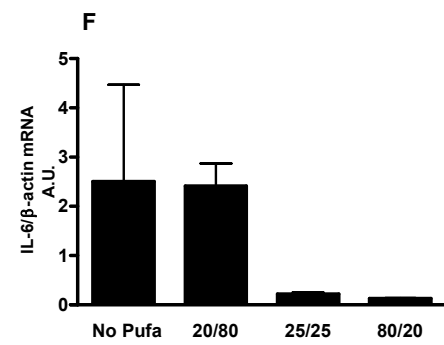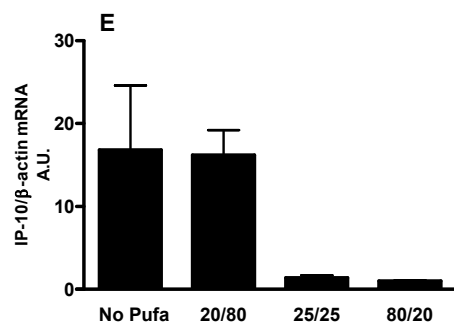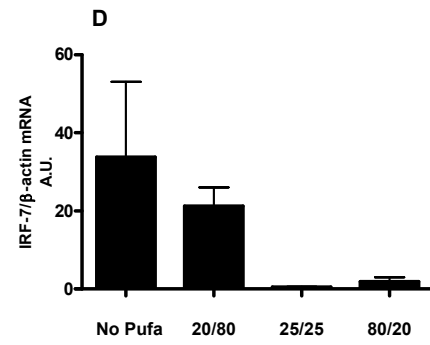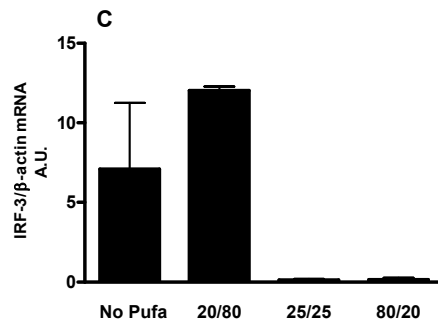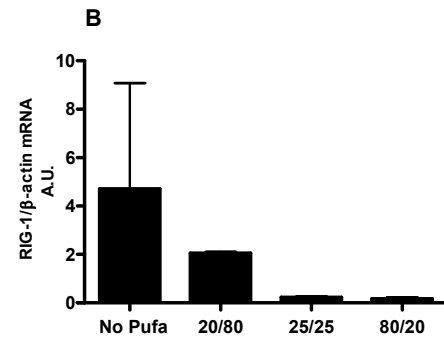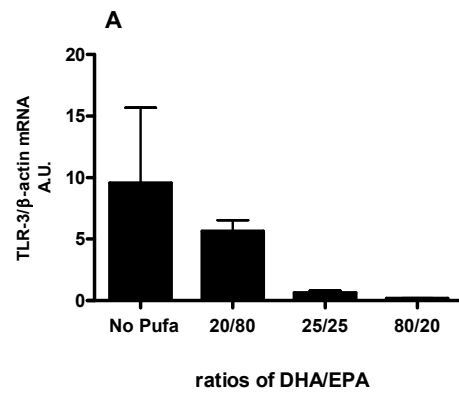
3.5 Discussion

The anti-inflammatory properties of n-3 PUFA have been described in many systems (174, 228). However, during infection, reduced inflammation could actually harm the host. Indeed, our previous study demonstrated that fish oil feeding increased morbidity and mortality of influenza-infected mice (214). To further characterize the effect of PUFA on the response to viral infection, we utilized both a mouse model of influenza infection as well as primary human bronchoalveolar cells.

Fish oil feeding has been shown previously to modify lipid membrane composition (46, 229, 230). However, it is not known if infection can modify membrane content of EPA and DHA. For the first time, we demonstrated that influenza virus infection can alter the lipid membrane profile of bronchoalveolar cells, inducing a significant increase in EPA and AA content in the infected lung. However, infection had little effect on DHA content. Interestingly, the DHA and EPA content of control fed mice were not altered during infection.

DHA is the most abundant n-3 PUFA in most tissues and its turnover is relatively low (231, 232). EPA exerts its anti-inflammatory function by serving as a competitive precursor for eicosanoids synthesis (13, 62). It is therefore possible that the increase in EPA during infection is a response of the cells need to control the inflammatory response induced by a viral infection (13, 62). The increase in AA levels, on the other hand, may reflect induction of pro-inflammatory prostaglandin synthesis which is needed to induce the inflammatory responses (233). However, the increased level of EPA in the infected fish oil fed mice led to an over-reduction in inflammation compared with control fed mice, resulting in less inflammation along

with increased viral titers (11). In contrast, control diet fed mice showed no alteration in the n-3 fatty acid profile, suggesting that increased incorporation of EPA into cell membranes during infection only occurs when n-3 PUFA is in excess.

During infection with influenza, DCs provide a critical role in activating T cells necessary for fighting the infection. Given that n-3 PUFAs have been reported to inhibit the antigen-presentation function of KLH–sensitized rat splenocytes and dendritic cells (45, 53, 228) in addition to decreased IFN-γ production during viral infection (37), we were surprised to find no functional changes in regard to effectiveness of DCs from fish oil fed mice to stimulate T cells, or of pulmonary CD8^{+} T cells from fish oil fed mice to be activated by dendritic cells from control fed mice. There are several explanations for our findings. DHA is considered to be more effective at altering cell membrane functions such as membrane fluidity and immunological synapse formation (234, 235). Because DHA levels were not altered during infection, perhaps a threshold amount was not reached to induce these changes. Many studies that used fish oil diets had a much higher amount of fish oil compared to our study diet (109, 120, 236, 237). In our study, we utilized a 4% menhaden fish oil diet that corresponds to a blood level of ~68μM DHA and ~ 28μM EPA. Previous studies that have demonstrated effects of fish oil on antigen presentation and/or T cell function used much higher levels of fish oil from 25-35% (26,27,33) Studies that used 20% or more fish oil feeding demonstrated decreased cytotoxicity of peritoneal exudate cells against P51 tumor cells (238) and decreased proliferation of Con A-stimulated spleen lymphocytes (236). In addition, our study

analyzed an antigen-driven response, rather than a mitogen response, which is more common in fish oil studies (109, 120, 236, 237).

Airway epithelial cells are the primary targets for influenza virus (180). In response to RNA virus infection, bronchoalveolar cells activate TLR-3 and RIG-1 (239) signaling, which ultimately results in the production of pro-inflammatory cytokines and type I interferons (16, 42). Additional activation of interferon regulatory factors by viral infection also enhances the transcription of type I IFN genes resulting in an amplified anti-viral response (240, 241). We found that specific n-3 PUFA ratios had a suppressive effect on mRNA expression for TLR-3, IRF-3 and 7 and RIG-1. PUFA treatment of ALI cells at DHA to EPA ratios of 25μM/25μM and 80μM/20μM significantly suppressed TLR-3, IRF-3 and 7 and RIG-1 mRNA levels. In addition, mRNAs for two pro-inflammatory cytokines, IP-10 and IL-6 were also suppressed by these PUFAs ratios. These data suggest that PUFA treatment suppressed the induction of antiviral and pro-inflammatory cytokines following influenza infection. This is the first study that demonstrates physiologically relevant levels of EPA and DHA can inhibit the influenza anti-viral response of primary bronchial epithelial cells.

Related studies have investigated the effects of individual n-3 fatty acids on the production of pro-inflammatory cytokine synthesis. For example Saedisomeolia *et al.* (242) reported that DHA at high concentrations (400μM) resulted in a significant reduction in pro-inflammatory cytokines in rhinovirus infected Calu-3 airway epithelial cells, whereas EPA at high (400μM) and DHA at lower concentrations (10-200μM) had no effect on cytokine production (242). Similarly,

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Bryan *et al.* (233) reported that supplementation of A549 cells with DHA at 50µg/ml (~200µM) resulted in a significant reduction of the chemokine regulated upon activation normal T cell expressed and secreted (RANTES) following histamine stimulation, whereas concentrations of EPA (high and low) and DHA of 10µg/ml (~40µM) did not elicit any suppression of RANTES or other pro-inflammatory cytokines measured. These studies all used high levels of EPA and DHA in transformed cell lines. It may be that much lower levels of DHA and EPA are effective in primary cell cultures.

DHA has been suggested to significantly alter basic properties of cell membranes, including acyl chain order and fluidity, phase behavior, fusion, rapid flip-flop of proteins in the cell membrane and resident protein function (7, 243). It is therefore plausible that DHA related lipid membrane modulations may have disrupted antiviral and pro-inflammatory cytokine signaling pathways. Lee *et al.* (244) used an LPS-stimulated macrophage culture model to show that TLR-4 and its inflammatory downstream cascade are affected by DHA, although membrane integrity was not determined. Thus, it is possible that DHA specifically modulated membrane receptor associated signaling events whereas EPA compromises anti-inflammatory effects by different mechanisms.

In our previous study, we found that fish oil feeding decreases immune cell trafficking in the lungs of infected fish oil fed mice (214). In this study we demonstrated that although dendritic and CD8+ T cell function was not affected by fish oil feeding, PUFA treatment inhibited influenza anti-viral responses of primary bronchial epithelial cells. Taken together, our data suggest that the fish oil related

decrease in inflammatory cell recruitment to the lungs of influenza infected mice (11) may have been a result of suppressed antiviral and pro-inflammatory cytokine induction in the bronchial epithelial cells following infection.

Further mechanistic studies are needed to determine how DHA and EPA could have differential effects on the immune response to infectious disease. For example, EPA has been shown to control the activity of transcription factors PPAR α/γ and nuclear factor-κB, (NF-κB), and depending on its concentration can induce anti-inflammatory effects (1, 245-247).

Because of the reported health benefits of fish oil, fish oil supplementation has increased in this country (27, 181). Given that fish oil supplementations have differing ratios of DHA and EPA (248), and that DHA and EPA have differing effects on different cell populations, there may be public health implications of fish oil supplementation during an infection. Further mechanistic investigations in n-3 PUFA effects on the response to viral disease is warranted.

Chapter IV

4 DIETARY FISH OILS MODULATE IMMUNITY TO INFLUENZA VIRUS INFECTION: A SYNTHESIS

The immunosuppressive properties of dietary *omega* -3 polyunsaturated fatty acids (PUFA) have been demonstrated to be beneficial in chronic inflammatory and autoimmune disorders (4, 5). However, in our studies we showed that these same anti-inflammatory properties can be detrimental by reducing the immune response to a viral infection when a fully intact immune system is essential for eradicating an invading pathogen. Intakes of *omega* -3, therefore, contribute to both beneficial and adverse host outcomes.

Infection with influenza virus is very common. On average, in any given year 5% to 20% of the population in the United States becomes infected, and during a pandemic, 20-40% of the world's population might be infected (34). Despite the availability of vaccines and antiviral agents, influenza virus continues to be a major cause of morbidity and mortality worldwide. Following infection, immune regulation is necessary for both clearance of the virus and host protection.

Given that fish oils have the ability to suppress immune functions; dietary fish oils have been hypothesized to reduce host resistance against influenza virus infection. The concept that suppression of inflammation can be associated with increased severity of infectious diseases was initiated by epidemiologists (249).

These investigators reported a lower incidence of autoimmune diseases in Greenland Eskimos, a population that traditionally consumes a diet rich in *omega*-3 PUFA from fish oils. However, these same populations presented grater incidence of infectious diseases, such as tuberculosis and recurring respiratory tract infections, than in populations on much lower intakes of fish (249).

Chapter 2 of this dissertation demonstrated that in the case of influenza virus infection in mice, the anti-inflammatory properties of fish oil were associated with a poor outcome post-infection. Results presented in Chapter 3 demonstrated that although dendritic and CD8+ T cell function was not affected by fish oil feeding, PUFA treatment inhibited influenza anti-viral responses of primary bronchial epithelial cells. Taken together, our data suggests that the fish oil related decrease in inflammatory cell recruitment to the lungs of influenza infected mice (Chapter 2) may have been a result of suppressed antiviral and pro-inflammatory cytokine induction in the bronchial epithelial cells following infection.

These results presented in those two chapters need to be considered in greater depth as to the mechanisms by which dietary fish oils can potentially increase susceptibility to viral infection, in general. Better understanding of how PUFAs modulate host resistance to infectious agents will allow us to devise better dietary strategies using *omega*-3 fatty acids for both the prevention and treatment of illnesses. Additional experimental designs using other animal models and human subjects are also needed to corroborate and support our current findings.

Specific topics investigated in our studies are covered next.

4.1 Impaired resistance to influenza infection in fish oil fed mice: Summary of results from chapter 2

Following influenza infection the immune system protects the host with coordinated responses against the virus. Disruption of such reactions may be detrimental. In our studies, we tested the hypothesis of whether the immunosuppressive properties of fish oil feeding could hinder immunity to influenza and found that fish oil feeding led to increased mortality in mice infected with influenza virus.

Despite a significant reduction of lung inflammation, fish oil fed mice had a 4-fold increase in mortality and surviving mice had prolonged recovery period following infection. The rapid recruitment of inflammatory cells to the site of infection is essential to eliminate the invading virus but these cells also contribute to additional tissue pathology. Since lung inflammation was greatly reduced, this decrease suggested that immune cell trafficking into the lung were absent in fish oil fed mice.

We initiated our studies by analyzing cells of innate immunity, i.e. NK and neutrophils and others. These present the first line of defense to viral infection, but they also stimulate the recruitment and activation of cell-mediated immunity that helps limit viral spread. We found that fish oil feeding resulted in lower numbers of neutrophils and NK cells in infected lungs, indicating that the recruitment and trafficking of these cells may not adequately occur in fish oil fed mice. Functionally,

NK cells retained their cytotoxicity in infected lungs, whereas in the spleen NK cytotoxicity was decreased, suggesting that fish oil feeding affects NK mediated target cell lysis differently depending on the type of infection and the resulting cytokine milieu present. Furthermore, cytotoxic CD8$^+$ T cells numbers were decreased with fish oil feeding in infected lungs, while CD4$^+$ T cells numbers did not change, indicating that fish oil feeding may have altered pathways required for CD8$^+$ T cell but not CD4$^+$ T cell trafficking. Thus, the increase in lung virus titer in the fish oil fed mice likely was attributed to the reduction in CD8$^+$ T cell number post-infection coupled with a reduction in the influx of immune cells during the early stage of infection.

In this study we also found that the induction of TNF-α, MIP-1α, and IL-6 mRNA was suppressed with fish oil feeding. Since these cytokines are primarily involved the expression of adhesion molecules, neutrophil trafficking (196), and activation of immune cells (208) the down-regulation indicates that the impaired recruitment of inflammatory cells to the site of infection may have been dependent on the cytokine response in infected lungs (Chapter 2).

These data demonstrate, for the first time, that fish oil feeding, at physiologically relevant levels, resulted in increased mortality in flu-infected mice. We showed that fish oil feeding restrained immune cell trafficking in conjunction with higher virus titer in influenza virus infected lungs. Thus, at least in the case of influenza virus infection, the immunosuppressive properties of dietary fish oils were

associated with poor outcomes post-infection, both increased death and slow recovery among survivors.

Molecular mechanisms by which *omega*-3 fatty acids modulate immune function include inhibition of the production of cytokines, membrane modulation, alternation of antigen presentation and enhancing eicosanoid production. Studies in chapter 2 focused on how the anti-inflammatory properties of fish oils can affect responses to viral infection. Specifically, focus was placed on changes in the innate immune components and their attempt to eliminate the invading virus. In chapter 3 we selectively determined if n3-PUFA treatment affects immune responses of primary epithelial cells towards viral infection.

4.2   Impaired virus release in omega-3 treated primary cultured human bronchial epithelial cells: Summary of results from chapter 3

In the previous study we found that fish oil feeding led to reduced CD8$^+$ T cells in lungs of infected mice. Since CD8$^+$ T cells play a critical role in actively killing virally infected cells, we analyzed their functional capacity. As reported in chapter 3, we found that CD8$^+$ T cells obtained from infected lungs of both diet groups were equally effective in responding to antigens presented to them.

Moreover, spleen dendritic cells play an important role in activating T lymphocytes (250). Because diminished antigen presentation of dendritic cells had been reported in rats fed high fish oil diets (45), we examined dendritic cell functional

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capacity and the subsequent T cell response in ex vivo splenocytes (Chapter 3). We found that splenic CD4$^+$ T cells from fish oil fed mice responded less strong to antigen presentation than control cells, although the difference from control fed mice was not significant. It is, therefore, possible that the newly discovered modulations of antigen presentation by n-3 PUFA treated T cells are dependent on differing diet treatment or experimental model used.

Additionally, fish oil feeding had been shown previously to modify lipid membrane composition. However, it was not known if infection can modify membrane content of EPA and DHA. For the first time, we demonstrated in chapter 3 that influenza virus infection can alter the lipid membrane profile of bronchoalveolar cells, inducing a significant increase in EPA and AA content in the infected lung. However, infection had little effect on DHA content. Interestingly, the DHA and EPA content of control fed mice were not altered during infection. These data suggested that diverse PUFAs are differently affected with infection.

Thus, we now had shown *in vivo* that dietary fish oils altered membrane lipid composition and decreased inflammatory cell trafficking and this decrease in recruitment was most likely due to a suppressed pro-inflammatory cytokine induction. Because the lung airway cells are the primary targets for influenza infection, we next determine if n-3 PUFA treatment can alter immune responses to influenza virus infection, *in vitro.*

In response to viral RNA infection, bronchoalveolar cells activate various signaling pathways which ultimately results in the production of pro-inflammatory cytokines and type I interferons. Additional activation of interferon regulatory factors by viral infection also enhances the transcription of type I IFN genes resulting in an amplified anti-viral response. We found that specifically DHA to EPA ratios of 25µM/25µM and 80µM/20µM suppressed the expression of pro-inflammatory signaling pathway intermediates. However, this was not the case in DHA to EPA ratios of 25µM/25µM. Our data suggested both to us, PUFA treatment, at physiologically relevant levels, suppressed the induction of antiviral and pro-inflammatory cytokines and that that DHA and EPA had differential effects on the immune response to influenza virus infection.

This dissertation studies demonstrate that n-3 PUFAs administered at physiological levels, significantly altered host immunity to influenza virus.
For the first time we have shown that influenza infection in fish oil fed mice lead to increase mortality and altered n-3PUFA membrane profiles. Thus, at least in the case of influenza virus infection, the immunosuppressive properties of dietary fish oils were associated with poor outcomes post-infection, both increased death and slow recovery among survivors.

4.3 Assessing alternate possibilities for decreased survival of influenza infection in fish oil fed mice.

Epithelial cells of the lung are central to respiratory gas exchange. Loss of functionality can lead to death. Since the ability of n-3 PUFAs to arrest cell growth and induce apoptosis has been reported previously (3, 251, 252), we determined if an increase in apoptotic lung cells could be accountable for the 4-fold increase in mortality illustrated in chapter 3. Using TUNEL staining to identify apoptotic cells, we found a direct correlation between levels of inflammation and apoptosis. Thus, fish oil fed mice had fewer apoptotic cells (and reduced inflammation) compared with control mice which had more apoptotic cells (and increased inflammation). Thus, the fish oil fed mice did not die from in increase in lung cell death.

We next evaluated if the cause of higher mortality in fish oil fed mice, may have been due to the virus infecting the brain and the resulting encephalopathies led to death (253, 254). Of the three regions examined for the presence of virus (by measuring influenza matrix RNA) olfactory bulb, hypothalamus and brain stem, we found virus predominately in the olfactory bulb, however, there were no significant differences between both diet groups in regard to viral RNA expression (data not shown). These data indicated that virus spread into the brain was not a cause for the decrease survival seen with fish oil feeding.

Lastly, we examined mouse liver and kidney function, since PUFAs have been linked to affect these organs in influenza associated Reye's syndrome (255, 256). We found that following influenza infection, alkaline phosphatase (liver function) was not affected by fish oil feeding (data not shown). Blood urea nitrogen

(kidney function test) levels on the other hand were decreased; indication that the general cacaxiatic state of fish oil feeding may have lead to dehydration of infected mice.

4.4   Potential further directions of investigation of n-3 PUFAs in immunity

Our study sets the stage for further investigations of how anti-inflammatory properties of nutrients can affect host immune function. Further mechanistic studies are needed to determine how PUFAs can influence the immune response to influenza infection.

4.5   Future directions

4.5.1   Immune cell trafficking

The migration of immune cells to the site of infection is dependent on the production of cytokines that stimulate endothelial cells to rapidly express adhesion molecules. Circulating phagocytic cells express surface receptors that bind to adhesion molecules. Given that fact that n-3 PUFAs decrease production of inflammatory cytokines and decrease adhesion molecule expression, suggests that the effects we see on decreased immune cell trafficking into the infected lungs may be mediated through PUFAs potential in down regulating adhesion molecule expression. The studies in chapter 2 clearly illustrate that alteration in immune cell recruitment may have been in part regulated by the cytokine/chemokine gradient. Therefore determining, the mediators of cell trafficking would provide a mechanism by which fish oil feeding leads to immune cell trafficking dysregulation.

4.5.2 Eicosanoid production and lymphocyte proliferation.

Although our findings in chapter 3 showed that antigen presentation was not affected by fish oil feeding, we found CD8$^+$ T-cells to be decreased with fish oil feeding. Studies have suggested that the link between fatty acid and lymphocyte function is the production of eicosanoids (60). Since n-3 fatty acids antagonize the production of inflammatory eicosanoid mediators from arachidonic acid, there is reason to hypothesize that during influenza infection, the production of prostaglandins from PUFAs is essential. For example, studies have demonstrated that the deficiency of COX-1 and COX-2 leads to contrasting effects in the host response to influenza infection, and these differences are associated with altered production of prostaglandins and leukotrienes following infection. COX-1 deficiency is detrimental whereas COX-2 deficiency is beneficial to the host during influenza viral infection (257). Thus, determining how fish oil feeding affects prostaglandin production in response to influenza infection, may determine if impaired resistance to influenza infection in fish oil fed mice is eicosanoid mediated.

4.5.3 Assessing viral and host factors involved in viral morphogenesis and budding.

In chapter 3 we demonstrated in cell culture experiments that DHA and EPA treatment resulted in dysregulated immune responses to influenza virus. For influenza virus to bud efficiently, viral hemagglutinin (HA) glycoprotein has to concentrate at membrane lipid aggregates (87, 102). Because PUFA affects membrane compositions it is hypothesized that viral release from the infected cell may be impaired. Thus it will be important to determine if n3-PUFA treatment can

alter viral morphogenesis and budding. Optical imaging technique (confocal microscopy) can be used to visualize host membrane associated viral M1 protein (258). Demonstrating visually the effects of n-3 PUFAs on viral replication and release will further enhance our understanding at what stage *omega-3* treatment can affect immunity.

4.6 Conclusions

Previous studies in our laboratory have demonstrated that the host nutritional status can impair immune responses to viral infection (183). This was shown in animal models for obesity (184) and deficiencies of vitamin E (259) , selenium (185) and vitamin C (260). The present study additionally provides evidence that n-3 PUFAs have to be considered when faced with infection. Given that fish oil supplementations have differing ratios of DHA and EPA and that DHA and EPA have differing effects on different cell populations, there may be public health implications of fish oil supplementation during an infection.

In conclusion, do we supplement with n-3 PUFAs or not ? - A two horned dilemma –

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