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**Effect of Dietary Treatment with Stearidonic Acid and
Eicosapentaenoic Acid on Membrane Phospholipid Fatty Acid
Composition and Glycogen-Elicited Peritoneal Neutrophil
Survival in Absence or Presence of Bacterial Endotoxin**

A Thesis
Presented for the
Master of Science Degree
The University of Tennessee, Knoxville

Jonathan Earl Phipps
December 2005

Dedication

This thesis is dedicated to my mother and father, Larry and Judy Phipps, who made it their first priority to provide me with the encouragement to accomplish my goals and dreams; also, to my grandparents; Charles and Louise Phipps, Charlie and Mardell Brewer; great grandparents, Earl Needham, Helen Brock, and especially Grace Phipps, all of whom have been beacons of guidance over the years; to Jada Shadden whose boundless compassion and dedication to her own dreams has been inspirational; finally, to my family of friends who, although are too numerous to mention all by name in the space allotted, have given me the courage and stamina to be where I am today.

Acknowledgements

I wish to thank all of those who took the time and patience to help me along the way of completing my research. First, I would like to thank my major professor Dr. Michael Karlstad, who provided me with the opportunity to pursue my graduate studies, and provided wisdom, friendship, and guidance along the way. I would also like to thank Dr. Jay Whelan and Dr. Joe Bartges for their assistance, advice and friendship.

I would also like to thank Dr. Mitchell Goldman, Dr. Blaine Enderson, and Dr. Brian Daley for their support of my research. I would like to thank Dr. Rob Gillis for his friendship and support throughout my graduate experience. I would also like to thank Richard Andrews, Lucy Simpson, Stacy Kirkpatrick, Steve Foster, Erica Johnson and Laura Jones for their expertise, friendship, and support throughout the research of this dissertation. I would especially like to thank Meghan Parrott for her selfless dedication to my research, as well as for her friendship and support during the endeavor.

Finally, I am grateful to my friends and family for all the love and support that they have provided over the course of my graduate studies. Above all, I thank God for the opportunity and blessings that have been provided me.

Abstract

We previously found that enteral nutrition with diets containing elevated levels of eicosapentaenoic acid (EPA; 20:5 n-3) reduced the number of neutrophils in bronchoalveolar lavage fluid and improved clinical outcome of patients with or at risk for adult respiratory distress syndrome (ARDS). We have also shown that the treatment of the neutrophil-like cell line, HL-60, with EPA can induce apoptosis. It is not known, however, if the addition of EPA to the diet would result in similar occurrences in neutrophils.

It has been suggested that the use of diets enriched with stearidonic acid (SDA; 18:4 n-3) may provide a novel source of n-3 fatty acids while offering similar beneficial effects on health as diets containing EPA. Therefore, we examined whether dietary SDA can be used as a precursor to increase EPA content in liver tissue.

It is well known that endotoxin increases neutrophil lifespan and that this may be important in maintaining innate immune response. The second part of this study incorporated the use of glycogen-elicited peritoneal neutrophils from the rat to examine the effect of dietary intervention with n-3 fatty acids (either EPA or SDA) on constitutive and endotoxin induced neutrophil survival. Male Long-Evans rats (n=18) were randomly divided into 3 dietary groups and fed a modified US-17 diet providing 200kcal/kg/day and containing 2% oleic acid (OA; 18:1 n-9) (n=6), 1% EPA/1% OA (n=6), or 1% SDA/1% OA (n=6) for 2 weeks. Liver phospholipids were analyzed using gas chromatography and neutrophil lifespan was evaluated using flow cytometry.

Dietary supplementation with EPA significantly increased the concentration of EPA and docosapentaenoic acid (DPA, 22:5n3), but not docosahexaenoic acid (DHA, 22:6n3) in liver tissue as compared with animals fed OA. Dietary supplementation with SDA also increased tissue contents of EPA and DPA, but not DHA as compared with OA fed controls. The increase in

tissue EPA was about one-half as effective in animals fed a diet containing SDA as those fed EPA. SDA was about two-thirds as effective as EPA at increasing tissue DPA content. These results indicate that, when fed at current dietary levels, SDA can increase the incorporation of EPA and DPA into the membrane phospholipids of liver tissue in a similar manner to dietary EPA. This provides evidence that SDA may provide an alternative to EPA for increasing tissue levels of EPA and DPA, but not DHA.

Flow cytometry analysis of neutrophils following 20h incubation in either presence or absence of endotoxin indicated that diet did not significantly affect constitutive or endotoxin-mediated neutrophil survival when fed at current levels. There was a non-significant trend towards an increase in both constitutive viability and endotoxin mediated cell survival ($p=0.069$) in both SDA and EPA fed animals as compared with control animals that were fed OA. While these results do not fully explain the role that EPA or SDA play in reducing neutrophil influx during ARDS, they do provide evidence that it is not through an increase of neutrophil apoptosis or an attenuation of the neutrophil response to endotoxin. These findings may indicate the importance of dietary EPA for maintaining host immune response, while providing beneficial effects in regards to inflammation. Because of similar action between dietary EPA and SDA these results might also provide further proof as to the potential for dietary SDA to be used as an alternative for dietary EPA.

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Part 1
Introduction

I. Introduction

Many studies have implicated the importance of the omega 6 (n-6) and omega 3 (n-3) families of fatty acids in their ability to affect a number of pathophysiological conditions. Both families of PUFAs have exhibited the ability to affect a variety of disease states such as, arthritis, coronary artery disease, cancer and acute lung injury (ALI)[1-5]. There have been several studies that implicate the role of PUFAs, especially members of the n-6 family, as regulatory factors for programmed cell death, or apoptosis. This may be particularly important in inflammatory diseases where the products of fatty acid metabolism, the eicosanoids, have been shown to activate inflammatory cells such as neutrophils[6] and increase their lifespan[7].

Neutrophils are the first line of defense in the response to bacterial infection. These immune cells are equipped with receptor sites that can detect components of bacteria such as endotoxin, which has been shown to attract and activate neutrophils[8]. Neutrophil activation by endotoxin has a wide variety of effects including; an increase in cell lifespan, the release of cytotoxic compounds, and the increased synthesis of eicosanoid products. The accumulation of activated neutrophils in the lung is an important feature in the development of ARDS[9]. Excessive neutrophil recruitment, activation, and release of their cytotoxic granules can lead to severe tissue damage in the lung. Neutrophil mediated tissue damage results in fluid accumulation and decreased gas exchange. This damage often results in the transformation of the lung into a non-compliant, end stage organ. Effective clearance of neutrophils from the lung while containing granule release is vital in the resolution of ARDS. This clearance is dependent on neutrophil apoptosis and the recognition of apoptotic bodies by resident macrophages[9-11].

Eicosanoids formed from the n-6 fatty acid arachidonic acid (AA, 20:4 n-6) are potent chemoattractants of neutrophils, and have also been correlated with an observed increase in neutrophil lifespan[12]. AA is found in abundance in

terrestrial food sources and is readily stored in the phospholipid portion of the cell membrane. The products of AA metabolism are the potent pro-inflammatory series-4 leukotrienes and the series-2 prostaglandins. The leukotrienes, particularly leukotriene B₄, has been the focus of interest in incidence of inflammatory diseases and adult respiratory distress syndrome (ARDS)[4] because of its ability to attract neutrophils and alter their lifespan.

Conversely, there has been much data supporting dietary intervention with members of the n-3 family of PUFAs, such as eicosapentaenoic acid (EPA, 20:5 n-3) or docosahexaenoic acid (DHA, 22:6 n-3), both of which are found primarily in fish oil, having suppressive effects on inflammation and attenuating tissue damage in conditions such as ARDS. This effect is attributed to a reduction of membrane phospholipid AA, and the products of EPA metabolism, e.g. the series-3 prostaglandins and the series-5 leukotrienes, which are on a scale of 30 fold less active than their n-6 derived cousins[13]. The ability of EPA to alter the concentrations and types of mediators present in an inflammatory response is apparent in several studies involving septic shock and ARDS[4;14;15]. Clinical trials have shown that ARDS patients receiving nutritional support with a high fat low carbohydrate diet containing fish oil as a major contributor of calories had significantly improved outcomes as compared with those given a traditional corn oil based nutritional formula. These effects were correlated with lower LTB₄ concentration and a decrease in the number of neutrophils present in bronchoalveolar lavage (BAL) fluid[15].

The effects of n-3 fatty acids in supportive therapy and disease prevention have led to recommendations from the American Heart Association for an increase in consumption of fatty fish and fish oil[16]. Unfortunately, due to a limit on natural fish stocks, new dangers associated with fish consumption due to environmental pollutants, and a resistance to the incorporation of fish into the established western diet these recommendations have been left largely unmet[17]. This has driven a new interest in identifying and developing alternative sources of n-3 fatty acids, particularly those that would increase tissue

levels of EPA efficiently when consumed at moderate levels. Recent development of genetically modified canola seeds that produce stearidonic acid (SDA, 18:4 n-3)[18] appear to be promising for this purpose.

II. Rationale and Significance of Study

It has been suggested that critically ill patients fed nutritional formulas containing high levels of n-6 fatty acids should be avoided, as excessive release of n-6 metabolites, particularly those derived from AA, may lead to a hyper-inflammatory condition that has been linked to the development of systemic inflammatory response syndrome and ARDS[4;19]. Dietary AA has been shown to increase membrane phospholipid content of AA in several tissues and cell types. Further, increased membrane phospholipid AA has been associated with an increased production of pro-inflammatory eicosanoids from stimulated peritoneal cells (i.e., macrophages, mast cells, neutrophils)[20]. During conditions such as ARDS, combined endogenous and exogenous factors such as cytokines and bacterial endotoxin stimulate cPLA₂ mediated AA cleavage from cell membrane phospholipids, resulting in an up-regulation of synthesis and extracellular release of products of cyclooxygenase (COX) and lipoxygenase (LO) metabolism from immune cells[11]. Products of COX and LO metabolism of AA have been shown to be potent chemoattractants and activators of inflammatory cells[21;22]. In particular, leukotriene B₄, a product of LO metabolism of AA, has been implicated as a neutrophil attractant and anti-apoptotic factor[12].

Neutrophils play a critical role in inflammation and tissue damage associated with inflammatory diseases such as ARDS[11]. Alternatively, the use of n-3 PUFAs, such as EPA from fish oil, has long been recognized as being beneficial because of the ability of EPA to decrease AA accumulation in circulating blood and tissue[23;24]. Incorporation of EPA at the expense of AA has been associated with a decrease in pro-inflammatory eicosanoid synthesis[25]. Feeding diets that contain n-3 fatty acids from fish oil have also been associated with an attenuation of symptoms of sepsis and ARDS in humans and animal models[4;26].

The effect of n-3 fatty acids on altering the products of eicosanoid metabolism has been shown to significantly improve patient conditions in a number of disease states of the lung[27]. Studies have shown that LTB₄ is an active mediator for neutrophil recruitment during pleural cavity inflammation, as well as in inflammatory lung diseases[28;29]. Work from our laboratory has demonstrated a reduction in levels of pro-inflammatory eicosanoid products as well as a decrease in neutrophil accumulation in bronchoalveolar lavage (BAL) fluid of ARDS patients fed enteral nutrition formulas containing n-3 fatty acids from fish oil[14;30]. However, these studies did not examine whether reduced neutrophil counts were due to an increase in apoptosis, or through a reduction of neutrophil chemotaxis. To address this question our laboratory examined the effect that coincubation with EPA would have on the lifespan of the promyelocytic human leukemia cell line, HL-60[31]. These studies found that direct treatment of HL-60 cells with EPA could significantly reduce cell viability through an increase in apoptosis. Using various inhibitors of AA metabolism, the importance of AA-derived eicosanoid products in the regulation of apoptosis in HL-60 cells was demonstrated[32].

Taken together, data from clinical trials and experiments with the HL-60 cell line suggest that dietary n-3 PUFAs may act by attenuating the increase in neutrophil lifespan associated with endotoxin activation through an increase in apoptosis; thereby, decreasing the number of neutrophils observed in BAL fluid of patients with ARDS receiving nutritional treatment with n-3 PUFAs. Further, the altered products of eicosanoid formation are responsible for this decrease in lifespan. However, due to limitations and different findings between immortalized HL-60 cells and neutrophils it is important that we examine this hypothesis in the neutrophil. The use of neutrophils from animals pre-fed modified US-17 diets enriched with EPA will allow us to determine the effect of pre-loading membrane phospholipids with EPA has on lifespan in either inactivated basal, or endotoxin activated neutrophils.

Many studies have suggested that the addition of n-3 fatty acids to the diet can have numerous beneficial effects[33]. Although these findings and subsequent recommendations from the American Heart Association have, until recently, been impractical because of supply and safety concerns of natural fish stocks[16;17]. The development of canola plants that accumulate SDA in their seeds[18] might provide a twofold benefit. First, this may offer a practical strategy for providing a dietary source of EPA without the supply limits and safety concerns that surround an increased intake of dietary fish oil. Second, in situations where it is desirable to provide dietary EPA as therapeutic support for critical care patients, an enteral formula containing modified canola oil would most certainly be a cost effective alternative as compared with diets relying on EPA derived from fish oil. This strategy is still in question, as previous work with another n-3 fatty acid, α -linolenic acid (ALA, 18:3 n-3), has indicated that the enzymatic fatty acid desaturation and elongation pathway responsible for the conversion to longer chain PUFAs might be too inefficient for the use of precursor fatty acids to increase tissue EPA content[18;34;35]. However, dietary SDA is the metabolic product of the Δ -6 desaturase enzyme action on ALA. It has been long held that Δ -6 desaturase is the rate limiting step in fatty acid metabolism; thus, the efficiency of conversion and storage of SDA as EPA may be greater than that observed in experiments that fed ALA[18].

There have been relatively few studies that compare feeding diets enriched with SDA to diets rich in EPA, and most of the studies involving these fatty acids have been designed using oil blends that contain high levels of EPA and SDA[36-38]. In light of this, comparing the effect of feeding diets containing purified SDA ethyl esters on altering tissue phospholipid fatty acid profiles with the effects observed when feeding a diet containing purified EPA ethyl esters may provide valuable insight into the potential for SDA to be used as a precursor molecule for increasing EPA levels in membrane phospholipids. Further, this experimental design will allow us to compare the

effects of purified SDA ethyl esters with that of purified EPA ethyl esters on neutrophil lifespan and apoptosis rates during endotoxin activation.

The hypotheses of this study are twofold; first, that diets supplemented with SDA ethyl ester may provide a means to increase EPA content in liver phospholipids, and that this increase will be similar to those observed in animals fed a diet containing EPA. Second, that dietary supplementation with SDA or EPA will impact the regulation of constitutive and endotoxin-induced survival in neutrophils by influencing the rate of apoptosis in both cell states.

The current study design will utilize glycogen-elicited neutrophils from male Long-Evans rats that were pre-fed diets enriched with purified EPA or SDA ethyl esters. Harvested neutrophils will be incubated overnight in either the presence or absence of bacterial endotoxin to induce an activated cell state similar to that seen in models of sepsis or ARDS. Following incubation, flow cytometry will be used to examine the effect of diet on altering neutrophil lifespan in both an inactive and LPS activated cell state. Gas chromatography analysis of liver tissue will be utilized to provide information on the incorporation of EPA and SDA from dietary sources into membrane phospholipids.

The significant features of this study are many fold; first, feeding purified EPA ethyl ester will allow us to confirm and examine the incorporation of EPA into membrane phospholipids. Using gas chromatography we can examine whether EPA ethyl ester provided in the diet is directly incorporated into membrane phospholipids, and if it is enzymatically altered and stored as other n-3 fatty acids. This design will also allow us to confirm previous studies that indicate EPA content in membrane phospholipids is increased at the expense of AA. Second, simultaneous feeding of diets containing purified SDA ethyl ester will allow us to examine the potential of dietary supplementation with SDA as an alternative to diets enriched with EPA for modifying membrane phospholipid fatty acids. Third, pre-feeding animals diets containing EPA

and SDA also allows us to examine the effect of fatty acid incorporation and membrane pre-loading with respect to neutrophil lifespan and apoptosis. *Ex vivo* treatment of neutrophils allows us to examine the effect diet may have on lifespan in both the inactive and endotoxin activated conditions in cells from individual animals. This design utilizes both endotoxin treated and untreated cells from the same animal; thereby, allowing each animal to act as its own control, minimizing the potential for genetic variability on the effect of diet or endotoxin treatment.

III. Objectives

Objective 1: To examine whether diets supplemented with SDA ethyl ester can be used as a precursor to increase EPA content in liver phospholipids and to determine the effectiveness of SDA in increasing tissue EPA and reducing tissue AA content in rat liver.

Objective 2: To evaluate the effect of dietary EPA and SDA on the regulation of basal and endotoxin-induced neutrophil survival using glycogen-elicited peritoneal neutrophils from the rat.

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Part 2
Literature Review

I. Essential Fatty Acids and Eicosanoid Synthesis

Essential Fatty Acids:

In 1929 Burr and Burr discovered that the exclusion of certain fatty acids from the diets of laboratory rats would produce a disease state. Animals developed scaly skin, swollen and scaly tails, and hair loss from the face, tail, and throat[1].

These fatty acids were later termed “essential” because higher mammals lack the enzymatic pathways responsible for their synthesis *de novo*, and they must be obtained from the diet[2]. Essential fatty acids are polyunsaturated fatty acids (PUFAs), meaning that they are 18 carbons or more in length and have numerous double bond positions throughout their carbon backbone. The positioning of the first double bond proximate to the methyl end of the fatty acid is used as a reference to classify essential PUFAs as members of either the omega-6 (n-6) or omega-3 (n-3) family of fatty acids[3]. The essential nature of these PUFAs is due to the lack of enzymes responsible for desaturation past the ninth carbon of the fatty acid chain from the carboxylic end of the molecule. Further, it has been shown that fatty acids from these families are not interchangeable[4].

The parent fatty acids of the n-6 and the n-3 families of fatty acids are linoleic (LA; 18:2n-6) and α -linolenic (ALA; 18:3n-3) acids, respectively. In the strictest sense of the term, only the parent compounds of the n-6 and n-3 are truly essential, as an enzyme system exists to elongate and desaturate the 18 carbon PUFAs to form the other members of the essential fatty acid family[5]. Although, as previously mentioned these fatty acid families are not interchangeable, they are metabolized by the same enzymatic elongation and desaturation pathways[3] (Figure 2.1). LA is desaturated and elongated to form arachidonic acid (AA; 20:4n-6), which is considered to be the bioactive n-6 fatty acid for the production of inflammatory compounds[6]. Along the same lines ALA can be converted to stearidonic acid (SDA; 18:4n-3) and then to

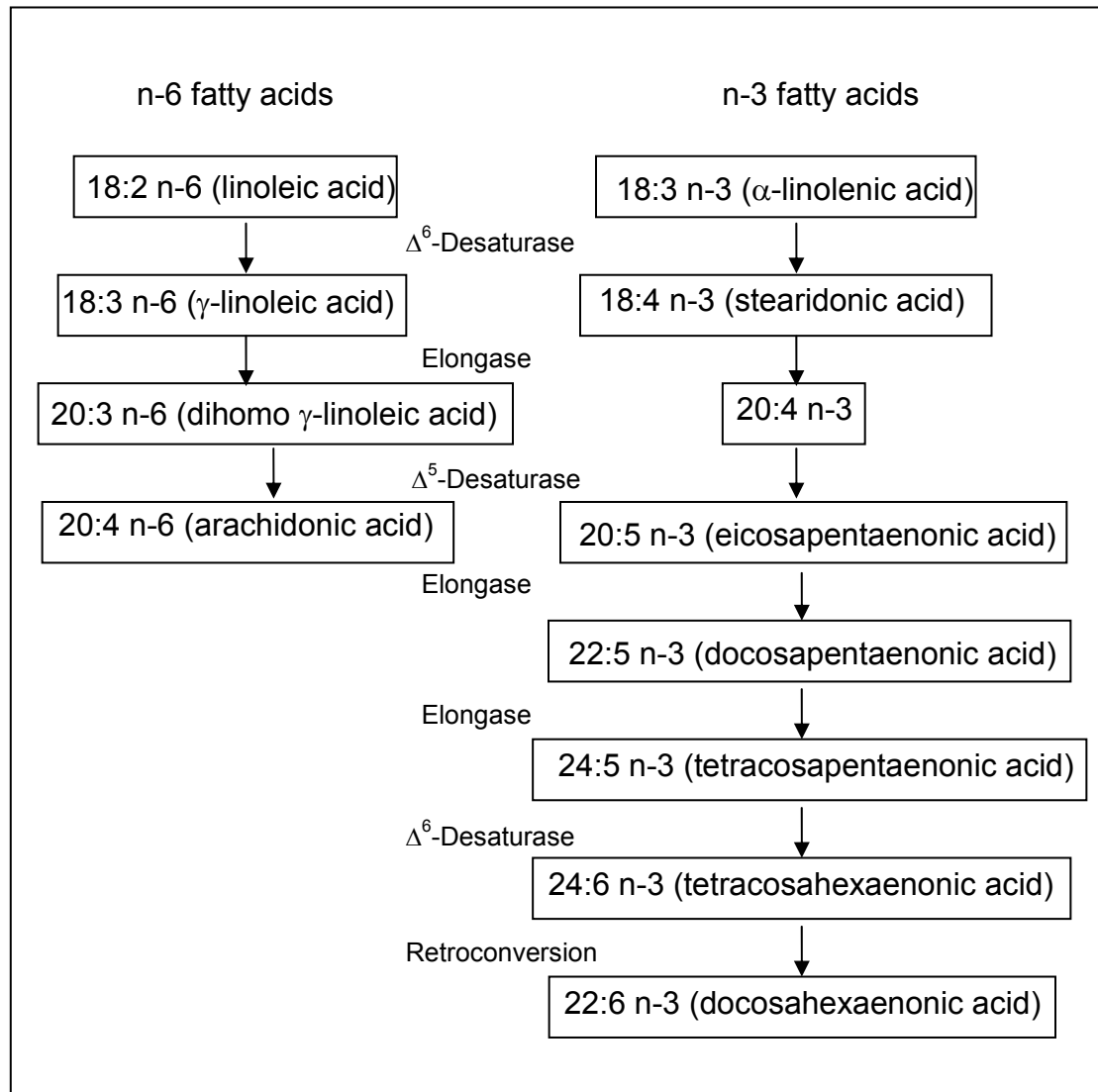


Figure 2.1 Pathways of Fatty Acid Synthesis.

eicosapentaenoic acid (EPA; 20:5n-3), which is the n-3 fatty acid counterpart to AA, and has been shown to attenuate the formation of proinflammatory products attributed to AA metabolism[7]. For this discussion we will focus on AA, SDA, and EPA. The main dietary source of AA is from consumption of animal products, although a small amount is synthesized by metabolism of LA from plant sources[8]. The beneficial effects of fish oil have been mainly attributed to the long chain PUFAs EPA and DHA. Typically, DHA nor EPA are not produced in terrestrial organisms, but found primarily in marine sources, such as cold water fish and fish oil[9;10]. SDA has been found in low abundance in certain seed oils, such as from *Echium plantaginium*, and recently has been produced in genetically modified canola plants[10;11]. Lipid derived eicosanoid products of AA metabolism have been ascribed a vital role in the development of inflammation, and have been implicated in the pathogenesis of sepsis, systemic inflammatory response syndrome, acute lung injury and acute respiratory distress syndrome[8;12].

Because of safety concerns associated with fish consumption, cost, and a limit on natural fish stocks the inclusion of fish into the western diet on a large scale is problematic and impractical[13]. Thus, there has been an urgent need for the location of alternative sources by which to provide n-3 PUFAs to the diet. Initially, the potential of ALA provided by diet to increase tissue levels of EPA and DHA was examined. These studies found that this reaction is too inefficient for dietary ALA to significantly alter the levels of n-3 fatty acids found in tissue membrane phospholipids[10]. The conversion of ALA to EPA is a three step enzymatic process, in which ALA is first desaturated by the Δ^6 desaturase enzyme to SDA and then elongated by 2 carbons to form EPA. The Δ^6 desaturase enzyme has been shown to be the rate limiting step in the conversion of ALA to EPA[14]. Stearidonic acid is the product of Δ^6 desaturase activity on ALA, and has gained interest as a precursor fatty acid for increasing n-3 PUFA content in tissue phospholipids, as it bypasses the rate limiting step in fatty acid

conversion to EPA and has been shown in some models to be more efficient at increasing tissue EPA as compared with ALA provided by diet[15].

Eicosanoids are biologically active products of either enzymatic or non-enzymatic oxidation of 20 carbon chain length PUFAs[6]. Synthesis of eicosanoid products begins with the cleavage of either AA or EPA from their storage position in the sn2 position of the cell membrane phospholipids. This cleavage is carried out by cytosolic phospholipase A₂ (cPLA₂), which is a member of a broad family of enzymes that are characterized by their ability to hydrolyze the center, sn2, position of substrate phospholipids[16]. Activation of cPLA₂ is a calcium dependant process, and yields a free fatty acid that can then be ferried to the nuclear membrane of the cell for further metabolism by cyclooxygenase, lipoxygenase, or cytochrome p450. For this discussion we will focus on the products of the lipoxygenase and cyclooxygenase enzyme pathways.

Cyclooxygenase Activity:

Prostaglandin endoperoxidase H synthases (PGHS; cyclooxygenase; COX) catalyze the committed step in the formation of prostanoid products from the 20 carbon fatty acids. There are two isoforms of COX enzymes, COX1 and COX2, both of which share approximately 60-65% sequence homology. All of the amino acids deemed necessary for COX1 function are present in the COX2 enzyme[17]. COX1 is a 576 amino acid length protein, while COX2 is a 587 amino acid protein. COX1 is considered to be constitutively active, and is involved in the production of housekeeper molecules, while COX2 is an inducible enzyme and is mainly activated in response to inflammatory stimulus which results in an enhanced prostaglandin production[18;19]. COX2 possesses an 18 amino acid sequence, inserted 6 residues in from the C-terminus that is not present on the COX1 enzyme[20]. Both isoforms of COX enzymes are located on the luminal face of the endoplasmic reticulum, and on both the inner and outer faces of the nuclear envelope. COX2 is more concentrated on the inner surface

of the nuclear envelope, possibly to facilitate the role of its products as nuclear regulators.

COX1 and COX2 utilize a two-step conversion process to metabolize AA to prostaglandin H₂ (PGH₂). This conversion is carried out by two discreet active sites on the enzyme. First, AA is combined with two molecules of oxygen in a *bis*-oxygenase reaction to form prostaglandin G₂ (PGG₂) in an enzymatic pocket on the hydrophilic surface of the enzyme. PGG₂ is then transported to a second pocket in the hydrophobic core of the COX1 enzyme where it is reduced to form PGH₂ through a peroxidase reaction that removes two electrons from PGG₂[17]. PGH₂ from COX1 is then released into the cytoplasm of cells to be converted to an active prostaglandin by other enzymes. Whereas PGH₂ formed by the COX2 enzyme most likely is transported to the nucleus[17].

Metabolism of AA by COX1 or COX2 results in the formation of the 2-series prostaglandins, where the subscript 2 denotes the number of double bonds in the molecule. Both COX1 and COX2 are capable of metabolizing EPA to prostaglandin products, although at a greatly reduced rate[21]. The additional double bond found in EPA as compared with AA results in the formation of prostaglandin products containing 3 double bonds along the carbon backbone of the molecule. These products are denoted by the subscript 3 (i.e., PGH₃), and are referred to as the 3-series prostaglandins.

Once released into the cell cytoplasm, the PGH products of COX activity can be metabolized by a number of enzymes to form prostaglandin products which are actively involved in cell signaling. These include PGE₂, PGD₂, PGF_{2α}, PGI₂, and thromboxanes. PGD₂ can be transformed by two steps of dehydration and one step of double bond migration to form Δ¹²-PGJ₂ and 15-deoxy-Δ^{12,14}-PGJ₂, which have been shown to induce apoptosis in neutrophils[22]. The reactions involved in transforming AA to cell signaling products are carried out by discreet heterogenous enzymes and non-enzymatic reactions. The prostaglandin products of AA metabolism have been linked with the pain, redness, and swelling associated with inflammation[19]. PGE₂ induces fever,

increases vascular permeability, vasodilation, as well as pain and edema enhancement[23]. However, on the cellular level PGE₂ has also been shown to exhibit anti-inflammatory actions such as reducing lymphocyte proliferation, and suppressing the production of pro-inflammatory cytokines such as TNF- α , IL-1, and IL-6[18]. PGI₂, or prostacyclin, exerts potent anti-thrombic and vasodilatory effects through interaction with vascular smooth muscle tissue and platelets[24]. Thromboxane A₂ (TXA₂) is produced by activated platelets and has strong thrombogenic, vasoconstrictive, and bronchoconstrictive properties[25].

As mentioned above, EPA can be metabolized by the COX enzymes, although at a reduced rate as compared with AA to form the 3-series prostaglandins. During prostaglandin formation there is further competition (aside from competing for position in the cell membrane) between the two classes of PUFA for COX activity which results in a decrease in the formation of AA derived COX products. Many of the products of EPA metabolism exhibit a reduced biological activity[26]. For example thromboxane A₃, a product of EPA metabolism, is a weak vasoconstrictor and weak platelet aggregator as compared with TXA₂ derived from AA[23]. The biological activities of PGE₃ are also muted as compared with PGE₂[27]. Besides the decreased biological activity of PGE₃, EPA has also been demonstrated to decrease the production of PGE₂[28]. There are exceptions, however, such as the case of the EPA prostacyclin product, PGI₃, which is as biologically active as PGI₂ as a vasodilator and platelet aggregator[23].

Prostaglandins exhibit their effects, with few exceptions, via a family of prostaglandin receptors. Each of the receptors has a high binding affinity for a specific prostaglandin, although there can be considerable cross reactivity among the receptors and ligands. The prostaglandin receptors are seven transmembrane domain receptors, belonging to the heterodimeric G-protein-coupled, rhodopsin-type superfamily[29]. The nomenclature of receptors is based on the ligand that they bind. For example, DP binds to PGD, TP binds to thromboxanes, IP binds to PGI, etc.. Within this superfamily of receptors the

branch dedicated to PGE binding is composed of four members (EP₁, EP₂, EP₃, EP₄), constituting a complete receptor sub-family[25]. Each prostaglandin receptor can be classified in one of three categories, based on the G-protein homodimer activated, and the cell response. These categories are the relaxant receptors, contractile receptors, and the adenylate cyclase inhibitors. The relaxant receptors consist of IP, EP₂, EP₄, and DP. Activation of these receptors stimulate cAMP production through adenylate cyclase activation[29-31]. The contractile receptors are TP, EP₁, and FP and are associated with increasing intracellular calcium levels[29;32]. EP₃ is the sole member of the adenylate cyclase inhibitor family[29]. Exceptions to the prostaglandin ligand-receptor motif are the PGJ products of PGD metabolism. These prostaglandins have no specific cell surface receptors, instead are actively transported across the cell membrane[33]. Once inside the cell they can interact with proteins directly via a cross conjugated dienone unit that can react with thiol nucleophiles, thereby altering protein function[22].

Lipoxygenase Activity:

The lipoxygenase enzymes are a class of non-heme iron containing enzymes that catalyze the incorporation of molecular oxygen into PUFAs[25]. This reaction is positionally selective, which is reflected by lipoxygenase nomenclature, as the enzymes are named after the carbon atom that they preferentially attack. Based on this system of classification the lipoxygenase enzymes are divided into three categories; the 5-, 12-, and 15-, lipoxygenases, respectively[25].

12-lipoxygenase (12-LO) was the first lipoxygenase enzyme to be discovered in the mammalian system, and has been found in the cytosolic fraction of a number of cell types. There are reports that state that 12-LO is translocated to the cell membrane in a calcium dependent mechanism, and that this translocation deactivates the enzyme[34-36]. 12-LO is primarily responsible for the synthesis of 12-hydroperoxyeicostetraenoic acid (12(S)-HPETE) from AA,

which is the parent compound of the hepoxilins and trioxilin[34]. Hepoxilins are monohydroxy-epoxy derivatives of PUFAs and consists of two known derivatives, hepoxilin-A₃ and -B₃. Hepoxilins can be hydrolyzed to form another metabolite group called trioxilins, which also has two derivatives, (trioxilin -A₃ and B₃) depending on the precursor molecule[34]. 12-LO can also metabolize EPA to 12-hydroperoxyeicosapentaenoic acid (12(S)-HPEPE). Although the biological activities of the 12-LO PUFA metabolites are much less well defined than those of the prostaglandins or the biologically active leukotriene products of 5-lipoxygenase activity, they have been shown to be involved in a number of processes, including the increase of intracellular calcium in neutrophils by hepoxilins A₃[34;37]. More work is needed to determine the effects of 12-LO products during pathological conditions.

15-lipoxygenase (15-LO) is another cytosolic protein member of the lipoxygenase family, which catalyzes the oxygenation of AA to 15-hydroperoxyeicosatetraenoic acid (15 (S)-HPETE), and similarly transforms EPA to 15-hydroperoxyeicosapentaenoic acid (15 (S)-HPEPE)[25;38]. 15-LO was first discovered in rabbit reticulocytes, and later two separate isoforms were identified in humans[39]. These isoforms are 15-LOa and 15-LOb, and are classified based on tissue distribution and substrate specificity. 15-LOa was the same protein that was first purified from rabbit reticulocytes, and is widely distributed in human eosinophils, bronchial epithelial cells, peripheral blood leukocytes, liver, kidney, spleen, thymus, testis, skeletal muscle, heart, and brain. In contrast, 15LOb has only been isolated in the prostate, skin, lung, and cornea[39]. 15-LO is also an active enzyme for shorter chain 18 carbon fatty acids, such as LA or ALA. A second feature unique to 15-LO is that it can also oxygenate metabolites that are esterified to phospholipids, independently of phospholipase activity[25]. It has been shown that each 15-LO isoform preferentially utilizes different substrates. 15-LOa preferentially metabolizes LA to 13(s)-hydroperoxyoctadecadienoic acid (13(S)-HODE), and metabolizes AA to 15(S)-HETE. In contrast, 15-LOb converts AA to 15(S)-HETE, but is a poor enzyme for

LA[39]. Products of 15-LO metabolism have been shown to possess various biological properties in a number of cell types. 15(S)-HETE has been shown to modulate pain response in rat skin, alters the growth of rat liver epithelial cells, regulates the secretion of prolactin in fibroblasts, induce a hypersensitivity to the β -adrenergic response in rat cardiomyocytes, and in erythroleukemia cells 15-HETE has been shown to regulate protein phosphorylation and cell proliferation[40]. 13-HODE has been linked with inducing histamine hypersensitivity in guinea pig airways, and modulating the EGF signaling tyrosine phosphorylation in fibroblast cultures[25;40]. There have been some studies that imply a particular importance for 15-LO in the lung, at least in epithelial cells. Several studies have demonstrated that tracheal and bronchial epithelial cells produce 15-LO products, and that the removal of the epithelial layer results in a reduction of 15-LO activity[38;41;42]. In other studies 15-HETE has been shown to induce mucus and chloride secretion in human lung epithelium, and mucus secretion and inflammation in canine airway epithelium[40]. The involvement of 15-LO in the inflammatory response has been illustrated in several studies in which it has been shown to reduce 5-lipoxygenase activity, reduce leukotriene mediated chemotaxis, and reduce superoxide anion generation in neutrophils[39]. In one of the most recent studies, the 15-LO product, 15-HETE was shown to have a regulatory effect on the LPS-induced production of tumor necrosis factor- α in human monocytic cell lines. 15-HETE exhibited this effect by degrading TNF- α mRNA[43].

Perhaps the most well studied of the lipoxygenase enzymes is 5-lipoxygenase (5-LO). This enzyme pathway is responsible for the production of the leukotrienes, which have been shown to have a wide range of effects on inflammation and innate immunity[44]. Intracellularly, 5-LO is located in the cytosolic fraction of resting leukocytes and upon activation associates with the nuclear envelope[45]. 5-LO requires an interaction with both phospholipase A₂ and 5-LO activating protein (FLAP)[46]. Following cleavage of AA or EPA from the cell membrane by PLA₂, FLAP along with PLA₂ and AA translocate to the

nuclear envelope to the site of 5-LO[45]. The translocation of all these participants of 5-LO enzyme activity are calcium dependant, based on studies using the calcium ionophore, A23187[47-49]. Following nuclear assembly of the 5-LO complex the enzyme can catalyze the synthesis of the leukotrienes from AA or EPA. Metabolism of AA results in the formation of the 4-series leukotrienes, while EPA metabolism results in the formation of the 5-series leukotrienes[50]. 5-LO first converts AA to 5-(S)-hydroperoxyeicosatetraenoic acid (5-HPETE), and then converts 5-HPETE to leukotriene A₄, which is the parent compound for leukotriene synthesis[51]. LTA₄ is an unstable epoxide that can either be enzymatically conjugated with reduced glutathione to form leukotriene C₄ (LTC₄), or hydrolyzed to leukotriene B₄ by LTA₄ hydrolase[52]. LTC₄ is the parent compound of the cysteinyl leukotrienes, which were originally described as the slow reacting substances of anaphylaxis. LTC₄ can be metabolized to leukotriene D₄ (LTD₄) by elimination of glutamic acid by γ -glutamyl transpeptidase. LTD₄ can be hydrolyzed by a dipeptidase to leukotriene E₄, removing the remaining peptide bond, forming LTE₄[53].

The cysteinyl leukotrienes are produced mainly by activated eosinophils, basophils, mast cells, and macrophages. They were first identified by their smooth muscle contracting properties, and were associated with asthma and the allergic response[54]. Although there have been some recent papers that implicate the cysteinyl leukotrienes with the release of pro-inflammatory cytokines, an increase in microvascular permeability, and the induction of pulmonary fibrosis[55].

LTB₄ is a leukotriene of major importance in regards to immune function and the inflammatory response. Structurally LTB₄ is similar to the cysteinyl leukotrienes, having a 20-carbon backbone, and multiple double bonds, although lacking the peptide side chain, but possessing an additional hydroxyl group at C-12[56]. LTB₄ is produced mainly by immune cells such as alveolar macrophages, and acts as a potent chemoattractant and stimulator of neutrophils to sites of infection and inflammation[44]. LTB₄ has been shown to promote neutrophil

chemotaxis, and to up-regulate the interaction between activated neutrophils and vascular endothelial cells[19]. LTB₄ is a stimulator of lysosomal enzyme and superoxide anion release in neutrophils. LTB₄ also increases the generation of the chemotactic agent interleukin-8 by neutrophils[56]. LTB₄ has also been shown to mediate survival in neutrophils by decreasing apoptosis[19]. There have also been reports that LTB₄-mediated cell survival is associated with the survival pathway observed when neutrophils are exposed to bacterial endotoxin, although this is debated[57;58]. LTB₄ is an important mediator for the immune system, especially in the lung, where alveolar macrophages have been shown to exhibit an enhanced release of LTB₄ as compared with other monocytes, indicating the role of LTB₄ as an early response mediator of innate immunity[41]. As previously mentioned, lipoxygenase enzymes can form EPA derived 5-series leukotriene products. This is the case with LTB₅, which is structurally related to LTB₄, but is less biologically active[59].

Like the products of the cyclooxygenase pathway, both LTB₄ and the cysteinyl leukotrienes exhibit their effects through interactions with members of the G-protein-seven-transmembrane receptor family. LTB ligands interact with two surface receptors; BLT1 and BLT2. These receptors are present in high numbers on inflammatory cells and vascular endothelial cells. Coupling of LTB to either receptor induces an increase in intracellular calcium levels as well as chemotactic responses in neutrophils[60]. The cysteinyl leukotrienes interact with two receptors as well. These are CysLT₁ and CysLT₂ receptors. These receptors have been found in bronchial tissue, and expressed on CD45+ leukocytes, macrophages, mast cells, neutrophils, and eosinophils in humans[55].

Another class of lipoxygenase products is the lipoxin family. The name lipoxin is an acronym for lipoxygenase interaction products. These eicosanoids are products of multiple lipoxygenase enzyme reactions that are carried out in cell-to-cell interactions[25]. The lipoxins were first identified by Serhan et al[61] from purified fractions of leukocyte suspensions that were coincubated with 15-

HETE and calcium ionophore A23187. Lipoygenase formation occurs by one of three pathways (two of which occur naturally and one of which involves the non-steroidal anti-inflammatory drug, aspirin) depending on the cells involved in interactions at the time of formation[62]. The earliest pathway to be described was for the formation of lipoxin A₄ (LXA₄) [63;64]. This formation involves the interaction between neutrophils and platelets. 5-LO activity in the neutrophil produces LTA₄, which is released from the neutrophil and taken up by the platelets. 12-LO activity inside of the platelets then transforms LTA₄ to LXA₄. A second pathway involves the interaction between 15(S)-HPETE or 15(S)-HETE formed by vascular endothelial cells. During activation neutrophils begin to roll along vascular endothelial cells in response to chemotactic stimuli, during this interaction they can pick up the 15-LO products from the endothelial cells. These products are subjected to metabolism by 5-LO in the neutrophil and forms an unstable intermediate, 5,6-epoxytetraene (5,6-ETE). 5,6-ETE is rapidly converted by epoxide hydrolases to three products, LXA₄, LXB₄, or 7-*cis*,11-*trans*-lipoxinA₄[25]. The final pathway for lipoxin formation involves the acetylation of COX-2 by aspirin. This shifts the activity of the COX-2 enzyme in monocytes to one that mimics a lipoygenase enzyme, forming the product 15(R)-HETE. 15(R)-HETE is structurally similar to 15(S)-HETE formed by 15-LO with the exception that the 15-C in the chain has its alcohol group in the R conformation. Once released, 15(R)-HETE can be taken up by neutrophils and metabolized by 5-LO, forming 15-epiLXA₄, or 15-epiLXB₄[65].

The pharmacological activities of the lipoxins are involved heavily in the resolution of inflammation. They have been shown to inhibit neutrophil and eosinophils trafficking by reducing sensitivity to LTB₄ and PAF, as well as to stimulate the macrophage mediated clearance of apoptotic neutrophils[66]. Lipoxins exert their influence in two ways. First there is a lipoxin-specific recognition site that has been localized on human neutrophils. This is thought to be a member of the G-protein-coupled receptor superfamily, although the downstream signaling has yet to be elucidated. Also, lipoxins are competitive

inhibitors of cysteinyl leukotriene receptors[62]. Substitution of AA with EPA leads to the formation of the 5-series lipoxins, which have been shown to be equally as active as signaling molecules as their AA derived counterparts[67].

Benefits of EPA and SDA:

As mentioned before, the addition of SDA to the diet may have beneficial effects on inflammatory mediator production because of its potential to be elongated to EPA[14;68]. SDA has also been shown to possess inhibitory effects on the oxygenation of AA to eicosanoid products[69]. This effect was later demonstrated to be due to an inhibition of Δ^5 -desaturase activity[70]. The inclusion of EPA into diet or nutritional formulas has been shown to have potent anti-inflammatory effects *in vivo*[71-73]. *Ex vivo*, dietary EPA has been shown to reduce the synthesis of interleukin-1 and tumor necrosis factor- α by activated monocytes[74]. EPA has been shown to reduce the formation of PGD₂ by inhibiting COX-2[75]. Besides inhibitory effects, EPA can be used as a substitute for AA in the formation of eicosanoids[8]. This substitution leads to the formation of products that are much less bioactive than their AA derived cousins. One example of this is LTB₅ which is much less active than LTB₄[76].

II. Apoptosis

Mechanisms of Apoptosis:

Apoptosis is an active process by which cells undergo a controlled breakdown of systems and molecules, leading to programmed death. Apoptosis was first observed in the late 19th century by C. Vogt during morphological examinations of amphibian cells. This process was lost and rediscovered over the years in a variety of cell types. The term apoptosis was coined in a paper by Kerr et al in order to describe non-necrotic cell death[77]. The apoptotic process begins in response to a stimulus, which triggers an energy requiring pathway leading to destruction of the cell by a characteristic set of reactions[78]. Necrosis is a passive process, typically in response to some insult to the cell leading to a loss of membrane integrity. The fate of the necrotic cell is fluid influx, swelling, and eventually rupture, emptying the contents of the cell into the environment[77]. The apoptotic pathway, on the other hand, leads to the inactivation and death of the cell without loss of the external membrane. As previously mentioned, signaling molecules that are expressed on the cell membrane during the apoptotic process target these cells for removal by phagocytic cells before the loss of membrane integrity can occur. Another important difference between apoptosis and necrosis is mitochondrial involvement. During apoptosis several studies have shown that the mitochondria exhibit a loss in the mitochondrial membrane potential ($\Delta\Psi_m$) which coincides with the appearance of the permeability transition pore (PT). This loss of mitochondrial membrane potential allows small pro-apoptotic proteins to leak into the cytosol, which amplifies and perpetuates the apoptotic signal[79]. The timing of mitochondrial participation has been used to elucidate the existence of two separate signaling pathways for apoptosis, which will be discussed further. Although apoptosis is an important process for the normal physiological functioning in numerous systems, this discussion will focus on the pathways and factors associated with apoptosis in the neutrophil and the inflammatory process.

Regardless of cell type, apoptosis is an ordered process which consists of 3 phases; initiation, execution, and termination. Initiation can occur by a number of intrinsic and extrinsic factors. Several examples of apoptotic inducers are growth factor withdrawal, ultraviolet or gamma radiation exposure, chemotherapeutics, or activation of membrane expressed cell death receptors[77]. Initiation by activation of cell death receptors is mediated through members of the tumor necrosis factor/ nerve growth factor (TNF/NGF) superfamily of receptors. These receptors all share a cysteine-rich extracellular domain, and a signal transducing intracellular death domain (DD). To date there have been six cell death receptors identified; TNFR1 (CD120a), CD95 (APO-1, Fas-1), DR3 (APO-3, LARD, Tramp, WSL-1), TRAIL R-1 (APO-2, DR-4), TRAIL R-2 (DR-5, KILLER, TRICK-2), and DR-6. Of these six, CD95 has been most well characterized. In the CD95 model of apoptotic induction, activation of the CD95 receptor by CD95 ligand (CD95l) leads to oligomerization of TNF/NGF receptors, forming an apoptotic signaling complex. Ligation and oligomerization of these receptors induces recruitment of the intracellular adaptor protein, Fas-associated death domain (FADD), to the cytoplasmic portion of the receptors. Association of FADD with the DD of the receptor complex sequesters molecules of pro-caspase 8 to the site, which interacts with other components to form the death induced signaling complex (DISC)[77;80;81]. Pro-caspase 8 is a member of an important family of proteases that are responsible for the execution phase of apoptosis; namely, the caspases. Caspases are a family of intracellular cysteine proteases with conserved sequence sites for binding and catalysis. Caspases cleave their substrates following accessible aspartic acid residues, which is where their name is derived (cysteiny aspartate specific proteases). Cleavage of substrates by caspases leads to a cascade of signals that result in controlled deconstruction of proteins and DNA, making these proteases vital initiators and effectors of programmed cell death[80;82].

In order to control caspase activity, the molecules are initially produced as inactivated zymogens, composed of three domains: an N-terminal pro-domain, a

p20, and a p10 domain. The active moiety is a heterodimer of the p20/p10 domains, formed by the cleavage of the pro-domain. The sites of cleavage required for caspase activation are all located following aspartate residues, making them candidate sites for autolytic cleavage. Aggregation of pro-caspase 8 molecules at the site of DISC formation increases their concentration sufficiently to allow autolytic activation to occur[83]. The proteolytic activation of caspase-8 begins a downstream cleavage of substrates, ultimately ending in apoptosis. The caspase cascade depends on three vital caspase family members; caspases-3, -6, and -7, which are responsible for cleavage of specific death substrates. These death substrates include various DNA repair molecules, ribonucleoproteins, signaling molecules, and structural proteins[77]. In the simplest model of caspase-mediated apoptosis, caspase-8 can be viewed as an initiator caspase, activated at the DISC, leading to activation of executioner caspases -3, and -7 which in turn activates caspase -6. This model is termed the “induced proximity model” of apoptosis[84]. In this model cleavage of caspase -3 occurs prior to loss of the mitochondrial membrane potential, indicating a lack of dependence on the mitochondria[77].

In a second form of receptor-mediated apoptosis, the role of the mitochondria is crucial to the apoptotic signal transduction cascade that occurs between caspase-8 and caspase-3[85]. This form of apoptosis is described in cell types with low levels of caspase-8 association with DISC. In this model the mitochondria act as signal amplifiers by releasing pro-apoptotic factors into the cell[86]. Mitochondrial dependent apoptosis involves interactions of several apoptotic signal regulating proteins, members of the BCL-2 protein family. These proteins share four BCL sequence homology regions (BH 1-4) and have either pro or anti-apoptotic effects. The BCL-2 family of proteins can be broken down into three archetypes. Type 1 BCL-2 family members contain the BH-4 domain and are anti-apoptotic. While the Type 2 and 3 BCL-2 family members lack this BH region and are pro-apoptotic[81]. The anti-apoptotic BCL-2 family members include BCL-2, BCL-X_L, MCL-1, BCL-W, A1, and BOO. The pro-apoptotic BCL-2

family members consist of two sub-types: Type 2 BCL-2 proteins contain BH regions 1-3, while type 3 BCL-2 proteins contain the BH-3 homology region only. The BH-3 region proteins include BAD, BID, BIM, BMF, BIK, BLK, HRK, NOXA, PUMA, NIX, and BNIP3. While the BH-4 family members have been shown to have other roles besides apoptosis inhibition, the BH-3 only proteins have been identified as exclusive cell death promoters[80]. Although the mechanisms are still being determined, members of the BCL-2 family are thought to interact with the mitochondrial membrane by formation of pores; the pro-apoptotic BH-3 members promote pore formation, while the BH-4 domain containing family members inhibit pore formation. The presence of these pores allows leakage of several compounds into the cytosol of the cell, including cytochrome-c, which is a key component in formation of a holoenzyme complex called the apoptosome[77]. Dimerization of pro- and anti-apoptotic BCL-2 proteins results in formation of heterodimeric neutral molecules[81]. This model emphasizes the importance of a balance between pro- and anti-apoptotic family members, as shift towards the apoptotic proteins would induce a change in mitochondrial membrane potential, allowing cytochrome-c leakage to occur. Regardless of mechanism, the role of the BCL-2 proteins can be simplified to the regulation of mitochondrial components, particularly cytochrome-c, into the cytosol.

In mitochondrial-dependant apoptosis, signal transduction from caspase-8 to caspase-3 requires activation of procaspase-9. As previously mentioned, activation of procaspase-9 requires formation of the apoptosome[81]. The apoptosome is a wheel like protein heptamer, made up of seven molecules of cytosolic protein, Apaf-1, and mitochondrial cytochrome c[87]. Apaf-1 contains a long C-terminal extension with 13 repeats of a WD40 motif. Apaf-1 also contains an amino-terminal caspase recruitment domain and nucleotide binding domain. Under normal circumstances Apaf-1 adopts a compressed conformation, which prevents oligomerization with other proteins. Cytochrome c released from the mitochondria binds to the WD40 motif, inducing the protein to adapt an open conformation. Following this unfolding process, Apaf-1 proteins can heptamerize

in an ATP dependent reaction to form a “seven spoke wheel” holoenzyme with the amino-terminal caspase recruitment domain facing the “hub”. This allows the collection of procaspase-9 enzymes to the hub region of the enzyme. Dimerization of procaspase-9 leads to the activation of a single monomer of the subunit[87;88]. The completed apoptosome then activates caspase-3, which in turn activates other executioner caspases, and induces apoptosis in the same manner as the induced proximity model.

Morphological Changes of Apoptosis:

Morphological characteristics were first described at great lengths in a 1972 paper by Kerr, Wyllie, and Currie following extensive electron and light microscopic observations[89]. The hallmark morphological characteristics occur during the termination phase of apoptosis, following initiation and activation of signaling molecules. These changes are nuclear condensation, cell shrinkage, formation of apoptotic bodies, and finally phagocytosis by other cells[90].

Morphologically, the onset of apoptosis begins cell shrinkage and membrane blebbing. Blebbing is thought to occur through proteolytic cleavage of structural components of the cell and nuclear membrane (cell death substrates), such as actin and fodrin cleavage by caspase-3 activated calpain[91]. While cellular shrinkage is a result of net loss of water from the cell to the extracellular environment from fusion of the golgi apparatus and endoplasmic reticulum with the cell membrane, and later the formation of apoptotic bodies[90]. Another very important change in the cell membrane is expression of phosphatidylserine on the outer membrane leaflet. Phosphatidylserine (PS) is a protein that is actively confined to the inner leaflet of the cell membrane during normal circumstances by the enzyme, aminophospholipid translocase. Relocation of PS to the outer leaflet of the cell membrane is brought about during the caspase-mediated cleavage of fodrin, which disrupts cell membrane symmetry and allows the PS molecule to “flip” to the outer cell membrane[92]. Expression of PS is vital for recognition and removal of apoptotic cells by macrophages[93].

Simultaneous to cellular shrinkage and membrane blebbing is a marked nuclear chromatin condensation. This chromatin condensation results in formation of compact, crescent shaped masses about the nuclear envelope. The nucleolus of the cell also undergoes shrinkage and chromatin aggregation. Eventually the nucleus itself begins to condense and break apart. Nuclear changes, like those observed in the cytosol, are a result of protease activity. Chromatin condensation may be a result of the cleavage of lamin-B1, which is responsible for connecting chromatin to the nuclear matrix. Loss of nuclear structural integrity is also the result of caspase-mediated lamin-A degradation[90;91]. Activated caspases are also responsible for DNA cleavage into 180-200bp double stranded fragments. This cleavage is mediated by DNA fragment factor (DFF40) and caspase-activated DNase (CAD). Both of these molecules are constitutively present in an inactive form. Cleavage of their inhibitor domains by caspase-3 activates their DNase properties[90].

In later stages of apoptosis, cell membranes form elongated extensions. These extensions eventually break off and the plasma membrane seals to form a separate membrane around the detached solid cellular material. These products of budding are termed apoptotic bodies. Apoptotic bodies contain well preserved, albeit crowded, cellular structures and organelles. These apoptotic bodies are usually quickly phagocytized by nearby macrophages; however, if allowed to remain they will degrade in a process that resembles necrosis, which has been termed secondary necrosis[90;94].

Apoptosis Versus Necrosis:

As previously mentioned, apoptotic cells that are not engulfed by phagocytic cells eventually degrade into a necrotic-like state. Necrosis is a modality of cell death that is typically induced by some insult. The process of necrosis is a passive one. Morphologic changes to the cell involve a loss in plasma membrane integrity, which allows influx of water and electrolytes. This fluid influx causes a rapid swelling of the cell leading to rupture of the cell membrane, and loss of cell

contents to the extracellular environment[90;95]. Necrosis also brings about an irregular chromatin clumping in the nucleus.

Apoptosis is an energy requiring, orderly process. The process of apoptosis can be disrupted or altered by deletion or perturbation of pathway members[81]. Histologically, apoptosis occurs in individual cells, whereas necrotic cells often occur in clumps of tissue and are often thought of as being indicative of a pathological condition[95]. Also, apoptotic cells do not induce an inflammatory reaction, owing to the maintenance of membrane integrity until clearance, whereas necrotic cells are associated with a spillage of cellular components that induces an inflammatory reaction[94].

Perhaps the most striking contrast between apoptosis and necrosis is the lack of surface changes in the cell membrane. The difference between apoptosis and necrosis membrane changes are best illustrated by the observation that necrotic cells are not engulfed by phagocytes and can induce an inflammatory response. Lack of recognition by phagocytic cells implies that the basic changes in expressed proteins and signaling molecules that become expressed during caspase activation are not present on necrotic cells[96]. The differences in surface membrane expression has been exploited by researchers as a way to differentiate cells that died via the apoptotic route from those that died by necrosis; i.e., as with the use of TUNEL or annexin-V staining combined with flow cytometry[97;98].

Role of Apoptosis in Inflammatory Cell Homeostasis:

Apoptosis in neutrophils (PMNs) is an important process in maintenance of homeostasis. In the non-disease condition approximately $1-2 \times 10^{11}$ PMNs are produced in the bone marrow of human beings and released into circulation daily[99]. Clearance by apoptosis is vital in order to avoid an overabundance of these cells. During infection numerous factors can not only sequester PMNs to the site of inflammation or infection, but also significantly decrease the rate of constitutive apoptosis. The apoptotic process in PMNs is very similar to that

observed in other cell types. It includes cytoplasmic condensation, aggregation and cleavage of nuclear chromatin, and formation of apoptotic bodies[100].

Apoptotic events in PMNs also lead to recognition of these cells by phagocytes, such as macrophages, which dispose of the neutrophil before loss of membrane integrity leads to the accidental release of cytotoxic compounds[101]. Of equal importance is loss of receptor mediated functional properties of apoptotic PMNs. Apoptotic PMNs have been shown to exhibit decreases in cytokine production, chemotaxis, phagocytosis, oxidative burst capabilities, and degranulation[102].

III. Bacterial Endotoxin

Bacterial endotoxin is a lipopolysaccharide molecule (LPS) found in the cell wall of Gram negative bacteria. LPS molecules are amphiphilic molecules consisting of a hydrophilic polysaccharide portion covalently linked to a hydrophobic lipid portion[103]. LPS is the predominant entity on the surface of bacterial cells; in fact one bacterial body contains approximately 3.5×10^6 LPS molecules, covering almost three-quarters of the bacterial surface[104]. When bacteria die and lyse, large quantities of LPS are released into the extracellular environment. Recognition of bacterial LPS by immune cells causes induction of the inflammatory response, and a physiological cascade that can lead to endotoxic shock and death[103].

Structure and Function of LPS Components:

As mentioned above bacterial LPS consists of a lipid portion covalently bound to a polysaccharide portion. The polysaccharide portion can be sub-divided into two regions; the O-specific chain and the core oligosaccharide. The O-specific chain is a polymer of oligosaccharide units that can have as many as fifty repeats[105]. The structure of the O-specific chain is highly variable, even from a single bacterial strain, which allows LPS to exhibit a large amount of functional variability. The core region of LPS is a hetero-oligosaccharide consisting of an inner and outer portion. The outer portion of the core region is proximal to the O-specific chain, while the inner portion is located adjacent to the lipid A portion of LPS[104]. Of the two core protein components, the inner portion of the core region is more highly conserved. This is reflected by the finding that regardless of bacterial species, the inner core typically contains a 2-keto-3-deoxyoctonic acid (Kdo) and *L-manno-D-mannoheptose* (*L,DHep*) portion. The outer portion of the LPS core region exhibits more variability in structure, as illustrated by the existence of at least 5 sub-types of outer core regions in *E. coli*[106]. Connected to the inner core region is the lipid A portion of the LPS molecule. Lipid A is the

most highly conserved component of LPS. Lipid A is an acetylated and phosphorylated disaccharide of glucosamine[107]. Lipid A is well known as the toxic and immunomodulating principle of bacterial LPS, as was demonstrated in studies where chemically synthesized lipid A, devoid of polysaccharide component, would induce biologic activities, both *in vitro* and *in vivo* that mimic those observed by bacterial LPS[105].

Recognition of LPS by Host Cells:

The biological activities of LPS in regards to eliciting an immune response from host cells is dependent on interaction between LPS, LPS binding protein (LBP), and the cell surface receptor, CD14[108]. The first step in recognition of bacterial LPS by immune cells is binding of LPS to LBP. LBP is a 60-kD serum glycoprotein that contains a high affinity binding region for the lipid A region of LPS[109]. Synthesized in hepatocytes, LBP is present in serum at <0.5µg/ml under normal circumstances, but can rise to 50µg/ml within 24h of induction of an acute phase response[110]. LBP opsonizes both LPS bound particles as well as intact bacteria, and mediates attachment to phagocytic cells such as macrophages by forming a complex with the cell surface receptor CD14[108]. There has also been evidence presented for a biphasic function of LBP. Some recent studies have indicated that lower concentrations of LBP will intercalate with the cell membrane of monocytes, enhancing the effects of LPS. While, at higher concentrations, LBP will bind to LPS aggregates in the extracellular milieu and inhibit their stimulatory properties[111]. Once LBP has bound to LPS it can complex with CD14. CD14 is expressed as either a glycosylphosphatidyl-inositol-linked membrane protein on macrophages, monocytes, and neutrophils or as a soluble protein that can act as an LPS receptor for CD14 negative cells[103]. It should be noted that LPS is capable of binding to CD14 without LBP, but the presence of LBP greatly enhances this process[112]. Structurally, CD14 lacks a transmembrane domain, which means that proper signal transduction requires participation of another protein, toll-like-receptor-4 (TLR-4).

This protein is a member of the toll-like receptor family and mediates the activation of nuclear-factor- κ B pathway[113].

Activation of immune cells leads to a number of cellular and physiological responses. Macrophage activation by LPS induces production of proinflammatory mediators such as tumor necrosis factor- α , interleukin-1, and pro-inflammatory eicosanoids. LPS induces a chemotactic response in neutrophils, as well as activates COX-2, and primes neutrophils for enhanced production of LTB₄[114;115]. Activation of the TLR receptor pathways have been shown to induce or prime cytokine production, superoxide production, and L-selectin shedding in human neutrophils[116]. Neutrophil exposure to LPS also induces a survival pathway through inhibition of constitutive neutrophil apoptosis[116]. Activation of the inflammatory response by LPS is linked with induction of numerous inflammatory diseases, such as endotoxemia or sepsis, the latter of which will be discussed in further detail [117;118].

IV. Systemic inflammatory Response Syndrome

Introduction to SIRS:

The clinical definition of sepsis is the systemic inflammatory response to documented infection[119]. It has since been recognized that other factors aside from bacterial infection can trigger an inflammatory response. The term Systemic Inflammatory Response Syndrome (SIRS) was put forth by the American College of Chest Physicians and the Society of Critical Care Medicine at a consensus conference in 1992 to define the inflammatory response classically referred to as sepsis, independent of cause[120]. Clinically SIRS is diagnosed by the presence of more than one of the following symptoms: body temperature greater than 38°C or less than 36° C, heart rate greater than 90 beats per minute, a respiratory rate greater than 20 breaths per minute, or hyperventilation indicated by PaCO₂ of less than 32mmHg. Hematologically, there is an alteration of white blood cell count; either greater than 12,000 cu/mm or less than 4,000 cu/mm, and possibly accompanied by an increase (10%) of immature neutrophils[120]. Further criteria describe the severity of the inflammatory reaction based on the presence of additional symptoms associated with the progression of sepsis. Severe sepsis is defined as organ dysfunction or sepsis-induced hypotension. The most severe form of sepsis, septic shock, meets the previous criteria along with sepsis-induced hypotension that persists regardless of fluid resuscitation and accompanied by sepsis induced organ dysfunction. Epidemiologic studies of sepsis indicate variable hospital and intensive care unit occurrence rates of 2-11% of all adult admissions, with a mortality rate of about 49%[121].

Etiology of Sepsis:

The initial onset of sepsis occurs with a bacterial infection. During their life cycle, bacteria shed numerous antigens, such as LPS, which are detectable by host immune cells. Sufficient numbers of bacteria may be able to release enough of these antigens to spill over into the host circulatory system. Once in the bloodstream antigens are recognized by host immune cells. Of particular importance for this step are macrophages[122]. Macrophages are specialized phagocytic white blood cells that are present in areas such as the sub-epithelium of the skin, alveolar tissue of the lung, or lining the blood sinusoids of the liver, spleen, and lymph nodes[119]. Macrophage activation leads to release of soluble factors, cytokines, that recruit and activate other cells; thus beginning the inflammatory cascade[122]. The proinflammatory cytokines also trigger release of several anti-inflammatory mediators that inhibit immune cell function and attempt to localize the inflammatory response[123]. This anti-inflammatory cascade provides a balance to the immune response to infection or injury.

Inflammatory Mediators in SIRS/Sepsis:

Macrophage activation by bacterial LPS initiates release of chemical mediators which are responsible for the pathology of sepsis. This process begins with formation of a complex between LPS with LPS binding protein (LBP). This complex is recognized and activates the CD14 complex found on the cell membrane. TLR4 then transduces this signal through a signaling pathway that mediates degradation of the inhibitor molecule for nuclear factor (NF) κ B.

Activated NF κ B is translocated to the nucleus of the cell, where it binds with DNA and promotes transcription of numerous genes responsible for production of a host of pro-inflammatory cytokines including; tumor necrosis factor- α (TNF α), interleukin (IL) 1, granulocyte-colony stimulating factor (G-CSF), interferon- γ (IFN- γ), IL-2, IL-6, and IL-12[122].

Tumor Necrosis Factor α : TNF α is a 17-kDa, 157 amino acid polypeptide that is produced mainly by monocytes and macrophages. TNF α exists in both a membrane bound and soluble form. Soluble TNF α is formed by cleavage of extracellular domain of the membrane bound molecule by metalloproteinase compounds, and is a 17-kDa molecule as compared with the membrane bound TNF α molecule which is 26-kDa[124]. Cleavage of soluble TNF α increases during infection or inflammation. Leading to an increase of circulating TNF α , which has been shown to peak approximately one hour following insult[125]. TNF α initiates its effect by binding with two distinct surface receptors; a 55kDa receptor (TNFRI) and a 75kDa receptor (TNFRII). Originally it was believed that TNFRI was the primary receptor by which signal recognition of TNF α was received because of its higher affinity for the soluble form of TNF α . However, it was later found that TNFRII plays an important role in sensing the presence of membrane bound TNF α in autocrine and paracrine interactions[126;127]. Although TNF α binding to either of these receptors triggers activation of NF κ B, other factors along the signal transduction pathways allow the molecule to exhibit different results. For example, activation of TNFRI and the proceeding signal transduction pathway initiates apoptosis, while activation of the TNFRII and the ensuing signaling cascade induces an increase in cytokine production. Transcription and expression of these receptors is under genetic control, which allows different levels of expression. These expression levels modulate the likelihood of interaction with ligands[128]. TNF α has a variety of activities in numerous tissue types. For example, NF κ B activation in vascular endothelial cells by TNF α up regulates expression of adhesion molecules such as E-selectin, ICAM-1, and VCAM[119]. Increased expression of these molecules alters the surface of vascular endothelium and causes immune cells such as PMNs, macrophages, and CD₄⁺ T cells to adhere and roll along the endothelium. These molecules also alter the structure of vascular endothelium allowing diapedesis of inflammatory cells from circulation to sites of infection, as well as increasing

vascular permeability[122], all of which are important factors with sepsis associated disorders such as acute respiratory distress syndrome (ARDS). $\text{TNF}\alpha$ injection induces the production and release of other proinflammatory cytokines such as $\text{IL-1}\beta$, IL-6 , IL-8 , and IL-12 , as well as inducing SIRS-like symptoms including: fever, hemodynamic abnormalities leukopenia, coagulation disorders, and increased production of liver enzymes[129].

Interleukin-1: IL-1 is another cytokine produced by monocytes in the acute phase of SIRS. IL-1 has two subtypes; $\text{IL-1}\alpha$, and $\text{IL-1}\beta$. $\text{IL-1}\alpha$ is mainly found in the cellular compartment, while $\text{IL-1}\beta$ is the predominant form found in circulation during sepsis[130]. There have been two receptors ($\text{IL receptor type I}$ and $\text{IL receptor type II}$) identified for $\text{IL-1}\alpha$ and $\text{IL-1}\beta$. The structural similarity of the molecules allows them both to activate each receptor type, although $\text{IL-1 receptor type II}$ is thought to be biologically inactive[119;119;130] which means that it may serve to negatively regulate IL-1 function. IL-1 is not normally found in human plasma, and when endogenously or exogenously administered induces many of the same symptoms as $\text{TNF}\alpha$ [129]. The overlapping role of these cytokines is thought to create either a synergistic, or additive effect that amplifies the severity of the inflammatory response[123]. One effect of IL-1 is rapid activation of cytosolic phospholipase A_2 , which is an important molecule in conversion of tissue polyunsaturated fatty acids such as arachidonic acid (AA) to lipid-based inflammatory mediators[131]. This activation has been linked with an increased production of prostaglandin E_2 (PGE_2), which is recognized by the central nervous system triggering induction of fever[132]. Pretreatment with cyclooxygenase inhibitors decreases some of the symptoms, such as fever and headache, that are induced when IL-1 and $\text{TNF}\alpha$ are coadministered[133]. IL-1 also induces production of other cytokines such as IL-1 , IL-6 , IL-8 , as well as $\text{TNF}\alpha$. Blocking activity of IL-1 using receptor antagonists can prevent the increase of other interleukins, but not $\text{TNF}\alpha$ [134]. IL-1 also up-regulates

production and expression of TNFRII which increases the effect of $\text{TNF}\alpha$ on cells[135].

Interleukin-6: IL-6 is a 21-kDA protein produced in many cell types, including monocytes. IL-6 activates B and T lymphocytes[119]. IL-6 release from resident liver macrophages (Kupffer cells) signals the liver to produce acute phase proteins. This results in increased circulating coagulatory, transportation, and tissue remodeling factors, as well as decreased proteins from the cytochrome p450 complex. IL-6 also lowers circulating levels of cholesterol and fatty acids. IL-6 alters the carrier molecules of fatty acids by increasing their affinity with activated macrophages, thereby providing more energy to the immune response[122]. IL-6 has been shown to induce fever by stimulating PGE_2 production in the central nervous system[136]. IL-6 has also been shown to increase cytotoxicity of PMNs by augmenting release of elastase[137]. Although the role of IL-6 in sepsis is not as clearly defined as IL-1 or $\text{TNF}\alpha$, an increase in IL-6 has a significant correlation with mortality in septic patients and severity of inflammation-associated organ dysfunction[119].

Interleukin-8: IL-8 is a small protein that is a member of the chemokine family of cytokines, is produced by a variety of cells, and is up-regulated by various factors such as LPS, $\text{TNF}\alpha$, and IL-1[138]. The primary function of IL-8 is to activate and chemoattract PMNs to sites of inflammation[129]. IL-8 operates through CXCR1 and CXCR2 receptors, which are in the seven transmembrane G-coupled protein receptor groups. IL-8 interaction with PMNs causes shape change and increases cell surface expression of integrin molecules, increasing PMN adherence to vascular endothelium, as well as increasing production of reactive oxygen metabolites in PMNs[119].

Interleukin-12: Proinflammatory IL-12 is a heterodimer consisting of a p35 and a p40 subunit, both of which are encoded by separate genes. IL-12 production occurs predominantly in macrophages and monocytes and targets T cells and natural killer cells. Activation of these cells by IL-12 induces production and release of interferon γ (IFN- γ), which acts on monocytes to increase production of proinflammatory cytokines including IL-12[119;130]. The role of IL-12 heterodimer may enhance the inflammatory response by creating a positive feedback loop for cytokine production between T cells, natural killer cells, and macrophages.

Anti-inflammatory Cytokine Cascade in SIRS/Sepsis:

In order to prevent host mortality from an uncontrolled inflammatory response, an intricate system has evolved to attenuate the effects of the inflammatory cascade. This system involves cytokines and signaling pathways that reduce production of proinflammatory mediators, soluble receptors that can bind inflammatory stimuli before it can interact with target cells, as well as cellular adaptations that reduce ability of cells to respond to stimuli.

IL-10 is a homodimeric 160 amino acid protein produced by a variety of cells. IL-10 inhibits production of TNF α , IL-1, and IL-8[129]. In PMNs, IL-10 reduces production of these proinflammatory cytokines in LPS-stimulated cells, enhances production of IL-1 receptor antagonists (IL-1ra), and reduces PMN migration during LPS induced lung injury[139].

Interleukin-4: IL-4 is a 20-kDa glycoprotein produced by activated Th2 cells, basophils, and mast cells. IL-4 targets monocytes and PMNs and inhibits secretion of proinflammatory cytokines[119].

Interleukin-10: Of the anti-inflammatory cytokines, IL-10 is perhaps the most well studied in models of bacterial infection. IL-10 is an 18-kDa polypeptide that is synthesized by T cells, B cells, and monocytes. IL-10 synthesis is stimulated by

TNF α , IL-1, IL-6, and IL-12, as well as bacteria and bacterial products such as LPS[130]. IL-10 production occurs maximally after about 24 hours in patients with severe sepsis, and has been shown to prevent LPS-associated mortality in laboratory animals[119].

Other anti-inflammatory cytokines: Certain cytokines seem to serve dual roles in inflammatory regulation. IL-6 for example, stimulates differentiation and proliferation of T cells, B cells, and induces acute phase protein synthesis. It inhibits IL-1 and TNF α production in monocytes[140], and induces production of IL-1 receptor antagonist and soluble TNF α receptors, which blocks effects of their proinflammatory ligands[141]. The p40 subunit of IL-12 has the ability to dimerize with the p35 subunit, or to form a homodimer with another p40 subunit. This homodimer of p40 subunits can bind to the IL-12 receptor with similar affinity as the p40/p35 complex without inducing a cellular response[130].

Soluble Inhibitors of Proinflammatory Cytokines: Anti-inflammatory cytokines function by inhibiting production of their proinflammatory cousins. However, there are also mechanisms to inhibit action of potentially toxic proinflammatory cytokines after they have been released from the cell.

Soluble TNF α : As previously mentioned, TNF α plays a major role in induction and maintenance of the inflammatory response. TNF α works synergistically with IL-1 to activate other pro-inflammatory cytokines and to direct the host response to infection and injury; however, excessive tissue production of TNF α can cause detrimental effects. Therefore, aside from a decrease in production caused by presence of anti-inflammatory cytokines, other mechanisms exist that blunt the effect of TNF α that is already present. These natural inhibitors are the soluble TNF α receptors (sTNFR). They are produced by numerous cell types as they shed the extracellular domains of their membrane bound TNF α receptors[142]. This shedding can be induced by a number of stimuli, including IL-10, IL-1, and

TNF α itself[130]. There are two varieties of sTNFR, a 30-kDA and a 40-kDA species, which share sequence homology with extracellular domains of TNFR I and TNFR II, respectively[142]. The presence of sTNFRs serves a twofold purpose. First, shedding the extracellular portion of their TNFR effectively desensitizes cells to TNF α . Second, sTNFRs can still bind to the extracellular TNF α , preventing it from acting as a ligand for membrane bound TNFRs.

Soluble and membrane bound IL-1 inhibitors and receptor antagonists:

Like the mechanisms that exist for TNF α , there are several methods for limiting biological activity of circulating IL-1. First, is the existence of a non-functional receptor, IL-1 receptor, type II, which binds to IL-1 β with high affinity[130]. IL-1 receptor type II exists in two forms, either as a membrane bound molecule, or as a soluble form. Like sTNFR soluble IL-1 receptor type II is made up of the cleaved portion of the extracellular domain of its membrane bound counterpart[143]. This cleavage can be induced by a variety of stimuli including LPS and TNF α [144]. IL-1 receptor type II is a unique cytokine receptor because it does not provide signal transduction once bound to IL-1 β . This along with the high affinity to IL-1 β allows both the soluble and membrane bound forms of IL-1 receptor type II to act as a decoy to IL-1 β [143].

Another mechanism for decreasing the biological impact of IL-1 is IL-1 receptor antagonist (IL-1ra). IL-1ra is a 22-kDa protein that shares sequence homology with IL-1 β . IL-1ra binds preferentially to the active IL-1 receptor (IL-1 receptor type I). However, crucial sequence differences in IL-1ra prevent proper formation of the complex required to incite signal transduction once IL-1ra is bound[143].

V. Complications Secondary to Sepsis

Multiple Organ Dysfunction Syndrome:

As the severity of sepsis progresses it is not uncommon for organ dysfunction to occur. In fact, the cause of death in most sepsis patients is not from infection, but from organ failure. The occurrence of multiple organ failure during sepsis is so prevalent that the condition has been termed multiple organ dysfunction syndrome (MODS). Organ dysfunction occurs when the functional status of that organ has been altered to the point that it is no longer able to maintain homeostasis. This change is attributable either primarily or secondarily to insult, and this route determines whether MODS is defined as primary or secondary MODS. In most cases of sepsis, MODS would be defined as secondary, as it is typically a result of host response to insult[120].

Lung Injury in MODS:

Lung dysfunction occurs in about 60% of septic patients with MODS[145]. The high incidence of lung involvement is due to several factors related to the organ and its function. Because of the large interface of the lung with the external environment, it has developed an intricate and highly sensitive defense mechanism to protect against microbial invasion. In this system two cell types have been identified as being vital for host defense; the alveolar macrophage and the PMN. Resident alveolar macrophages have perhaps the most important role of any monocyte cell type in host defense of microbial invasion. These cells are positioned within the surfactant layer of the alveolus. They are directly responsible for detection and clearance of bacteria in the alveolar space[146]. Aside from the direct antimicrobial role of the alveolar macrophage, this cell can recruit circulating PMNs to the alveolar lumen through a series of chemical mediators. These mediators include protein derived cytokines as well as lipid derived eicosanoids. Several recent studies have suggested that in models of

lung injury eicosanoid products play the predominant role in cell signaling. This is based on findings that alveolar macrophages are capable of enhanced eicosanoid release as compared with monocytes or macrophages from other tissues[147]. Alveolar macrophages are primed for an increased production of 5-LO eicosanoid products, specifically LTB₄[41]. It is thought that this difference exists as a mechanism to expedite sequestration of PMNs to the site of bacterial invasion, as the enzymes responsible for eicosanoid synthesis are constitutively present and do not require *de novo* synthesis, as is the case for cytokine production enzymes. Release of eicosanoid mediators swiftly induces the chemotaxis of circulating PMNs from the lung microvasculature to the alveolar lumen. Once recruited, PMNs become activated by cytokines and eicosanoids. This activation leads to release of bactericidal and cytotoxic compounds, resulting in PMN-mediated tissue damage, setting the stage for lung injury. During sepsis the sensitivity and enhanced response of immune cells in the lung places this organ at higher risk of being involved in development of secondary MODS. Like sepsis, lung dysfunction follows along a spectrum of severity. The progression of tissue damage may lead to conditions known as acute lung injury (ALI), and in the most severe cases, acute respiratory distress syndrome (ARDS).

Acute Lung Injury and Acute Respiratory Distress Syndrome:

ALI is defined as a syndrome of inflammation and increased permeability that is associated with a constellation of clinical, radiologic, and physiologic abnormalities that cannot be explained by, but may coexist with, left atrial or pulmonary capillary hypertension[148]. Clinical diagnosis of ALI is determined by acute onset, PaO₂/FiO₂ levels of less than 300mm Hg regardless of positive-end expiratory pressure (PEEP), bilateral infiltrates observed on frontal chest radiographs, and a pulmonary artery wedge pressure of greater than 18mm Hg. As with SIRS, ALI is considered to be a disease that covers a broad spectrum of severity. The most severe form of ALI is acute respiratory distress syndrome

(ARDS). ARDS follows the same definition as ALI with the exception that the $\text{PaO}_2/\text{FiO}_2$ level must be less than 200mm Hg[148].

Pathology of ARDS:

ALI/ARDS progresses through three overlapping phases; exudative, proliferative, and fibrotic phases. The exudative phase of ALI/ARDS is denoted by influx of protein-rich edema fluid into alveolar spaces, as well as increased local production of proinflammatory cytokines and eicosanoids such as LTB_4 , $\text{TNF}\alpha$, IL-1, and IL-8 by pulmonary macrophages[149]. Release of these inflammatory signals is important for the development of ARDS. Lungs extracted from patients that died during this phase are heavy and rigid, often weighing over 2000g. The lungs also become discolored, turning from their normal color to a reddish-blue or grey hue[150;151]. Alterations in endothelial cell function begin to allow proteinaceous fluid to seep from the vasculature to the interstitial space. Fluid accumulation widens the interstitial space between the pulmonary epithelium and vascular endothelium. This space is normally $2.2\mu\text{m}$, which includes the basement membrane as well as specialized type 1 alveolar epithelial cells. Type 1 alveolar epithelial cells are flattened in appearance and typically compose 8% of lung epithelial parenchyma, while covering a surface area of approximately $5098\mu\text{m}^2$. Type 1 alveolar epithelial cells operate in conjunction with type 2 alveolar epithelial cells, which are larger cuboidal cells that typically comprise about 16% of the alveolar parenchyma while only covering a surface area of about $183\mu\text{m}^2$ [151]. Type 2 alveolar epithelial cells produce surfactant, which is responsible for reduction of surface tension at the alveolus[150;152;153]. Both alveolar epithelial cell types are connected by tight cell junctions that form a relatively impermeable barrier. Sodium and potassium pumps found on type 2 alveolar epithelial cells actively transport ions from alveoli to pulmonary capillary beds. These pumps operate in tandem with aquaporins found on type 1 alveolar epithelial cells facilitating fluid clearance during normal functioning[150;151;154]. The interstitial distance and normal functioning of alveolar epithelial cells is

crucial for proper gas diffusion and fluid exchange. As fluid accumulation in the interstitial space continues, the alveolar ducts themselves begin to dilate, decreasing the air spaces to less than 1mm. Eventually, edema fluid infiltrates the alveolar lumen. Histologically, this first appears as a hyaline membrane along the alveolar ducts. This membrane consists of condensed plasma proteins and cell debris that have leaked into the alveolus. This hyaline membrane is often associated with a selective early loss of adjacent epithelial cells[151]. As ALI/ARDS develops, activated PMNs appear in the pulmonary vasculature, interstitial spaces, and eventually in the alveolar lumen[149;150;155]. PMN activation leads to release of nitric oxide (NO) and reactive oxygen species, proteases, elastase, and collagenase. These cytotoxic compounds are responsible for the damage caused to type 1 and type 2 alveolar epithelial cells. Type 1 alveolar epithelial cells are very susceptible to PMN-mediated damage, and significant loss leads to areas of exposed basement membrane and disruption of normal fluid exchange contributing to alveolar flooding[150-152].

Following the exudative phase of lung injury is a period of tissue restructuring and overall lung remodeling known as the proliferative phase[150]. The overall appearance of the lung during this phase is described as being a glistening pale grey color, having a very solid and slippery feel to it, and having very small air spaces that may even be absent in some areas[151]. A key feature of this phase is increased accumulation of interstitial myofibroblasts. Increased production of transforming growth factor β (TGF β), and autocrine IL-1 β -dependant IL-6 up-regulation activates fibroblasts which, deposit collagenous connective tissue along the wall of the alveoli[156;157]. The increased amount of fibrous tissue in the alveolar septa leads to decreased airspace in the alveolar ducts and in the space of individual alveoli, further decreasing efficiency of gas exchange. Lesions in alveolar epithelium from loss of type 1 alveolar epithelial cells allow fibroblast infiltration into the alveolar lumen. Here the fibroblasts begin to convert the proteinaceous edema fluid into connective tissue through collagen deposition. This effectively converts areas that were previously air

spaces in the alveolar lumen to interstitial tissue. Type 2 alveolar epithelial cells rapidly proliferate, and begin to differentiate into type 1 alveolar epithelial cells in an attempt to cover old basement membrane and newly deposited fibrotic connective tissue as well[150-152]. Another mechanism of lung remodeling is alveolar collapse. This is observed after injury to the alveolocapillary unit, when the actual alveolar walls collapse either partially or completely. Fibroblast infiltration and conversion of edema fluid to fibrotic tissue followed by epithelial cell growth effectively seals these areas from the rest of the lung. This tissue remodeling leads to a lung that contains significantly fewer, but larger alveoli with dilated alveolar ducts as compared to the uninjured lung.

The final stage, or the fibrotic stage, occurs 3-4 weeks following the onset of ARDS. The lungs of surviving patients become heavily scarred, non-compliant, end-stage organs, and is described as having a coarse, cobblestone surface due to heavy scarring and restructuring[150;151]. The parenchymal surface is pale and spongy, consisting of a honeycomb network of scar tissue and microcystic air spaces. Microscopically alveolar walls are sparsely cellular and thickened by collagenous connective tissue[151].

VI. The Neutrophil

The polymorphonuclear neutrophil (PMN) is considered to be the primary effector cell in innate immunity. This cell type has evolved a variety of biological agents to aid in tissue transit and bacterial killing. The PMN contains more than 30 histotoxic agents, and has been implicated in the pathogenesis of numerous inflammatory disease states[158].

Role of PMNs in ARDS:

The tissue damage and pathophysiology of ARDS is driven by an aggressive inflammatory response. The initiation of this response is choreographed by the alveolar macrophage, which acts through various inflammatory mediators to rapidly recruit PMNs to lung tissue. Recruited PMNs actively migrate to the site of infection or inflammation. Bactericidal action of PMNs occurs by phagocytosis of the invading organism, or by release of cytotoxic granules and reactive oxygen species into the extracellular environment[159]. During normal immune function specialized complexes within the PMN allow the cell to produce highly reactive oxygen species such as superoxide radicals, hydrogen peroxide, and hydroxyl radicals. These compounds along with numerous proteases found in specialized granules and secretory vesicles are used to kill ingested microbes[160;161]. PMNs can also release these granules and reactive oxygen species into the extracellular environment. These compounds interact with microbes in a variety of manners, eliciting damage to their cell membranes, disabling key metabolic pathways, and causing damage to their DNA[159;162]. The critical role of the neutrophil in ARDS was first suggested in studies in which LPS injections were shown to increase PMN sequestration to pulmonary tissue[163], and that PMN depletion prior to insult could prevent ARDS symptoms[164]. Under normal circumstances PMNs constitute approximately 0.08% of the cell population found in the lung. However, bronchoalveolar lavage fluid cell counts from patients with ARDS have demonstrated that the PMN population increases to constitute as

much as 70% of the cell population[165]. The release of potent neutrophil attractants and activating factors such as LTB₄, IL-8, TNF α , IL-1, and IL-6 by resident alveolar macrophages stimulate the pulmonary microvascular sequestration and activation of PMNs. There is some debate as to the control that the PMN has on the release of cytotoxic compounds upon activation. There have been some reports in the literature that would indicate PMNs typically exert a fair amount of control in their response to stimulation[162], while others report that damage caused by PMNs is due to their lack of control mechanisms[145]. This makes the increased presence of PMNs in pulmonary tissue a key factor in the tissue damage and lung injury during SIRS/sepsis and ARDS [145;150;152;165]. Resolution of ARDS is highly dependent on clearance of PMNs from the lung, while preventing the release of cytotoxic contents of the cell. Programmed cell death, or apoptosis plays a critical role in accomplishing this task. Apoptotic PMNs lose their ability to produce and release cytotoxic compounds, and alveolar macrophages recognize specific signaling molecules that are present on the extracellular membranes of these dying cells. Macrophages phagocytize the apoptotic PMNs, preventing granule and superoxide release[166].

Granules and PMN Function:

The specialized nature of PMNs as highly adaptable cells of immune function stems from their granules and secretory vesicles. For decades it was believed that these structures, unique to PMNs, were simple storage compartments for bactericidal compounds. Indeed, PMN granules contain a vast arsenal of proteolytic and oxidative chemicals that, when released into the extracellular environment or fused with the phagocytic vesicle, efficiently kill bacteria[162]. However, the membrane portion of PMN granules and secretory vesicles contain numerous protein receptors and ligands that become expressed upon fusion of the granule with the cell membrane[161]. The expression of these factors allows

the PMN to effectively interact with the extracellular environment, transforming it from a passively circulating cell to an agent of the innate immune system.

Neutrophil Activation:

Neutrophils are highly specialized cells of immune function. Features of these cells allow them to rapidly shift from a passive role as a component of the blood milieu, to aggressive and effective cytotoxic cells in presence of infected or inflamed tissue. This process is mediated by several signaling cascades, including cytokine, chemokine, and eicosanoid release. These factors initiate cellular changes that recruit, prime, and induce the cytotoxic activity of neutrophils.

Neutrophil sequestration and activation occurs in a series of steps that alter form and function to allow them to first migrate to the site of infection or inflammatory stimulus and then to release their cytotoxic components to effect bacterial killing. There are a variety of stimulating factors that can interact with PMNs to effect these changes. These can include bacterial products such as LPS, or cytokine or eicosanoid products released by macrophages. Regardless of source, activation of PMNs induces cytokine production as well as physiological changes in the cells such as the up-regulation of adhesion molecules, alteration of PMN shape, and killing potential[167]. One of the effects of PMN activation by LPS is a change in the cytoskeletal structure of the cells that prevents them from being able to alter their shape to squeeze through small capillary beds. It has been suggested that cytoskeletal alterations may play a role in the influx of some of the PMNS in the primary phase of ARDS. These PMNs become trapped in the pulmonary vasculature and begin releasing cytokines. This cytokine release recruits more PMNs to the area, where they become activated and begin emigrating to the pulmonary parenchyma[167]. Although LPS can directly exert an effect on PMN function, alveolar macrophages play a more prominent role as effectors of PMN function during sepsis and ARDS. For example, IL-1 and TNF- α have both been shown to be

potent activators of PMNs. These two cytokines operate through different surface receptor families that converge with induction of NF κ B translocation to the nucleus leading to PMN activation[145;145;168]. PMN exposure to TNF- α leads to production and release of elastase, superoxide ion, hydrogen peroxide, soluble phospholipase A₂ (sPLA₂), platelet aggregating factor (PAF), thromboxane A₂, and leukotriene production[145]. IL-1 has several overlapping effects on PMNs, stimulating production and release of elastase, collagenase, and prostaglandins. Another factor in PMN infiltration of lung tissue is IL-8. As previously mentioned IL-8 is a member of the chemokine family that is produced primarily in the lung by pulmonary macrophages and is a potent chemoattractant of PMNs. IL-8 concentrations were higher in BAL fluid from hospitalized patients that subsequently developed ARDS as compared with those who did not fully develop the condition[169].

Neutrophil Migration:

The crucial role of PMNs as the primary defensive cell against microbial infection comes from the attribute of directed action and controlled release of cytotoxic granules at the site of inflammation. The PMN inflammatory response is considered to be a multi-step process. The first phase of PMN activation is the recruitment phase. During this phase PMNs undergo an alteration of receptors and adhesion molecules expressed on the surface of the cells. These alterations in the PMN membrane change the cells from passive circulating cells to active and potent participants in innate immunity. PMN recruitment occurs in three distinct phases; rolling, firm adhesion, and finally extra-vascular migration[169]. PMN recruitment involves a series of regulated exocytotic events involving intracellular granules and secretory vesicles that gradually change the functional state of PMNs[162].

The rolling phase of PMN migration involves co-stimulation of vascular endothelium near the site of inflammation. Activated vascular endothelial cells translocate P-selectin to their luminal membranes. This endothelial cell ligand

activates P-selectin glycoprotein ligand-1 (PSGL-1) on the PMN. The interaction between these ligands promotes PMN rolling along the endothelium[170]. Activated endothelial cells also up-regulate E-selectin and the glycosylated cell adhesion molecules (glycans) that are ligands for PMN adhesion molecules CD15 and L-selectin, respectively[145;162;167].

After the velocity of the circulating PMN has been slowed by interactions between selectins, a second series of adhesion molecules are expressed that form a firm adhesion between PMNs and activated vascular endothelial cells. This adhesion is mediated primarily by increased expression of the $\beta 2$ integrin CD11/CD18 on the surface of the PMN. CD11/CD18 is found in high concentrations along the membranes of secretory vesicles. The mobilization of these vesicles during this phase enriches the surface of the PMN with CD11/CD18, while inducing shedding of L-selectin. These changes allow interaction between the $\beta 2$ integrins and intercellular adhesion molecules (ICAMs) found on the vascular endothelial cells, forming a firm adhesion between the PMN and vascular endothelium. Interactions between CD11/CD18 and the ICAMs also prepare PMNs and local endothelial cells for extravasation[145;167;171].

The final step in PMN migration is extravascular migration to the site of inflammation. Migration is directed by a number of factors, primarily the existence of a concentration gradient of chemoattractants that draw the PMN to the tissue. This process is localized at endothelial cell junctions and involves platelet-endothelial cell adhesion molecule-1 (PECAM-1). PECAM-1 is found in high concentrations in junctions between vascular endothelial cells. It has been shown to act as a direct adhesion molecule for PMNs, and may also provide a measure of protection to preserve the permeability of vascular endothelium. Blocking PECAM-1 by monoclonal antibodies decreases PMN extravascular migration significantly[171;172]. Following migration through the junctions of vascular endothelial cells, PMNs release gelatinases, and collagenolytic metalloproteases that degrade the basement membrane surrounding blood

vessels. As PMNs proceed toward the site of inflammation special matrix-sensing receptors are expressed on the cell membrane. These receptors choreograph release of collagenases and serine proteases that allow the PMN to effectively “drill” through the tissue toward chemotactic stimuli[145;162;165].

Cytotoxic Activity of PMNs:

PMN mediated bacterial killing can occur by one of two mechanisms. Primarily, PMNs act by engulfing microbes in a phagocytic vacuole. Once internalized granules mobilize and merge with the membrane of the vacuole. This allows release of granular contents into a controlled microenvironment. Secondly, mobilized granules and secretory vesicles interact with PMN cell membranes to exocytose their contents into the extracellular environment. This second type of granule release is not only involved with bacterial killing, but is also an important mechanism by which the PMN migrates to sites of infection across extracellular tissue[162;167].

Upon reaching the site of infection PMNs have several tools at their disposal to kill invading microbes. First, is an arsenal of proteolytic enzymes, which are stored in the granules of the PMN. Granular storage protects the PMN from its own arsenal of cytotoxic compounds while providing a mechanism for a controlled response upon stimulation[158]. Fusion of these granules with either the cell membrane or the phagocytic vacuole causes release into the microenvironment. As previously mentioned there are several granule types, each having different protein constituents for bacterial killing. Primarily azurophilic granules contain high concentrations of myeloperoxidase, but also contain α -defensins, and bacterial permeability increasing protein[173]. These granules also have high concentrations of proteases such as proteinase 3, cathepsin G, and elastase[161]. The α -defensins are small cationic antimicrobial and cytotoxic peptides that perforate the cell membrane leaving small multimeric holes[174]. Myeloperoxidase plays a vital role in the formation of reactive oxygen metabolites from NADPH oxidase-derived hydrogen peroxide[159].

Bacterial permeability increasing protein binds with membrane LPS on gram negative bacteria and causes a rearrangement of the lipids in the outer and inner membranes of the bacteria[175]. Proteases are examples of PMN components directed against tissue of the intercellular matrix. These compounds quickly degrade components such as type IV collagen, vitronectin, fibronectin, and elastin.

Specific and gelatinase granules contain high levels of bactericidal and matrix degrading proteins. These include lysozyme, lactoferrin, and matrix metalloproteases[176-178]. Lysozyme is a protease that is present in all granules; it is found in highest concentrations in the specific granules. Lysozyme cleaves the peptidoglycan polymers of bacterial cell walls. Lysozyme also acts as an anti-inflammatory agent by binding to LPS, and reducing its ability to bind with CD14 receptors. Lactoferrin is found in specific granules, and impairs bacterial growth through a sequestration of Fe^{2+} . Matrix metalloproteases are found primarily in gelatinase granules. These compounds include leukolysin and gelatinase, both of which are responsible for the degradation of the extracellular matrix. These proteases are particularly effective against collagens, fibronectin, proteoglycans, laminin, and gelatin. Gelatinase granules are mobilized in response to activation of specialized matrix sensing receptors, allowing the PMN to tunnel to the site of infection[179]. Gelatinase, in particular, has been implicated for its role in the disruption of the alveolar basement membrane, and edema formation in ARDS[180].

The second mechanism by which PMNs deal with infection is by production of reactive oxygen species (ROS). ROS production is managed by the NADPH oxidase enzyme complex[160]. In its native state the NADPH oxidase enzyme is stored in four separate parts. The primary component, cytochrome b_{558} is found in abundance on the surface of specific granules. Cytochrome b_{558} becomes expressed on the phagocytic vacuole and the cell membrane following fusion with specific granules. Cell activation also induces relocation of other constituents of the NADPH oxidase complex, p47^{phox} , p67^{phox} ,

and p40^{phox}, along with GTP binding protein to b₅₅₈, thereby completing the complex[181]. Once assembled, the NADPH oxidase complex facilitates transfer of electrons from cytosolic NADPH to molecular oxygen in either the phagocytic vacuole or the extracellular environment. This transfer is dependent on phosphorylation of p47^{phox} at several serine residues. This phosphorylation changes the structure of the molecule to allow a low affinity binding of NADPH.

Activation of the NADPH complex results in formation of superoxide (O_2^-) radicals, which are bactericidal in their own right, but interact with the myeloperoxidase enzyme complex to form hydrogen peroxide (H_2O_2). This reaction accounts for the majority of the oxygen consumption observed during the respiratory burst of the PMN[159;182]. Myeloperoxidase can then use H_2O_2 to oxidize a number of other molecules to form other reactive species. One of the more unique ROS formed by myeloperoxidase activity is hypochlorous acid (HOCl). HOCl is a strong non-radical oxidant of a wide range of biological compounds, and accounts for much of the bactericidal activity of ROS generation[183]. Further, HOCl along with superoxide radicals are used by myeloperoxidase to form an even more reactive compound, the hydroxyl radical. There is some debate as to the actual *in vivo* formation of these molecules by PMNs; however, they have been shown to be highly reactive with most biological compounds causing DNA modification and strand breaks, enzyme inactivation, and lipid peroxidation[159]. Myeloperoxidase can also combine catalytically HOCl with amine groups to form chloramines. Chloramines are long lived oxidants that react with thiols and thioesters. The degradation of chloramines leads to the formation of toxic aldehyde compounds[159;160].

As illustrated here, PMNs possess a number of systems designed to destroy invading microorganisms. During inflammatory conditions such as ARDS, in which there is an overabundance of PMNs these same compounds can cause massive damage to host tissue as well. There are a number of theories as to why this occurs, one of which is the “Proteinase-antiproteinase balance theory” of PMN inflammation[158]. In order to avoid PMN-mediated tissue damage

during infection, host cells have developed several counteracting compounds that inactivate PMN-derived proteases and ROSs. During acute PMN influx release of cytotoxic compounds effectively overwhelms the counterbalancing compounds and cause damage to host tissue. Release of ROS and proteases by PMNs can either be intentionally via granule exocytosis or by loss of PMN membrane integrity during necrosis. Apoptotic PMNs retain membrane integrity as assessed by vital dye exclusion assays. Further, initiation of apoptosis in PMNs leads to a decrease in granule mobilization in response to stimulus. In these regards, apoptosis plays a major role in PMN clearance without release of cytotoxic granules[102;184].

Neutrophil Apoptosis and ARDS:

The clearance of PMNs from the lung during ARDS or ALI without the release of cellular content is a vital step in the resolution of inflammation. Recognition of apoptotic PMNs by alveolar macrophages triggers phagocytic engulfment of the PMN before membrane rupture occurs, resulting in spillage of cytotoxic compounds into the extracellular environment[185;186].

Neutrophil apoptosis represents the first step in clearance of inflammatory cells from areas of inflammation. In order to prevent accidental release of cytotoxic compounds into the lung, alveolar macrophages can detect PMNs that are undergoing apoptosis. Detection of these cells induces phagocytosis of PMNs by macrophages. If not detected and engulfed, PMNs will undergo secondary necrosis. This is associated with a loss in membrane integrity and a release of cytotoxic compounds. This release is associated with detachment and death of human lung epithelial cells *in vitro*[187]. Phagocytosis of senescent PMNs prevents release of cytotoxic compounds into host tissue. Apoptotic cell recognition by macrophages is still poorly understood, and several models have been suggested[186;188;189]. Macrophages have a large capacity for engulfing PMNs, which make them a major source of clearance for inflammatory cells. Phagocytosis of microbial particles induces production of proinflammatory

mediators such as $\text{TNF}\alpha$, IL-1, etc., whereas phagocytosis of apoptotic PMNs by macrophages has also been shown to inhibit proinflammatory cytokine production, while increasing production of anti-inflammatory compounds such as PGE_2 , $\text{TGF-}\beta$, and PAF[188]. These findings emphasize the importance of apoptosis in the clearance of inflammatory cells and the resolution of inflammation. This system of recognition is prone to disruption, however, and studies have shown that recognition of apoptotic PMNs can be decreased in low pH environments, or in presence of cationic molecules. During conditions such as ARDS the presence of high numbers of PMNs can create such an environment, which could be one explanation for progression of the disease state[166].

VII. Fatty Acids in ARDS

Experiments from our lab by Mancuso et al. demonstrated that feeding diets supplemented with 20% fish oil for 21 days significantly attenuated lung permeability and hypotensive effects compared with control animals fed diets containing 20% corn oil for 21 days in LPS induced acute lung injury[72;73]. These experiments also demonstrated that diets containing fish oil were able to significantly reduce concentrations of LTB₄, PGE₂, and TXB₂ produced by macrophages collected from BAL fluid of animals and stimulated *in vitro* by the calcium ionophore, A23187. These reductions were accompanied by decreased accumulation of AA in tissue phospholipids, along with increased EPA in animals fed diets containing fish oil[71]. Further studies by Mancuso demonstrated that fish oil diets could significantly reduce levels of myeloperoxidase activity in the lung when compared with animals fed a corn oil diet with LPS acute lung injury. Animals fed diets containing fish oil and administered LPS to induce lung injury also exhibited a reduction in the number of PMNs found in the BAL fluid when compared with animals fed the corn oil diet[72]. These studies culminated in clinical trials where patients were fed a custom high fat, low carbohydrate enteral nutrition formula containing a combination of fish and borage oils as well as antioxidants. Patients fed this formula demonstrated a significant decrease in PMN accumulation in recovered BAL fluid as compared with patients fed an isocaloric, isonitrogenous diet deriving its main lipid content from corn oil. Accompanying the reduction in PMNs, patients fed the fish and borage oil diets demonstrated significant improvements in oxygenation, and significantly decreased dependence on ventilatory support. These improvements resulted in a decreased stay time in the intensive care unit for patients fed the fish oil and borage oil supplemented diets when compared with controls[190]. Since eicosanoid products have been shown to be involved in regulation of PMN apoptosis, and their diets were shown to reduce the concentration of pro-inflammatory eicosanoid products interest was raised as to the role that these

fatty acids might have on PMN lifespan[57]. Later work from our laboratory by Gillis investigated the possible interaction of diet and apoptosis using the human promyelocytic cell line HL-60[191;192]. The results of these experiments showed that direct treatment of HL-60 cells with either EPA or GLA ethyl esters alone or in combination would significantly decrease cell viability and that this decrease was through an increase in apoptosis[192]. Since previous work from our laboratory had demonstrated that decreased PMNs and symptoms of lung injury from endotoxic rats were accompanied by a significant reduction in eicosanoid products, we examined whether the effects of fatty acid treatment at inducing apoptosis in HL-60 cells could be replicated by inhibiting the pathways of eicosanoid metabolism[191]. These studies found that blockage of the 5-LO pathway, but not the COX pathways could induce apoptosis, implying a critical role for the products of 5-LO metabolism in the regulation of HL-60 cell apoptosis. Taken together, these studies demonstrate the potential for n-3 fatty acids to influence the course of the inflammatory response in ARDS, and suggest that this impact could be due to an interaction between diet and neutrophil viability.

VIII. Summary of Literature and Introduction to Current Study

Fatty acids of the n-6 and n-3 families are important substrates for the formation of inflammatory mediators. The 20-carbon members of these families; AA and EPA, respectively, are competitive substrates for storage in cell membrane phospholipids and for the formation of eicosanoid products. Eicosanoid products of AA are considered to be pro-inflammatory, and have been shown to have numerous effects including the chemotaxis and activation of PMNs. Eicosanoid products of EPA are less biologically active than those from EPA, and are considered to be anti-inflammatory. The addition of n-3 PUFAs to the diet reduces concentrations of n-6 PUFAs in the cell membrane and reduces the production of pro-inflammatory n-6-derived eicosanoids.

It has been suggested that an increased intake of n-3 fatty acids into the typical western diet may provide numerous health benefits. However, due to concerns with safety and supplies of natural fish stocks these suggestions have been left largely unmet. This has led to interest in identifying alternatives to fish oil for increasing n-3 fatty acid content in the diet. Addition of ALA to the diet has been explored, but the conversion of ALA to EPA has been shown to be inefficient. Thus, there is doubt as to whether dietary supplementation with ALA provides a suitable alternative to fish oil for increasing n-3 incorporation into tissue. Recent developments in the agricultural industry have yielded crop plants that are capable of producing SDA in appreciable quantities, providing a potential renewable resource of n-3 PUFAs.

Dietary supplementation with n-3 PUFAs from fish oil has demonstrated the ability to decrease eicosanoid concentrations and neutrophil accumulation in BAL fluid in patients and animals with ARDS or LPS-mediated acute lung injury. Reduction of neutrophil numbers in the lung has been correlated with improved outcomes in both patient and animal models. It is not currently known if diets containing n-3 fatty acids affect neutrophil numbers by reducing neutrophil chemotaxis or by increasing neutrophil apoptosis.

This thesis has been designed to test two hypotheses; first, to examine if diets supplemented with SDA ethyl ester increases EPA content in liver phospholipids and if this increase is similar to those observed in animals fed a diet containing EPA. Second, if dietary supplementation with SDA or EPA will impact the regulation of constitutive and endotoxin-induced survival in neutrophils by influencing the rate of apoptosis in both cell states.

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Part 3
Materials and Methods

I. Materials and Methods

Animals: Male Long-Evans Rats (N = 18) weighing between 200-250g were acquired from Taconic (Germantown, NY). Animals were housed at the animal care facility at The University of Tennessee Medical Center Knoxville at temperature ~24° C and on a 12h light: dark cycle. After arrival at the animal care facility, rats were fed Harlan/Teklad 22/5 Rodent Diet (W) 8640 (Harlan Industries Indianapolis, IN) for 4 days before being placed into individual metabolic chambers for the feeding period of the study. Fresh water was provided *ad libitum*. Animal weights and urine volumes were recorded daily. Food was withheld overnight before animals were killed. All animal procedures were approved by The University of Tennessee Animal Care and Use Committee and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Diets: Animals were randomly divided into 3 dietary groups (n=6 animals/group) and were fed 200 kcal/ kg body weight/ day for 14 days. All experimental diets were based on a typical western diet with the following caloric distribution: 20.62% of calories from protein, 47.18% of calories from carbohydrates, and 32.19% of calories from lipids. This experimental diet mix was predicated on the US17 diet formulated by the Monsanto corporation (St. Louis, MO)[1] (Table 3.1). The experimental diets contained either 10g/kg (1%) eicosapentaenoic acid (EPA, 20:5; n-3, 95% pure) and 1% Trisun (ACH Humko, Cordova, TN) high oleic acid sunflower oil (OA, 18:1; n-9, 85% pure), or 10g/kg (1%) stearidonic acid (SDA, 18:4; n-3, 85% pure) and 1% Trisun high OA sunflower seed oil. This design allowed fatty acids to be added at the expense of Trisun high oleic acid sunflower oil[1-3] without altering caloric density among diets. Control diet, or OA diet, was prepared by adding 20g/kg (2%) Trisun to the base diet.

Table 3.1:
Experimental Diet Composition

Component	OA (g/kg)	EPA (g/kg)	SDA (g/kg)
Casein	229	229	229
L-Cystein	3	3	3
Cornstarch	274	274	274
Maltodextrin 10	86	86	86
Sucrose	114	114	114
Cellulose	57	57	57
Cocoa Butter	43	43	43
Linseed Oil	5	5	5
Palm Oil	60	60	60
Safflower Oil	33	33	33
Sunflower Oil (Trisun)	20	10	10
20:5(n-3) ethyl ester (EPA)	0	10	0
18:4(n-3) ethyl ester (SDA)	0	0	10
Salt Mix RD-96	11	11	11
Dicalcium Phosphate	6	6	6
Calcium carbonate	19	19	19
Vitamin mix V134014	11	11	11
Choline bitartate	2	2	2
α -Vitamin E acetate	0.15	0.15	0.15
TBHQ	0.03	0.03	0.03
kcal/kg protein	928	928	928
kcal/kg carbohydrates	2124	2124	2124
kcal/kg lipids	1449	1449	1449
total kcal/kg	4501	4501	4501
kcal/g chow	4.501	4.501	4.501
	Carbohydrates	Protein	Lipids
Energy distribution (%)	47.2	20.6	32.2

Diets had a final caloric density of 4.5kcal/g. All diets were mixed by hand under yellow light, and daily feedings were aliquoted into whirl-pak (Nasco, Ft. Atkinson, Wisconsin) containers, flushed with N₂ gas to prevent peroxidation, and stored at -80° C until time of use. Gas chromatography was used to confirm the fatty acid content of each diet[1-3] (Table 3.2).

Cells: All chemicals were purchased from Sigma Chemical (St. Louis, MO) unless otherwise noted. Neutrophils were obtained by lavage from the peritoneal cavity after glycogen elicitation[4-6]. Rats were anesthetized with isoflurane (Abbott Labs, North Chicago, IL) and given a 30ml intraperitoneal injection of 1% type 2 oyster glycogen dissolved in 1x Dulbecco's PBS without Ca²⁺ or Mg³⁺. Animals were allowed to recover for 4h and then re-anesthetized with isoflurane. A cardiac puncture was performed and animals were exsanguinated. The musculature of the abdominal cavity was exposed through an incision in the skin and peritoneal lavage was performed using a 30ml syringe fitted with an 18 gauge blunt tipped needle to flush the peritoneal cavity with 50ml of ice cold 1x Dulbecco's PBS without Ca²⁺ or Mg³⁺ and with 1mM EDTA (Fluka Chemical, Switzerland) and 0.01% v/v heparin (American Pharmaceuticals Partners, Inc., Schaumburg, IL). Lavage fluid was briefly agitated by massaging the abdominal wall and withdrawn from the opposite side. Fluid was pooled in a 50mL conical centrifuge tube (Becton-Dickinson, Franklin, NJ) and peritoneal cells were pelleted by centrifuging at 700 x g for 7min in a Sorvall RT6000B refrigerated centrifuge (Sorvall, Newtown, CT). Supernatant was carefully aspirated and contaminating red blood cells were removed by hypotonic lysis using 1ml pyrogen free cell culture water. After 1 min of lysis, 0.5ml of 3x Dulbecco's PBS was used to restore tonicity[7]. Cells were then resuspended in 50ml of 1x Dulbecco's PBS. Cell number and viability were assessed using a Neubauer hemacytometer and trypan blue dye exclusion assay. Glycogen elicitation yielded > 98% pure neutrophil populations as evaluated during hemacytometry. Cell concentrations were adjusted to 1×10^6 cells/ml. Cells for each incubation

Table 3.2:
Experimental Diet Fatty Acid Composition.

Fatty Acid	Dietary Groups		
	OA	EPA	SDA
	g/100g total fatty acids		
12:0	0.36	0.33	0.34
14:0	0.56	0.52	0.53
16:0	26.09	25.21	25.15
16:1	0.17	0.17	0.16
17:0	0.13	0.12	0.12
18:0	12.75	12.16	12.13
18:1 (n-9)	35.06	31.2	31.21
18:2 (n-6)	20.23	19.82	19.69
18:3 (n-3)	2.1	2.05	2.05
18:4 (n-3)	ND	ND	5.26
20:0	0.56	0.54	0.53
20:1	0.14	0.13	0.12
20:4 (n-6)	ND	0.18	ND
20:5 (n-3)	ND	5.44	ND
22:0	0.24	0.2	0.2
22:6 (n-3)	0.13	0.11	0.11

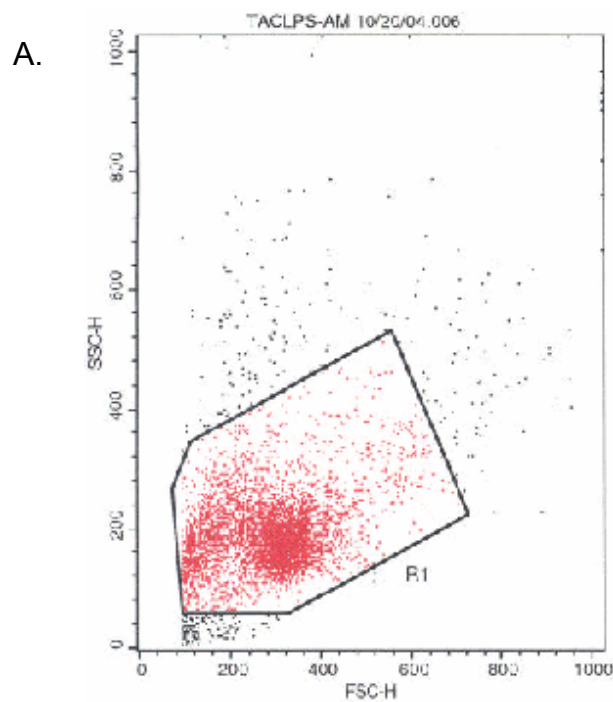
ND indicates not detectable.

were separated and placed into 15ml conical centrifuge tubes (Becton Dickinson Labware, Franklin Lakes, NJ) and were pelleted by centrifugation at 700 x g for 7min and resuspended at 1×10^6 cells/ml in RPMI 1640 without phenol red and supplemented with 10% heat inactivated fetal bovine serum, 1% glucosamine, and 1% penicillin/ streptomycin. Cells were transferred to 12x75mm polypropylene tubes (Becton Dickinson Labware, Franklin Lakes, NJ) containing pre-warmed media and allowed to equilibrate.

Cell Treatment and Incubation: Cells from each feeding group were incubated for 20h in either the presence or absence of $1 \mu\text{g/ml}$ of *Salmonella enteritidis* lipopolysaccharide (LPS, Sigma Chemicals, St. Louis, MO). A positive control for apoptosis was obtained by incubating cells for either 4 or 20h in presence of $5 \mu\text{M}$ camptothecin (CAM, Sigma Chemicals, St. Louis, MO). All treatment and control tubes were prepared in triplicate and contained a final concentration of 250,000 cells/ml. Atmosphere was maintained at 37°C and 5% CO_2 / 95% room air mix in a NAPCO 5410 incubator (Precision Scientific, Chicago, IL).

Flow cytometry: Cell preparation and staining for flow cytometric analysis of apoptosis was performed using an Annexin-V FITC Apoptosis Detection Kit (BD Pharmingen, San Diego, CA). At 0 and 20h, cells from control and LPS treatment groups were pelleted by spinning at 700xg for 7 min using a Sorvall RT6000B refrigerated centrifuge. CAM treated cells were pelleted at 4h or 20h but otherwise were handled identically to other treatments. Supernatants were carefully removed and cells were resuspended in 1ml of PBS containing 0.1% bovine serum albumin (BSA). Cells were transferred to 2ml polypropylene tubes and pelleted using a model 5415 D eppendorf centrifuge (Brinkmann Industries, Westbury, NY). Supernatant was removed and $100 \mu\text{l}$ of annexin-V binding buffer (kit) was added to each tube. Cells were mixed by flicking the side of the tube followed by addition of $5 \mu\text{l}$ of annexin-V protein and $10 \mu\text{l}$ of propidium iodide (PI) (both from kit) in the dark. Cells were then allowed to incubate for 15 min at

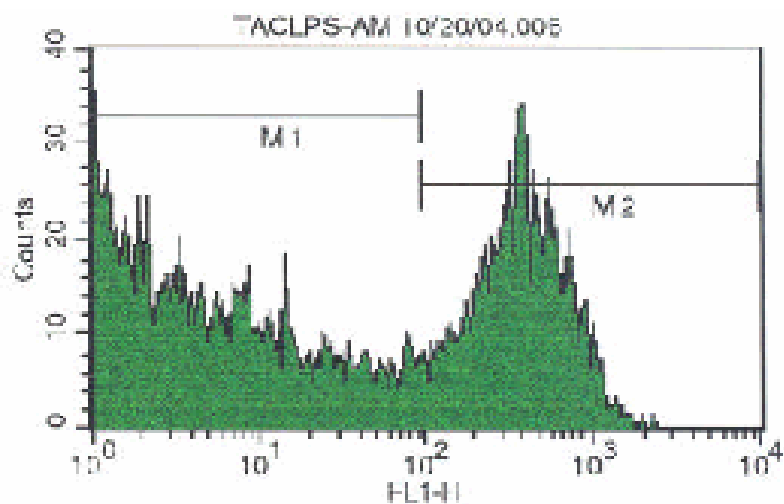
room temperature in the dark. Following incubation, 400 μ l of annexin-V binding buffer was added to each tube. Cells were transferred to 12 x 75mm polystyrene tubes (Becton Dickinson Labware, Franklin Lakes, NJ), placed on ice and kept in the dark until analysis. Flow cytometry was performed using a Becton-Dickinson FACScan flow cytometer and analyzed using Cell Quest software v. 1.2 (Becton Dickinson, San Jose, CA). Unstained, PI positive/ annexin-V negative, and annexin-V positive/ PI negative cells were used to adjust gain and compensation levels, as well as to properly adjust scatter plot and gate settings of the flow cytometer (figure 3.1). The main cell population was isolated based on size and complexity from the forward scatter and side scatter dot-plot. This population was liberally gated to encompass viable, necrotic, and apoptotic PMNs, while excluding smaller aggregates and debris components. FL-1 represents a typical histogram representation of annexin-V fluorescence, while FL-2 represents a typical histogram of PI fluorescence. Annexin-V positive and PI positive cells were defined by histogram markers. M1 represents cells that exhibit either negative or dim fluorescence, which are considered negative for annexin-V or PI, respectively. M2 represents brightly stained cells that are considered to be positively stained for either annexin-V or PI. M3 region was used to calculate the number of cells that were only dimly stained (data not shown). Figure 3.1 also illustrates a typical scatter-plot of cell fluorescence. Each quadrant is labeled as being either annexin-V +/- or PI +/-, and represents cell condition. Cells in the lower left quadrant are considered viable since they were not stained with either PI or annexin-V. The lower right quadrant contains cells that are PI negative and annexin-V positive and are considered apoptotic. Cells in the upper right quadrant are cells that are positively stained for both annexin-V and PI. Since dual staining indicates cells with DNA damage representative of apoptosis as well as PI permeable membranes these cells are defined as being secondary necrotic cells. *In vitro*, secondary necrotic cells have been defined to be in the



(A) Flow cytometric representation of forward scatter versus side scatter.
¹Red cells in Region R1 represent gated cell population used for analysis.

Figure 3.1. Settings and Representative Report From Flow Cytometry Analysis (A-D).

B.

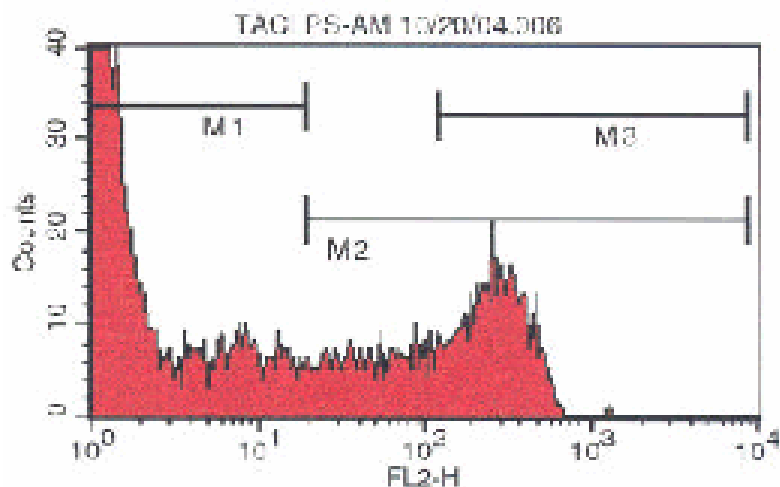


(B) Fluorescence histogram of Annexin-V

M1 represents unstained or dimly stained cells that were considered annexin-V negative.

M2 represents brightly stained cells that were considered annexin-V positive.

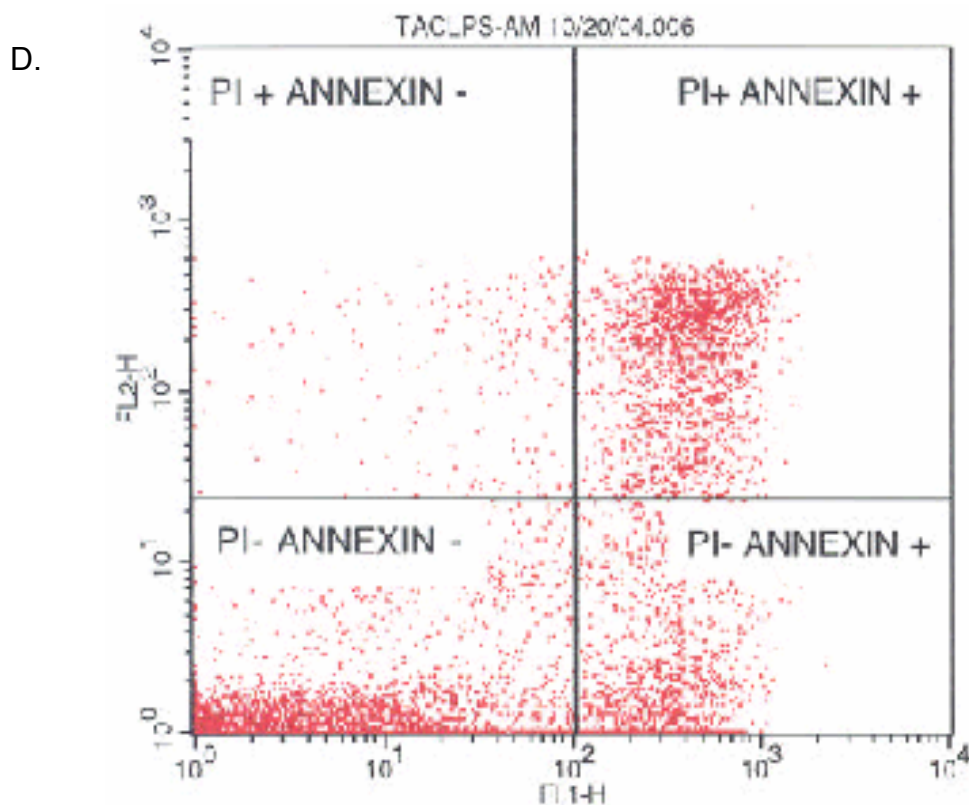
C.



(C) Fluorescence Histogram of propidium iodide.

M1 represents unstained or dimly stained cells that were considered PI negative. M2 represents brightly stained cells that were considered PI positive. M3 was used to calculate dimly stained cells (data not shown).

Figure 3.1. Continued.



(D) Scatter-plot representation of cell staining.
 PI- ANNEXIN - represents unstained viable cells.
 PI- ANNEXIN + represents annexin stained apoptotic cells.
 PI+ ANNEXIN + represents dually stained secondary necrotic cells.
 PI+ ANNEXIN - represents PI stained necrotic cells.

Figure 3.1. Continued.

phase following apoptosis[8]. Finally cells in the upper left quadrant are considered to be necrotic, as their membranes have been permeablized to PI without occurrence of DNA cleavage. The lower right and upper right quadrants were also combined to determine the total number annexin-V stained cells regardless of PI staining. These cells were considered to represent those both actively undergoing apoptosis, as well as those that had previously died through the apoptotic route. This data set was termed “cell death”.

DNA Laddering: Presence of apoptosis in peritoneal cells from animals from each feeding group (n=2) was confirmed by agarose gel electrophoresis[9]. For each gel 1×10^6 cells from 0h or from 20h treatment groups were transferred into 2ml snap-top eppendorf tubes. Cells were pelleted at 700 x g for 7min using an eppendorf centrifuge, model 5415D (Brinkmann instruments, Westbury, NY), the supernatant was carefully removed, and cell pellets were flash frozen in liquid nitrogen. Cells were stored at -80° C until analysis. On day of analysis, cells were allowed to thaw for ~5min and 20 μ l of lysis buffer (20mM EDTA (Mallinckrodt, St. Louis, MO), 100mM TRIS, pH 8.0, 0.8% (w/v) sodium lauryl sarcosine (Bioworld, Dublin, OH)) was added to each tube. Immediately following addition of lysis buffer 10 μ L of 1mg/ml RNase/T1 cocktail mix (Ambion, Austin, TX) was added, and samples were mixed and incubated for 75min at 37°C. Proteinase K 10 μ l (Ambion, Austin, TX) was added to cell lysates which were then incubated for 120min at 50° C. Samples were mixed with 5 μ l loading buffer and loaded onto a 1.5% agarose gel. Gel was run for 200min at 30v in 1x TAE buffer using Bio-Rad Mini-Sub Cell GT (Bio-Rad, Hercules, CA) and a Fisher Biotech FB300 (Fisher Scientific, Pittsburgh, PA) power supply. Molecular marker XIV (Roche, Mannheim, Germany) was used as a reference for DNA fragment size. Cells were exposed to a 4h CAM treatment as a positive control for apoptosis. DNA bands were visualized using ethidium bromide (Promega, Madison, WI) on a Fluorchem 8000 Imager (Alpha Innotech, San Leandro, CA).

TUNEL Assay: Detection of apoptosis by TUNEL assay was performed using the *In Situ* Cell Death Detection Kit, Fluorescein (Roche, Mannheim, Germany). At 0h and for both control and LPS treatment groups at 20h approximately 20,000 cells were cytopun for 5 min at 500 rpm (Cytospin3, Shandon, England) and placed onto glass microscope slides (Fisher Scientific, Pittsburgh, PA). Slides were air dried and fixed for 1h using Carson's solution. Slides were then rinsed using PBS and placed in drying racks and stored until analysis. On day of analysis, slides were rinsed with PBS and 100 μ l of cytofix/cytoperm solution (Pharmingen, San Diego, CA) was applied. Slides were incubated for 20min at 4° C and washed with 100 μ l perm/wash buffer (Pharmingen, San Diego, CA). Slides were rinsed with deionized water, allowed to air dry, and stained for apoptosis using 50 μ l TUNEL reaction mixture (kit) and incubated for 60min at 37° C in a humidified chamber. 100 μ l of Hoechst stain (Molecular Probes, Eugene, OR) was applied to each slide as a counter stain to detect total DNA. Slides were incubated for 30min at 37° and rinsed with PBS. Protective 12 x 50 – 1.5 glass coverslips (Fisher Scientific, Pittsburgh, PA) were applied using Dako fluorescent mounting medium (Dakocytomation, Carpinteria, CA). Slides were viewed using an Olympus BX51 fluorescent microscope (Olympus Optical Co., LTD., Japan) under both FITC and DAPI filters. Images were captured using a Magmafire SP CCD camera (Optronics, Goleta, CA). Image overlay and analysis was performed using Image-Pro Plus software v 4.5 (Media Cybernetics, Silver Spring, MD).

Liver: Following peritoneal lavage a central incision was made along the linea alba exposing the abdominal cavity. The portal vein was located and separated from surrounding connective tissue; ligated using Silk suture (3-0, Ethicon, Somerville, NJ), and liver was perfused with 30cc ice cold PBS using an 18 gauge needle. Liver sections ranging from 1-1.5g were removed and placed in 50ml conical centrifuge tubes, labeled, and flash frozen using liquid nitrogen. Liver sections were stored at -80° C until time of analysis.

Total Liver Fatty Acid Extraction and Phospholipid Separation: Total liver fatty acids were extracted using a modified Bligh and Dyer technique[10-12].

Approximately 200mg of liver from individual animals was placed in a tissue homogenizer along with 1ml 0.9% cold saline/ 100mg liver. Tissue was thoroughly homogenized and 0.8ml of homogenate was transferred into 125x16mm Teflon coated screw top tubes (Corning, New York, NY).

Chloroform:methanol (3ml,1:2) was added to each tube along with 44 μ g of 1,2-diheptadecaenoic-sn-glycerol phosphorycholine (Matreya Biochemicals, Pleasant Gap, PA) as an internal standard. Homogenate solution was vortexed and incubated for 30 min on ice in the dark. Following incubation, 200 μ l saturated NaCl and 1mL chloroform was added to each tube. Tubes were vortexed and incubated for 15 min on ice in the dark followed by centrifugation for 4 min at 900x g to ensure complete phase separation. The bottom chloroform layer was transferred to a 12x75mm borosilicate glass tube (Fisher Scientific, Pittsburgh, PA) using a glass Pasteur pipette. The extraction and transfer process was repeated and samples were completely evaporated under nitrogen. Once both extractions had dried completely they were reconstituted in 50 μ l chloroform, vortexed, and added to pre-scored silica gel 60 HP-TLC plates (Merck, Darmstadt, Germany) for separation of the phospholipid portion by thin layer chromatography (TLC). Plates were placed in a TLC chamber containing approximately 1 inch of chloroform:methanol (8:1) as a mobile solvent system and ran for 15 min. Plates were removed and allowed to dry fully under nitrogen. The phospholipid portion of each lane was scraped onto weighing paper and added to 16x125mm screw top tubes.

Analysis of Phospholipid Fatty Acids: Liver phospholipids were prepared for analysis by gas chromatography (GC)[2;10;12]by first saponifying with 0.4ml of 0.5N NaOH/MeOH for 15 min in an 86° C water bath in tightly sealed 16 x 125 mm screw top tubes. Tubes were then removed from the water bath and allowed

to cool. Fatty acids were methylated by adding 1ml of boron trifluoride/methanol, tightly recapping tubes, and incubating in an 86° C water bath for 10 min. Samples were removed from the water bath, allowed to cool, and acidified with 0.4ml of 0.7 N HCl in MeOH. Following acidification, fatty acid methyl esters were extracted once again by adding 2ml of hexane and 2ml of saturated NaCl solution to each tube. Tubes were vortexed and allowed to settle for approximately 5 min for phase separation to occur. Samples were centrifuged for 4 min at 900 x g for complete separation. The hexane (top) layer was carefully transferred to a new 12 x 75mm borosilicate tube for each sample. A second hexane extraction was performed and pooled with the first and both extractions were completely evaporated under N₂ gas. Dried samples were reconstituted in 100µl of hexane and vortexed vigorously. Samples were analyzed using a Hewlett-Packard model 5890 series II gas chromatograph equipped with flame ionization detector and DB23 fused silica capillary column (0.25 mm i.d. x 30m x 0.25µm film; J&W Scientific, Folsom, CA). Fatty acid separation was achieved by increasing temperature from 160-250° at a rate of 3.5° C / min using hydrogen as the carrier gas. Gas chromatography standards 461 and 68B (Nu-Chek Prep, Elysian, MN) were included in each analysis procedure. Retention times for the known standards were used to identify fatty acid methyl esters.

Lipid Analysis of Diets: The fatty acid composition of each diet was confirmed using gas chromatography. Approximately 50mg of diet was placed into a 16 x 125mm tube along with 3ml chloroform:methanol (8:1) and 44µg of 1,2-diheptadecaenoic-sn-glycerol phosphorycholine as an internal standard and incubated on ice in the dark for 30 min. Solid portions of food were separated by centrifugation and fatty acids from diets were extracted using the modified Bligh and Dyer technique[11] and analyzed using gas chromatography following the same protocol as outlined for liver phospholipids.

Statistical Analysis: Data were expressed as mean $\pm$ SEM. Fatty acid data were analyzed by one-way ANOVA followed by Ryan-Einot-Gabriel-Welch based on ANOVA F test (REGWF) to determine differences among groups. Analyses were performed using CRUNCH v. 4.0 statistical package (Crunch Software Corp., Oakland, CA). Animal weight data were analyzed by repeated measures ANOVA followed by Wilk's Lambda multivariate post-hoc test. Flow cytometry data were used to determine effect of LPS treatment, diet, and interaction between diet and LPS treatment in altering the number of viable cells, cells undergoing early or late apoptosis, or cell death after 20h. Flow cytometry data were analyzed using repeated measures ANOVA followed by Wilk's Lambda multivariate post-hoc test. Analyses were performed using SPSS v. 13.0 for windows (SPSS inc., Chicago, IL). $P \leq 0.05$ was considered significant.

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Part 4

Results

I. Results

Animal Weights: Body weights were recorded daily and reported as percent of body weight taken on the first day. Repeated measures ANOVA indicated no differences in weight during the feeding period (figure 4.1).

Liver Fatty Acid Composition: Liver phospholipid fatty acid composition was determined after 14 days of feeding and reported as mol % (table 4.1). Alterations in liver phospholipids reflected fatty acid supplementation. As compared with dietary OA, animals fed the EPA diet demonstrated a significant decrease in tissue AA levels ($p \leq 0.05$). There was a significant increase in tissue EPA content for animals in the EPA group, as compared with those from the OA group ($p \leq 0.05$). EPA-fed animals demonstrated a significant increase in tissue DPA, as compared with controls ($p \leq 0.05$). Supplementation of the diet with EPA failed to increase DHA levels as compared with OA fed animals. EPA animals exhibited a significant increase in total n-3 fatty acid content, as compared with control animals ($p \leq 0.05$). This was accompanied by a significant decrease in total n-6 fatty acid content of membrane phospholipids as compared with controls ($p \leq 0.05$). These changes were reflected by an increased ratio of (n-3)/(n-6) fatty acids for animals in the EPA group as compared with OA animals ($p \leq 0.05$). The increased ratio of (n-3)/(n-6) PUFAs coincided with a significant increase in the membrane phospholipid ratio of EPA/AA in animals from the EPA group as compared with controls ($p \leq 0.05$).

Dietary SDA produced similar results to those observed in EPA-fed animals in regards to fatty acid composition of membrane phospholipids. Dietary SDA supplementation exhibited a slight, although not significant, decrease in AA content of membrane phospholipids as compared with OA diets. SDA did, however, produce a significant increase in phospholipid levels of EPA as compared with dietary controls ($p \leq 0.05$). SDA also significantly increased the

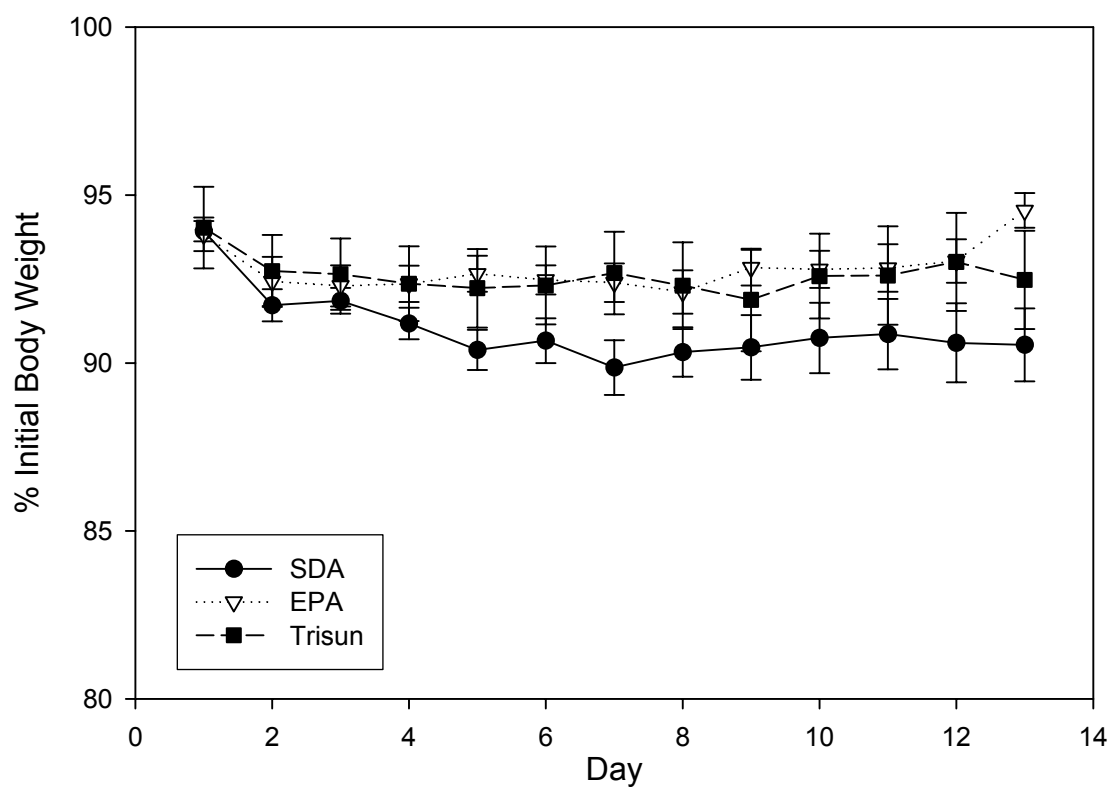


Figure 4.1. Effect of Diet on Animal Weight
¹Data are expressed as mean % initial body weight $\pm$ SEM

Table 4.1:
Fatty Acid Composition of Rat Liver Tissue After Feeding Experimental Diets for 2 Weeks.

Fatty Acid	OA (n =7)	EPA (n = 8)	SDA (n = 7)
16:0	16.08 ± 0.37	15.99 ± 0.41	15.93 ± 0.42
16:1	0.06 ± 0.03	0.10 ± 0.27	0.14 ± 0.03
18:0	26.04 ± 0.25	25.78 ± 0.33	26.07 ± 0.23
18:1 n-9	3.73 ± 0.2	3.50 ± 0.13	3.32 ± 0.23
18:1 n-7	1.55 ± 0.05	1.50 ± 0.05	1.49 ± 0.03
18:2 n-6	15.06 ± 0.55	16.41 ± 0.12	15.5 ± 0.51
18:3(n-6)	0.11 ± 0.02	0.11± 0.02	0.1 ± 0.02
18:3 (n-3)	0.09 ± 0.02	0.07 ± 0.02	0.08 ± 0.01
20:0	0.01 ± 0.01	0.02 ± 0.01	ND
20:1	0.07 ± 0.01	0.07 ± 0.02	0.08 ± 0.02
20:2	0.3 ± 0.05	0.29 ± 0.01	0.26 ± 0.01
20:3 n-6	0.56 ± 0.04	0.49 ± 0.02	0.52 ± 0.03
20:4 n-6	28.35 ± 0.56 ^a	25.64 ± 0.39 ^b	27.48 ± 0.47 ^a
20:5 n-3	0.21 ± 0.05 ^a	1.18 ± 0.17 ^b	0.62 ± 0.07 ^c
22:4 n-6	0.30 ± 0.02 ^a	0.15 ± 0.01 ^b	0.14 ± 0.04 ^b
22:5 n-6	0.14 ± 0.02 ^a	0.04 ± 0.05 ^b	0.09 ± 0.02 ^{ab}
22:5 n-3	0.51 ± 0.04 ^a	1.69 ± 0.10 ^b	1.03 ± 0.02 ^c
22:6 n-3	6.84 ± 0.36	6.96 ± 0.25	7.12 ± 0.42
Total (n-3) FA	7.65 ± 0.41 ^a	9.9 ± 0.35 ^b	8.86 ± 0.37 ^b
Total (n-6) FA	44.52 ± 0.28 ^a	42.84 ± 0.45 ^b	43.83 ± 0.25 ^{ab}
(n-3)/(n-6) ratio	0.17 ± 0.01 ^a	0.23 ± 0.01 ^b	0.2 ± 0.01 ^c
EPA/AA ratio	0.01 ± 0.00 ^a	0.05 ± 0.01 ^b	0.02 ± 0.00 ^a

Data are expressed as mean mole % ± SEM. ND indicates not detectable.

Values with different superscripts indicate significant difference (p<0.05). n indicates number of animals.

DPA content of membrane phospholipids of the liver as compared with animals from the OA group ($p \leq 0.05$). Dietary supplementation with SDA did not result in a significant increase in tissue DHA levels. Although diets containing SDA significantly altered some of the n-6 fatty acids, these changes did not result in a significant decrease of total n-6 levels as compared with controls. However, there was a significant increase in the total n-3 PUFA content of membrane phospholipids of SDA animals as compared with OA fed animals ($p \leq 0.05$). This was reflected by a slight, but significant, increase in the n-3/n-6 fatty acid ratio in membrane phospholipids of SDA-fed animals as compared with controls ($p \leq 0.05$). SDA did not significantly alter ratio of EPA/AA in the membrane phospholipids as compared with control animals.

Addition of SDA or EPA to the diet resulted in an increase in the amount of EPA incorporated into membrane phospholipids in the liver. Dietary SDA was approximately half as effective as dietary EPA at increasing membrane phospholipid EPA levels. Diets supplemented with SDA were about two-thirds as effective as those containing EPA at increasing tissue DPA content above those observed in controls. Neither the SDA nor the EPA diet produced a significant increase in tissue DHA levels.

DNA Laddering: During apoptosis DNA is degraded by endogenous DNases into 180-200bp fragments, and presents a distinct banding pattern when examined using gel electrophoresis. In this study, gel electrophoresis was used to confirm the presence of DNA cleavage, which is indicative of cellular apoptosis. Results shown in Figure (4.2), although not quantitative, provide evidence that after 20h of incubation a number of cells had undergone apoptosis. This banding was present regardless of diet or treatment with LPS. The only lanes in which banding patterns indicative of DNA fragmentation were absent were those containing cells that were collected at 0h.

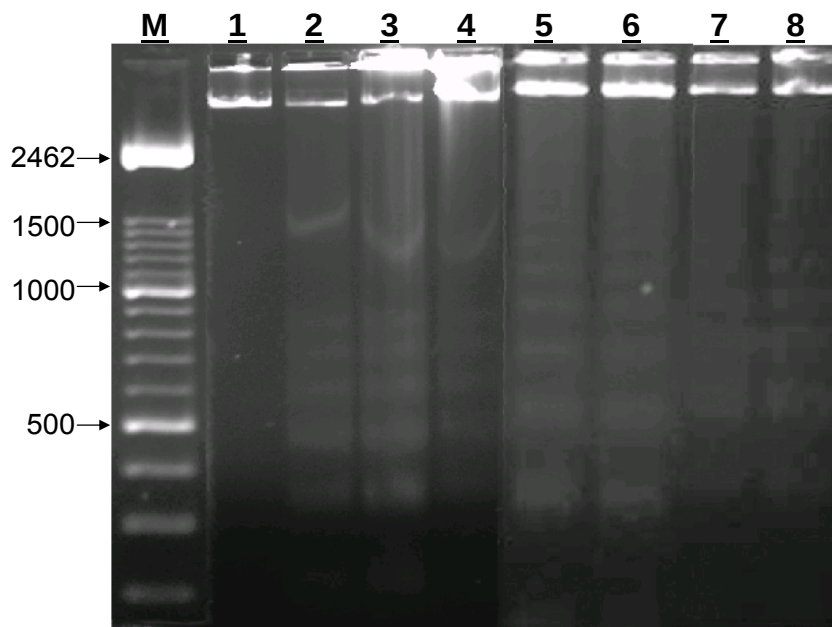


Figure 4.2. Effect of Diet and LPS Treatment on DNA Fragmentation.

Lanes: M=marker lane containing a 100bp ladder of DNA fragments from 2642bp, 1500-100bp; 1=PMNs from OA fed animal at 0h (negative control); 2=OA 20h -LPS; 3=OA 20h+ LPS; 4=OA cells 4h + CAM (positive control); 5=EPA 20h -LPS; 6=EPA 20h +LPS; 7=SDA 20h -LPS; SDA 20 +LPS

TUNEL assay: Examination of slides prepared with both TUNEL and Hoechst stains confirmed the presence of apoptotic bodies among cell populations from all feeding groups. Representative images from fluorescence microscopic examination of stained slides are shown in figure 4.3. These images were created by overlaying digital pictures of a single area of a microscope slide taken under both DAPI and FITC filters. Hoechst stained cell nuclei were used to provide a reference point for regions of the slide that were TUNEL positive. Images shown were taken from slides prepared with cells that had been incubated for 20h in either presence or absence of LPS. Gross examination of slides indicated that treatment of cells with LPS for 20h led to an apparent decrease in the amount of bodies that were positive for TUNEL stain. Slides of cells treated for 20h with LPS also appeared to be more densely populated with Hoechst positive bodies. Under gross examination, slides prepared with cells from animals that were fed either the EPA or the SDA diets also appeared to have fewer apoptotic bodies as compared with those from animals fed the OA diet.

Flow Cytometry: The effect of LPS on viability, apoptosis, secondary necrosis, and cell death in glycogen-elicited neutrophils after dietary treatment is shown in Figure 4.4 (A-D). After 20h incubation diet did not significantly alter viability or LPS-mediated viability in neutrophils. Because there was no interaction between diet and neutrophil viability we examined whether treatment with LPS had any effect on neutrophil survival, irrespective of diet. The effect of LPS treatment, regardless of diet, on viability, apoptosis, secondary necrosis, and cell death are shown in figure 4.5 (A-D). We show that, independent of diet, LPS significantly increased viability and decreased cell death due to a decrease in apoptosis and secondary necrosis. Since apoptosis, secondary necrosis, and cell death at 20 hours of incubation are directly related to freshly isolated (basal) values of these same parameters (basal data not shown), the absolute differences between the basal and 20 hr in absence and presence of LPS were calculated irrespective of

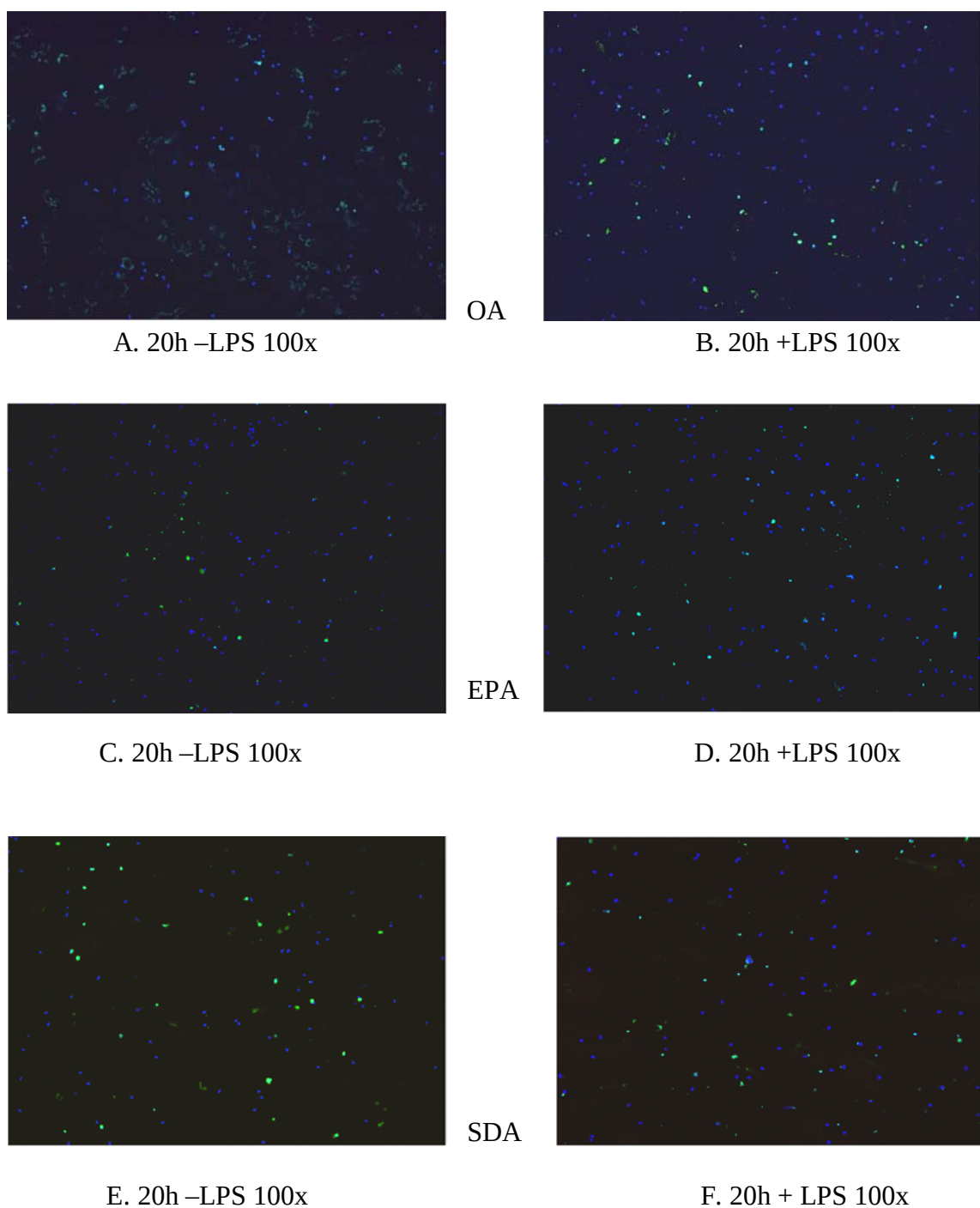
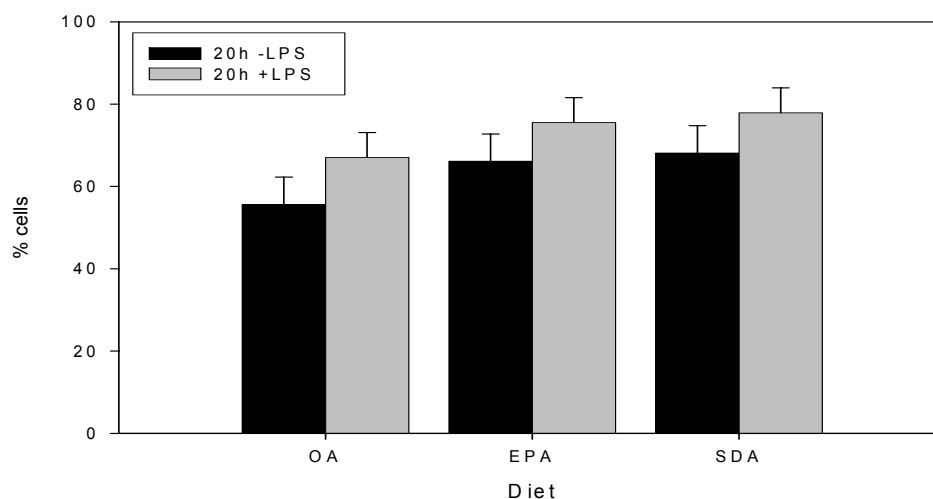


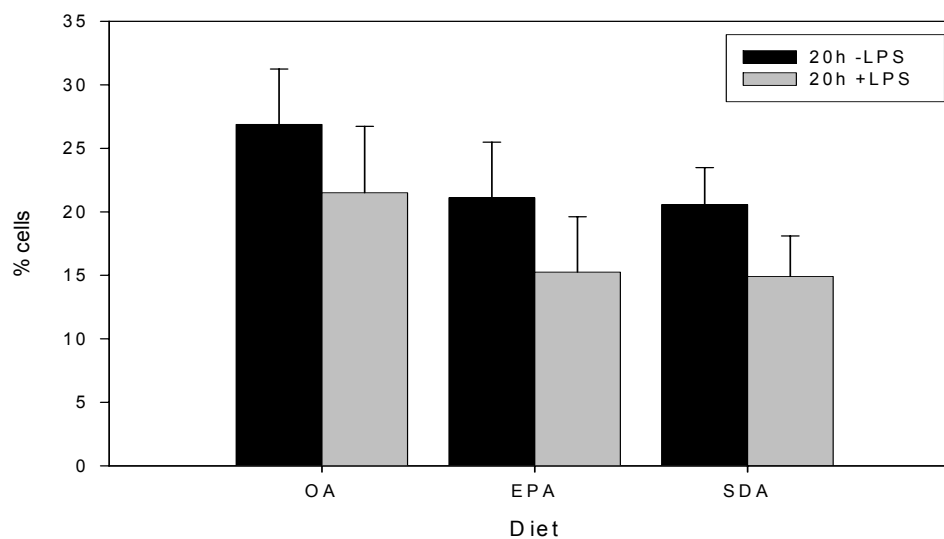
Figure 4.3. Fluorescence Images of TUNEL and Hoechst Stained Cells From Each Feeding Group After 20h Incubation in Absence or Presence of LPS. A. Oleic acid group 20h -LPS; B. Oleic Acid group + LPS; C. EPA group -LPS; D. EPA group +LPS; E. SDA group -LPS; F. SDA group +LPS

Figure 4.4. (A-D). Effects of Diet and LPS Treatment on Cell Viability, Apoptosis, Secondary Necrosis, and Cell Death in Glycogen Elicited Peritoneal Neutrophils at 20 Hours in Presence or Absence of LPS.

A. Effect of diet on viability at 20h in presence or absence of LPS



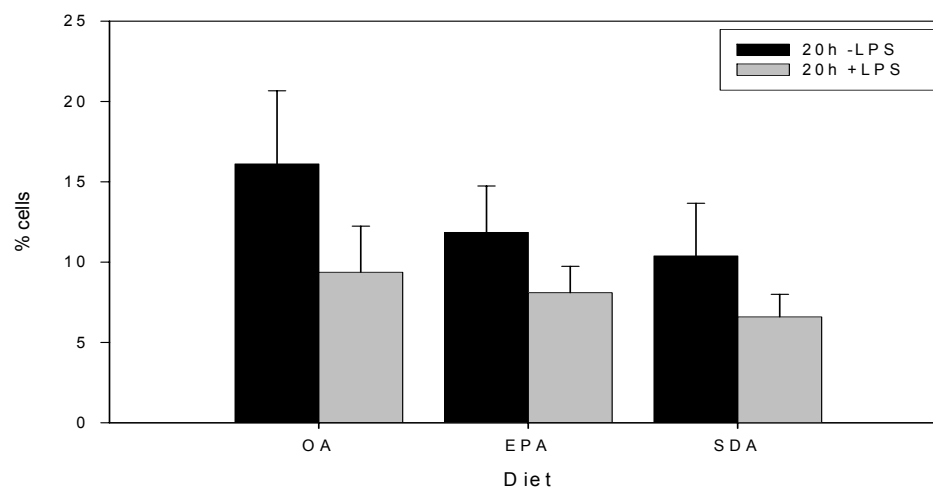
B. Effect of diet on apoptosis at 20h in presence or absence of LPS



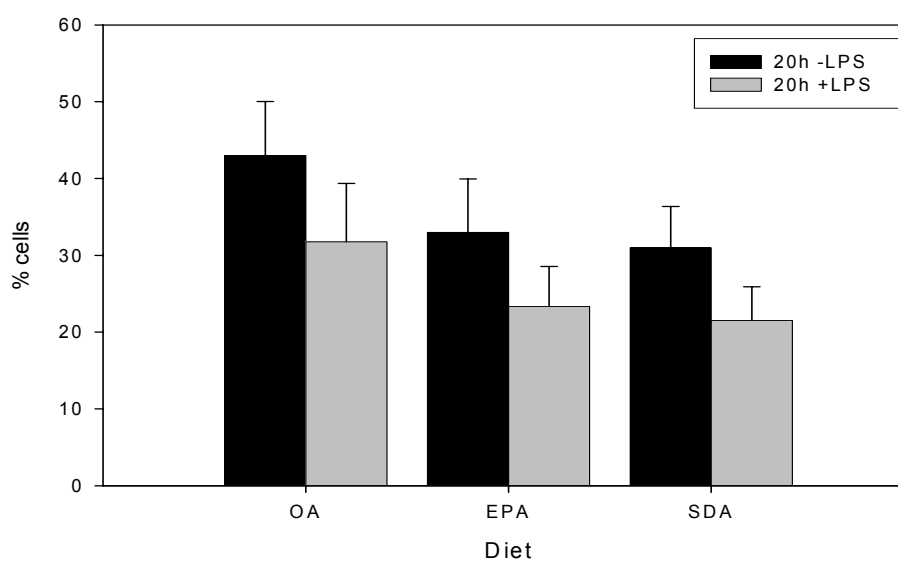
(A-B) Effects of diet and LPS treatment on cell viability and apoptosis in glycogen elicited peritoneal neutrophils at 20 hours in presence or absence of LPS.

¹Mean $\pm$ SEM of cell population at 20h in presence or absence of LPS for cell viability and apoptosis as measured using flow cytometry with propidium iodide and annexin-V staining (N=6/group).

C. Effect of diet on secondary necrosis at 20h in presence or absence of LPS



D. Effect of diet on cell death at 20h in presence or absence of LPS

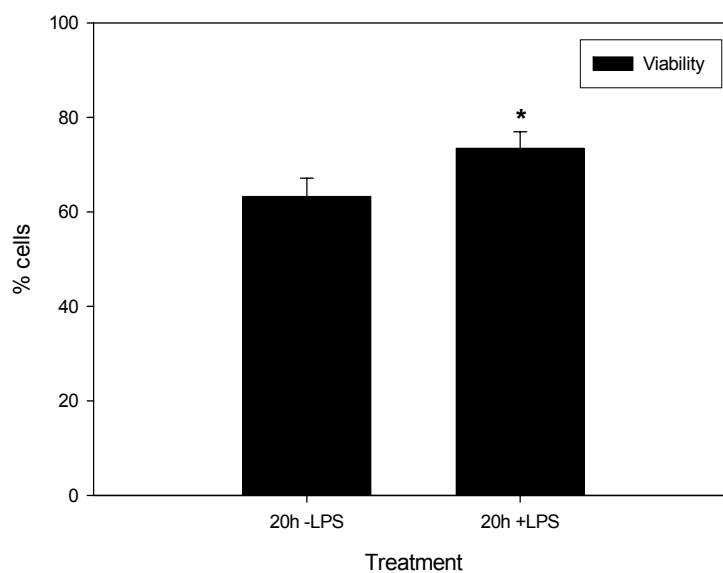


(C-D) Effects of diet and LPS treatment on secondary necrosis and cell death in glycogen elicited peritoneal neutrophils at 20 hours in presence or absence of LPS.

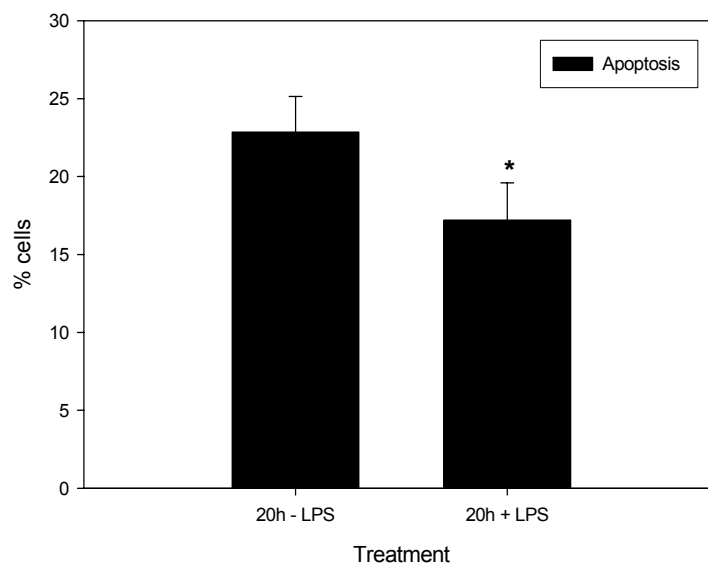
¹Mean $\pm$ SEM of cell population at 20h in presence or absence of LPS for secondary necrosis and cell death as measured using flow cytometry with propidium iodide and annexin-V staining (N=6/group).

Figure 4.5 (A-D). Effect of LPS on Viability, Apoptosis, Secondary Necrosis, and Cell Death Following 20h Incubation.

A.



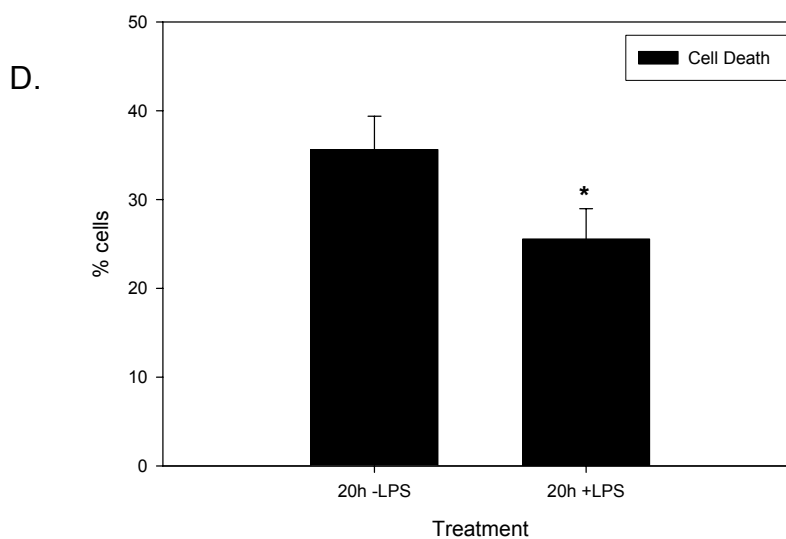
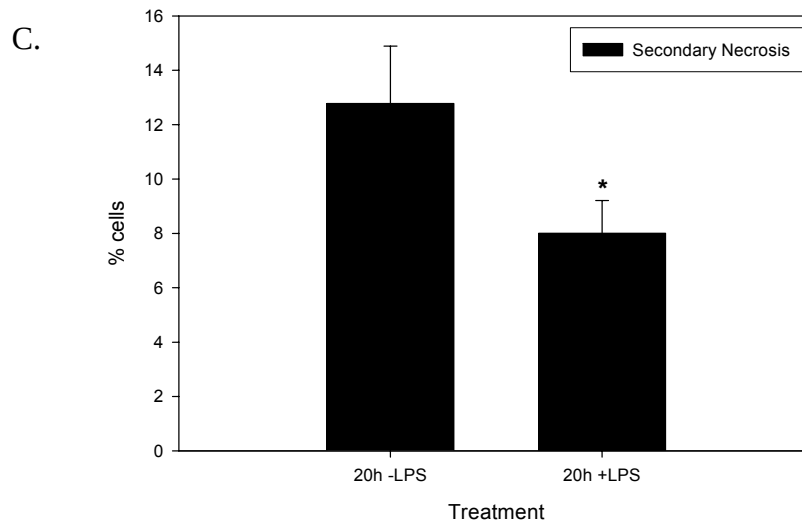
B.



(A-B) Effect of LPS on viability, apoptosis, secondary necrosis, and cell death following 20h incubation.

*Values sharing different superscripts are different ($p \leq 0.05$)

¹Mean $\pm$ SEM of % cell population as measured by flow cytometry using annexin-v and propidium iodide staining (N=18).



(C-D) Effect of LPS on viability, apoptosis, secondary necrosis, and cell death following 20h incubation.

*Values sharing different superscripts are different ($p \leq 0.05$)

¹Mean $\pm$ SEM of % cell population as measured by flow cytometry using annexin-v and propidium iodide staining (n=18).

diet. Figure 4.6 (A-C) shows that apoptosis, secondary necrosis, and cell death were significantly decreased in the presence of LPS after correction, or normalization, for basal values and are in agreement with the findings shown in Figure 4.5 (A-D).

The effect of dietary treatment on viability, apoptosis, secondary necrosis and cell death of freshly extracted glycogen-elicited neutrophils is shown in Figure 4.7 (A-D). These results show that after two weeks of dietary treatment there were no significant changes in viability, apoptosis, secondary necrosis, and cell death between the groups. It is important to note that although it appears that secondary necrosis and cell death with EPA were increased as compared with OA, statistical significance was not reached ($P = 0.053$). Since apoptosis, secondary necrosis, and cell death at 20 hours of incubation are directly related to the basal values of these same parameters, the absolute differences between basal and 20 hr incubations in the absence and presence of LPS were calculated for each dietary treatment group. The results indicate that neither SDA nor EPA significantly affected viability, apoptosis, and secondary necrosis as compared with OA in the absence or presence of LPS (Figure 4.8 (A-D)). Although, there appears to be an increase in cell viability with EPA and SDA as compared with OA in the presence or absence of LPS, these changes were not significantly different ($p=0.069$)

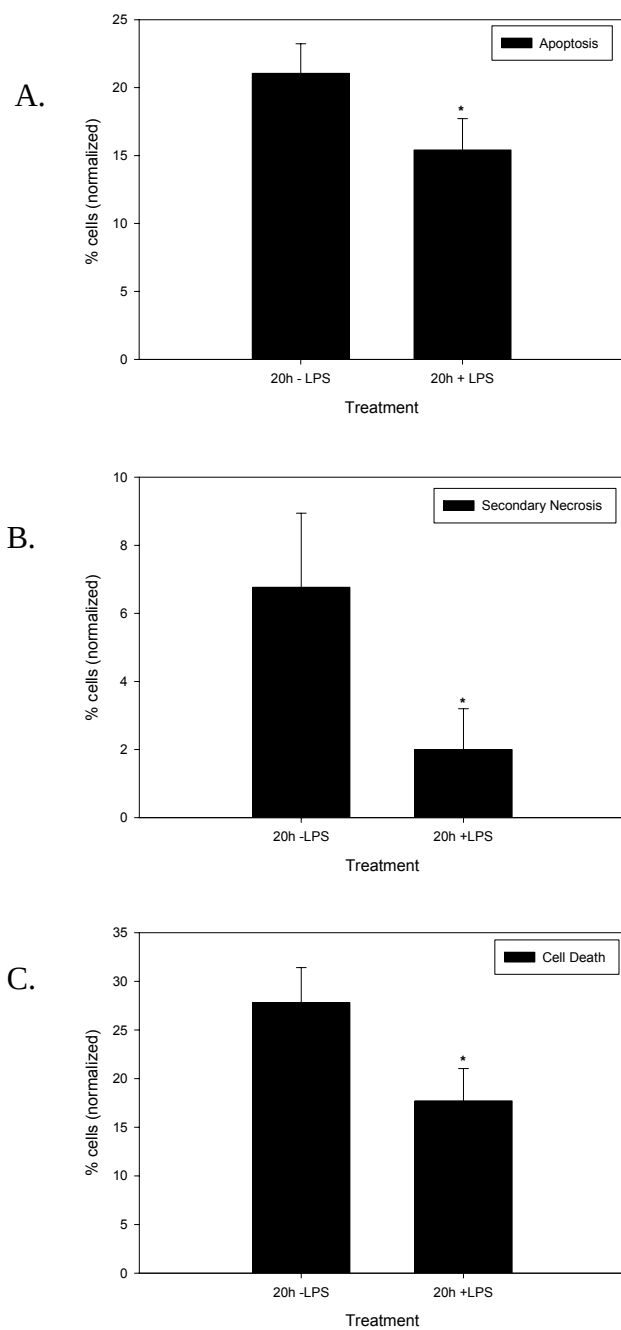


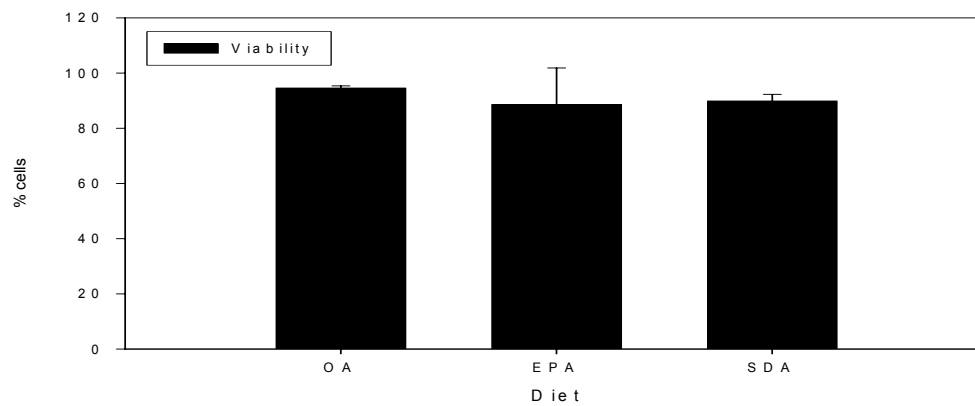
Figure 4.6. Effect of LPS on the Absolute Difference Between 0h and 20h for Apoptosis, Secondary Necrosis, and Cell Death Following 20h Incubation.

*Values sharing different superscripts are different ($p \leq 0.05$)

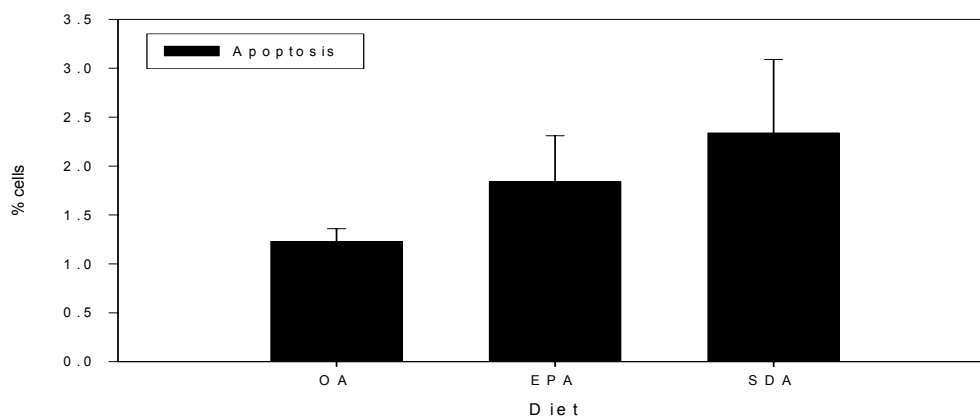
¹Mean $\pm$ SEM of the difference of cell population % from time 0h for apoptosis, secondary necrosis, and cell death as measured using flow cytometry with propidium iodide and annexin-V staining (N=18).

Figure 4.7 (A-D). Effect of Diet on Cell Viability, Apoptosis, and Secondary Necrosis in Glycogen-Elicited Peritoneal Neutrophils at 0 Hours.

A.



B.

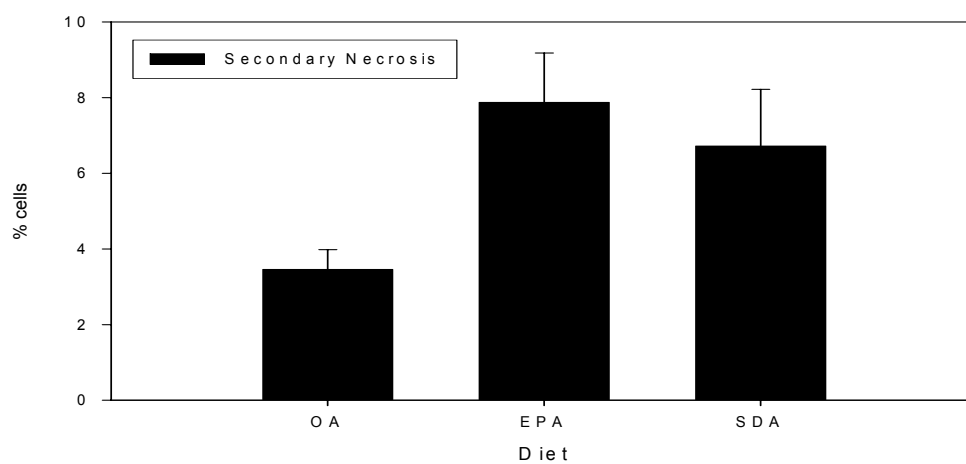


(A-B) Effect of diet on cell viability, apoptosis in glycogen-elicited peritoneal neutrophils at 0 hours.

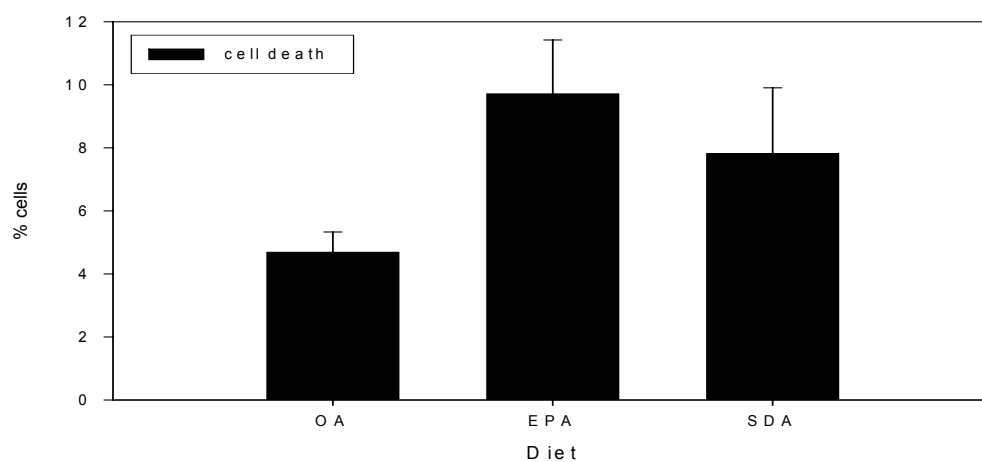
^{a,b} Values sharing different superscripts are different ($p \leq 0.05$)

¹ Mean $\pm$ SEM cell population % at 0h for cell viability, apoptosis, and secondary necrosis as measured using flow cytometry with propidium iodide and annexin-V staining (N=6/group).

C.



D.



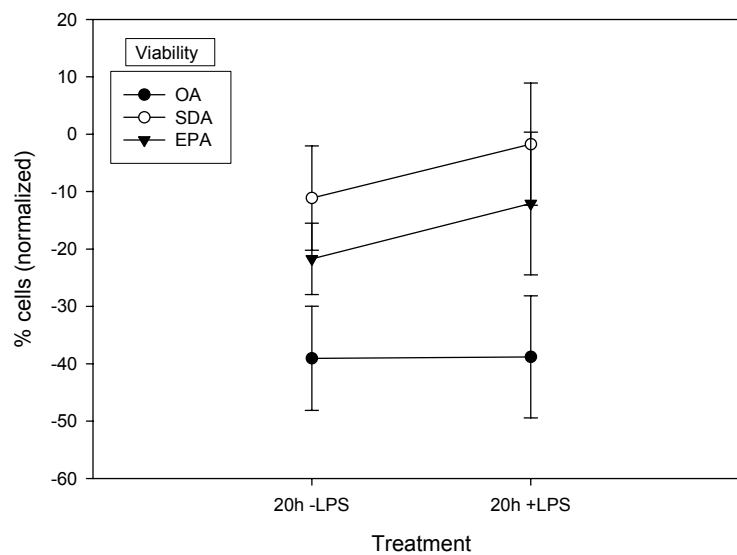
(C-D) Effect of diet on secondary necrosis and cell death in glycogen-elicited peritoneal neutrophils at 0 hours.

*Values sharing different superscripts are different ($p \leq 0.05$)

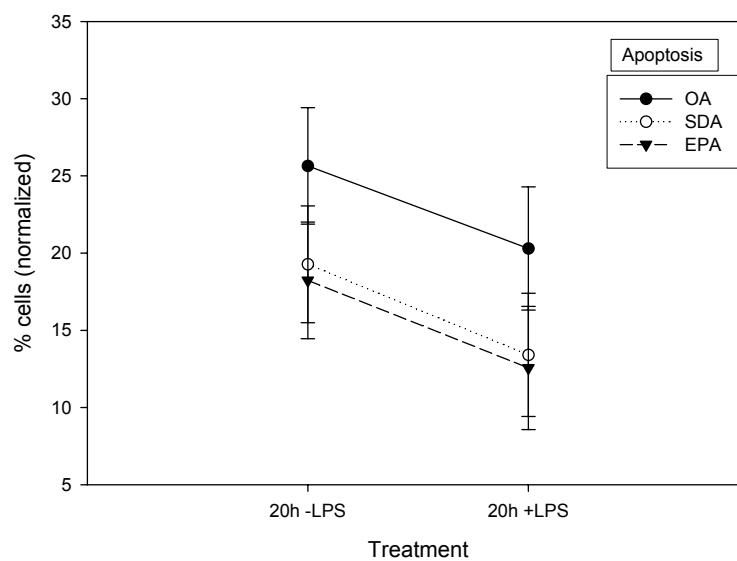
¹Mean $\pm$ SEM cell population % at 0h for cell death as measured using flow cytometry with propidium iodide and annexin-V staining (N=6/group).

Figure 4.8. (A-D). Effect of Diet on the Absolute Difference From Time 0h to 20h for Viability, Apoptosis, Secondary Necrosis and Cell Death Following 20h Incubation in Presence or Absence of LPS.

A.

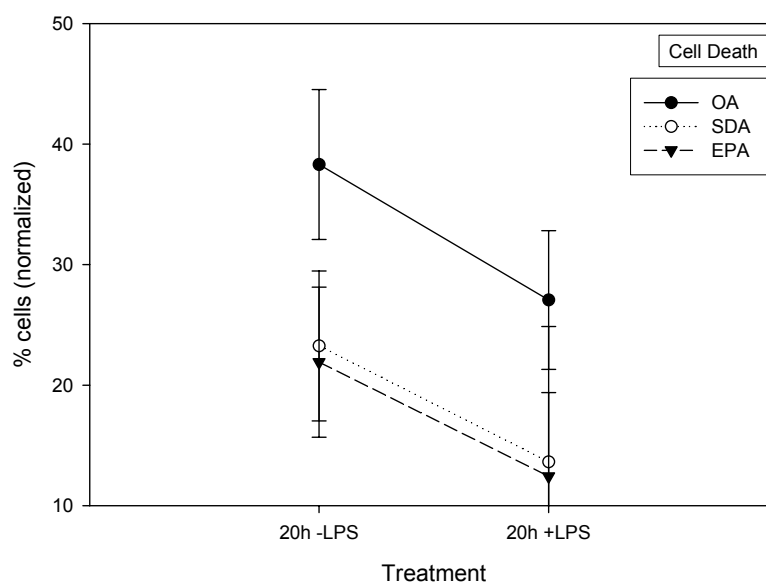
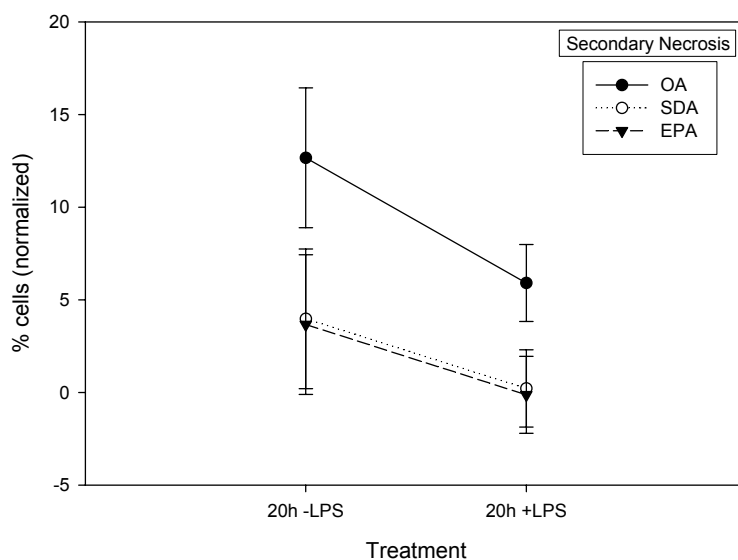


B.



(A-B) Effect of diet on absolute difference from time 0h to 20h for viability and apoptosis following 20h incubation in presence or absence of LPS.

¹Mean $\pm$ SEM of the difference of cell population % from time 0h for apoptosis, secondary necrosis, and cell death as measured using flow cytometry with propidium iodide and annexin-V staining.



(C-D) Effect of diet on absolute difference from time 0h to 20h for secondary necrosis and cell death following 20h incubation in presence or absence of LPS.

¹Mean $\pm$ SEM of the difference of cell population % from time 0h for apoptosis, secondary necrosis, and cell death as measured using flow cytometry with propidium iodide and annexin-V staining.

Part 5
Discussion

I. Discussion

The results of this study show that feeding purified ethyl esters of EPA and SDA in the context of a typical western diet for 14 days resulted in the incorporation of n-3 fatty acids in the phospholipids of rat liver. The diets used in this study were designed based on the macronutrient and fatty acid composition of a typical western diet. Petrik et al[1] showed that by using oleic acid (OA) as the control fatty acid, they could maintain constant levels of n-6 fatty acids in their diets. They previously showed that OA is neutral in respect to prostaglandin formation[2]. OA is an n-9 fatty acid, and is not interconvertible with n-6 or n-3 fatty acids. We also designed our experimental diets using OA as the control fatty acid. Our experimental diets were supplemented with purified ethyl esters of EPA or SDA instead of fish or an SDA containing oil such as echium oil[3]. Thus, our design allowed us to vary fatty acid intake in the context of the typical western diet and avoid the problems of one dietary oil substituted for another. The results of this study can be attributed to substitution of one fatty acid for another.

Dietary supplementation with purified SDA and EPA ethyl esters resulted in a significant increase in tissue phospholipid incorporation of n-3 PUFAs, such as EPA and DPA, in liver. We found that supplementation with SDA resulted in a significant increase in tissue EPA levels as compared with control animals, and that this increase was about one-half of that seen when animals were fed EPA. SDA was also effectively converted and stored as DPA, as compared with control animals. This increase was about two-thirds as effective as that demonstrated by the inclusion of EPA in the diet. Neither EPA nor SDA provided by diet were metabolized to form DHA in the membrane phospholipids. These results are consistent with others that report either a lack of or very low conversion of either SDA or EPA to DHA[3;4]. This lack of conversion could be due either to the low level of activity of the Δ^6 desaturase enzyme, or the presence of ALA in our basal US17 diet mix. In mammalian cells the conversion of DPA to DHA is not a direct

one. Instead, DPA is first metabolized by two rounds of elongation to form 24:5n-3. The Δ^6 desaturase enzyme then desaturates 24:5n-3 to 24:6n-3. The 24 carbon chain length fatty acid is then shortened by β -oxidation to form DHA[5]. This pathway is referred to as the “Sprecher pathway”. The reaction rate of this pathway is slow, and it has been reported that DPA is preferentially used as a substrate for β -oxidation to form EPA rather than being metabolized to DHA[6]. This may provide one possible explanation for the lack of DHA accumulation in liver phospholipids of our animals. The presence of ALA in our basal US17 diet may provide an alternative explanation for the lack of significant increase in tissue DHA seen in animals fed EPA or SDA as compared with controls. DHA plays an important role in formation of the cell membrane, and because of this shorter n-3 fatty acids are converted to DHA until the membranes become saturated, only then is it used to form EPA or DPA. This leads to the possibility that our basal diets may have provided enough ALA to saturate the concentration of DHA in the cell membranes regardless of the addition of EPA or SDA.

Studies that suggest that the inclusion of n-3 fatty acids in the diet can have beneficial effects on health aspects from cardiovascular function to inflammation[7-9]. As previously mentioned the American Heart Association has recommended an increased intake of n-3 fatty acids as a means to reduce the risk for heart disease[10]. It has also been accepted that nutritional formulas containing n-3 fatty acids may be useful as therapeutics for critically ill patients in numerous disease states[11;12]. Most evidence for the benefits associated with n-3 fatty acids applies to the long-chain members of the n-3 family EPA and DHA, both of which occur in fish and fish oil. Suggestions that patients or communities at large increase intake of fish are problematic for numerous reasons, including; consumption of contaminated fish, food related allergies, general resistance to dietary change, and limitations on natural fish stocks[8;13]. Given the utility of n-3 fatty acids in human health and nutrition, identification of a suitable alternative source for dietary n-3 fatty acids is urgently required.

The use of the Δ^6 desaturase precursor fatty acid ALA may be used as an alternative means to increase tissue EPA levels; however, the conversion from ALA to EPA is too inefficient to be a practical suggestion[4;14]. Conversion of ALA to EPA is a three step enzymatic process, and it has been suggested that the first step in this metabolism, the Δ^6 desaturase enzyme, may be the rate limiting step in the conversion[15]. The inefficiency of this conversion process has driven an interest in the use of SDA, which is the fatty acid product of Δ^6 desaturase activity on ALA, as a possible source for increasing tissue EPA[4]. Feeding oil blends containing SDA can efficiently increase membrane phospholipid n-3 content [3;16;17]. Studies in which SDA was added to cell culture also resulted in incorporation of EPA and DPA into cell membranes[18]. As a result, agricultural companies have been working towards designing plant species that can produce high levels of SDA in their seeds in hopes of providing a sustainable alternative source for increasing tissue content of beneficial n-3 fatty acids[19].

Results from our current study confirm that addition of purified EPA ethyl esters to the diet can increase tissue content of EPA and DPA, but not DHA. These findings also demonstrate that dietary supplementation with SDA can significantly increase content of EPA and DPA, but not DHA in liver membrane phospholipids in a comparable fashion as animals fed a diet containing EPA. These findings suggest that dietary supplementation with SDA might provide a reasonable alternative to EPA provided by diet to increase the accumulation of beneficial n-3 fatty acids in tissue phospholipids.

Animals were fed $200 \text{ kcal} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ and diets were typically fully consumed by the following day. Body weights were measured daily, and although it appears that there is a slight decrease in body weight for SDA fed animals as compared with the other dietary groups, this difference was not significant. This was an interesting trend as SDA animals readily consumed their food, which rules out a dietary preference. SDA animals also had similar starting

body weights, and there were no obvious physical differences among animals in this feeding group and those in the EPA and OA groups.

In order to ensure that our experimental model was appropriate for detecting apoptosis we utilized two independent tests, TUNEL and gel electrophoresis, to look for presence of apoptotic bodies and hallmark DNA banding patterns, respectively. Although not quantitative, our gel electrophoresis experiments provided evidence that cells were indeed undergoing apoptosis after 20h, regardless of LPS treatment. Prepared slides from all feeding groups contained bodies that fluoresce positively for TUNEL stain following 20h incubation. Regardless of feeding group, slides containing cells that were incubated in presence of LPS appeared to possess fewer TUNEL positive bodies than their untreated counterparts. Both of these tests provided evidence that cells were undergoing apoptosis. Fluorescence microscopy also showed that LPS reduced apoptosis.

Our results show that diet did not significantly affect neutrophil viability following 20h incubation in absence or presence of LPS. LPS is a well known activator of neutrophils and increases neutrophil lifespan[20]. The lack of difference among dietary groups is not likely due to fault with LPS treatment, as we tested whether our LPS model was sufficient to induce changes in neutrophil lifespan, regardless of diet. We found that, irrespective of diet, LPS increased neutrophil viability, and that this increase in viability was through a reduction in apoptosis, secondary necrosis, and cell death. Furthermore, we found that in freshly extracted neutrophils, secondary necrosis and cell death appeared to be increased in the EPA group, but this increase failed to reach significance ($p=0.053$). Although, these effects were probably due to the small sample size and high variability, there is a close association between basal viability, apoptosis, secondary necrosis, and cell death. Because of this association we normalized the data by calculating the absolute difference between basal neutrophils and those incubated for 20h in absence or presence of LPS. We ensured that the calculation had not altered the LPS treatment by comparing the

results shown in Figure 4.5 (A-D) with those in Figure 4.6 (A-C). Results from both figures showed that the addition of LPS resulted in decreased apoptosis, secondary necrosis, and cell death. We found that the lack of dietary interaction was also probably not due to differences in basal neutrophils, as normalization did not produce any significant changes among dietary groups regarding neutrophil viability in absence or presence of LPS. Analysis of normalized data revealed a trend towards an increase in viability in EPA and SDA animals as compared with OA animals. Although this trend failed to attain significance ($p=0.069$), this could be a dietary effect, as power analysis suggested the possibility of a statistically significant increase in viability with the addition of approximately 15 animals.

Despite numerous studies that demonstrate definitive health benefits from diets containing n-3 fatty acids, mainly attributed to EPA or DHA found in fish oil, and a large body of literature demonstrating the positive effects of n-3 PUFAs as therapeutic agents for inflammatory disorders, such as sepsis and ARDS, there is a growing concern in the literature regarding safety of feeding n-3 fatty acids as a means of controlling inflammation[21]. Several of these studies indicate that feeding high levels of n-3 fatty acids can lead to an immuno-compromised state by decreasing ability of the host to respond to infection[22;23]. PMNs play a vital role at maintaining host immunity, and have been described as being the first response to bacterial infection. Our current study may be of interest in light of these recent papers that have questioned the safety of dietary supplementation with n-3 PUFAs because of the potential to induce an immunocompromised condition in subjects[21]. Studies discussing the effect of n-3 fatty acids and immunosuppression have shown that long-term feeding (28d) of diets containing high levels of fish oil (18% by weight) leads to a diminished host response to bacterial challenge[22;23]. Our current findings show that EPA or SDA does not significantly alter neutrophil lifespan or LPS-mediated neutrophil survival. These findings show that 1% EPA or 1% SDA will not interfere with the ability of

neutrophils to increase cell survival in response to bacterial products such as LPS.

One of the objectives of this study was to investigate the hypothesis that the beneficial effects of dietary supplementation with n-3 PUFAs observed by Mancuso et al[24] was due to an attenuation of LPS-mediated PMN survival. The increased rate of apoptosis would facilitate a faster clearance of inflammatory cells from pulmonary tissue, thereby attenuating PMN accumulation. In this study SDA and EPA did not significantly increase PMN apoptosis or attenuate LPS mediated PMN survival. Previous work by Gillis et al[25] suggested that diet might interact with cell viability by increasing apoptosis, and that this increase in apoptosis may be the reason for the muted PMN response observed in ARDS patients, and animal models of ALI previously described by our laboratory[25;26]. However, findings from our current study do not support this hypothesis. There could be several reasons for the differences observed in our current work and previous results by Gillis et al[26]. First, HL-60 cells are a promyelocytic human leukemia cell line that has been adapted for use as a model of PMN function[27]. N-3 fatty acids such as EPA have been reported to have anti-tumorigenic properties, and decrease viability in a number of cell lines at least *in vitro*[1;28-32]. One review reports that in over 100 cancer cell lines, the addition of PUFAs to cell culture could reduce cell proliferation, or induce apoptosis, while having little or no effect on normal cells[33]. Thus, the increase in apoptosis observed in earlier studies from our laboratory may have been complicated by the anti-carcinogenic action of PUFAs. Differences between the findings from this study and those previously reported could also be due to a difference in treatments. HL-60 cell culture work from our lab was dependent on the addition of PUFAs directly to cell media. This could also induce unanticipated effects as studies have reported that some of the cytotoxic effects of n-3 PUFAs may be due to the formation of lipid peroxidation products *in vitro*[34].

These findings suggest that there may be other factors besides apoptosis involved in the beneficial effects observed in the studies by Mancuso[24]. A possible alternative mechanism for the reduction of PMN concentration in BAL fluid of ARDS patients fed nutritional formulas containing fish oil may be due to a reduced potential of alveolar macrophages to recruit circulating PMNs to pulmonary tissue by a reduction of LTB₄ synthesis. LTB₄ has been shown to be a potent attractant of PMNs to pulmonary tissue[35]. It has also been shown that LTB₄ is increased in the sputum of patients with COPD and ARDS, which results in an increased chemotactic potential for PMNs[36]. The importance of LTB₄ as a signaling molecule in the lung is further emphasized by the fact the alveolar macrophages have been shown to be capable of an increased production of leukotrienes upon stimulation as compared with non-alveolar macrophages and monocytes, and that alveolar macrophages preferentially produce leukotriene products of eicosanoid metabolism[37]. Stimulated alveolar macrophages also preferentially metabolize EPA as compared with AA[38;39]. Previous work from our laboratory demonstrated a significant reduction of LTB₄ concentrations in BAL fluid of animals and patients with ALI or ARDS fed nutritional formulas containing fish oil[24;40]. Reduction in LTB₄ could lead to a decreased chemotactic potential of resident alveolar macrophages, resulting in a decreased influx of PMNs rather than an attenuated lifespan of recruited PMNs.

One of the limiting factors of our current study was that we only examined the effect of feeding 1% EPA or 1% SDA for 14 days. For future studies it would be of interest to explore a dose-response of SDA and EPA on both membrane phospholipid profiles as well as PMN function. Feeding fatty acids at different concentrations and for different lengths of time might help clarify the differences and similarities between SDA and EPA, and further strengthen the case for SDA as a substitute for dietary EPA. An extension of current study conditions to include more animals in each feeding group may also elucidate whether diet supplemented with 1% EPA or 1% SDA is influencing PMN lifespan by

increasing PMN viability and LPS-mediated cell survival. Given the importance of LTB₄ in the sequestration of PMNs to pulmonary tissue, it would also be worthwhile to examine the chemotactic potential of alveolar macrophages following dietary supplementation with EPA and SDA, as well as the ability of PMNs with preloaded cell membranes to respond to chemotactic stimuli.

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Part 6
Conclusions

I. Conclusions

Based on findings from these experiments it can be concluded first, that the diets supplemented with SDA ethyl ester increased EPA and DPA, but not DHA content in the liver. These findings were similar to those observed in animals fed diets containing purified EPA ethyl ester, which increased membrane phospholipid concentrations of EPA and DPA, but not DHA in liver. These findings lead to the acceptance of our first hypothesis that diets supplemented with purified SDA ethyl ester increased EPA content in liver phospholipids and the increase was similar to that observed in animals fed diets containing EPA ethyl ester.

It is concluded, second, from these findings that the addition of 1% EPA or 1% SDA to the diet does not alter viability, apoptosis, secondary necrosis, or cell death in glycogen-elicited peritoneal neutrophils in absence or presence of LPS in the rat. This leads us to reject our second hypothesis that dietary supplementation with SDA or EPA will impact the regulation of constitutive and LPS-induced survival in rat glycogen-elicited peritoneal neutrophils by influencing the rate of apoptosis in both cell states.

The significance of this study is that it provides evidence that dietary SDA provides an alternative to EPA as a means to increase the concentration of EPA and DPA in liver membrane phospholipids. This study also provides evidence that the inclusion of 1% EPA or 1% SDA in the diet does not lead to an increase in constitutive neutrophil apoptosis and will not interfere with the ability of neutrophils to respond to bacterial endotoxin by increasing cell lifespan. This indicates that the addition of 1% EPA and 1% SDA to a standard western diet will not lead to an immunocompromised state.

Vita

Jonathan Earl Phipps was born on May 29th, 1979, in Knoxville, Tennessee to Larry and Judy Phipps. An only child, Jonathan grew up on his family farm, and attended Horace Maynard High School from 1993 to 1997. Upon graduating from high school, Jonathan attended Lincoln Memorial University in Harrogate, Tennessee from 1997 until 2001. While at Lincoln Memorial University, Jonathan majored in biology with a minor in chemistry. He graduated in the spring of 2002 with a Bachelor of Science degree in biology. Jonathan worked in the lab of Dr. M.D. Karlstad for 3 months during the summer of 2002 as a laboratory assistant before entering the University of Tennessee as a Master of Science student in the Comparative and Experimental Medicine program under the tutelage of Dr. Karlstad. During his time in Dr. Karlstad's lab Jonathan focused on physiology and pathology, particularly, the role that polyunsaturated fatty acids play in the inflammatory process. Jonathan completed the requirements for his Master's degree in the summer of 2005. In June, 2005, Jonathan will be continuing in the Comparative and Experimental Medicine program, pursuing his Doctor of Philosophy under the tutelage of Dr. J. Wall.