Published in final edited form as:

Immunol Endocr Metab Agents Med Chem. 2009 March 1; 9(1): 38–44. doi:10.2174/187152209788009841.

Chemotherapeutic Properties of n-3 Polyunsaturated Fatty Acids - Old Concepts and New Insights

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Abstract

Over the past several decades, data from both experimental animal studies and human clinical trials have shown that dietary n-3 polyunsaturated fatty acids (PUFA) exhibit anti-inflammatory bioactive properties, compared to n-6 PUFA. Collectively, these studies have identified multiple mechanisms by which n-3 PUFA affect immune cell responses. In this review, we discuss the putative targets of anti-inflammatory n-3 PUFA, specifically, cytokine production, antagonism of n-6 PUFA metabolism, binding to nuclear receptors as ligands, and the alteration of signaling protein acylation. In addition, we investigate the effect of n-3 PUFA on the coalescence of lipid rafts, specialized signaling platforms in the plasma membrane.

Keywords

Diet; n-3 PUFA; chronic inflammation; CD4⁺ T-cells

INTRODUCTION

With respect to the dietary manipulation of immune cell function, long chain n-3 polyunsaturated fatty acids (n-3 PUFA), which are enriched in dietary fish oil, have been studied in relation to their anti-inflammatory properties [1–5]. n-3 PUFA have their first double bond at the third carbon from the methyl end of the fatty acyl chain. α -linolenic acid (ALA; 18:3n-3), eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) are examples of n-3 PUFA. DHA is unique in that it is the longest and most unsaturated fatty acid commonly found in mammalian cell membranes [6]. In contrast, n-6 PUFA (e.g., linoleic acid (LA; 18:2n-6) and arachidonic acid (AA; 20:4n-6)), which have the first double bond at the sixth carbon from the methyl end, generally exhibit pro-inflammatory properties. Due to the degree of unsaturation and the low activation energy for rotation about the single C-C bonds

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that separate the unsaturated carbon atoms in the recurring methylene-interrupted (=CH-CH₂-CH=) pattern, DHA is thought to form a liquid-disordered structure in biological membranes [7–9]. The beneficial clinical effects of n-3 PUFA on the immune system have been well documented by numerous investigators examining both humans and animal models of inflammation [10–14].

FUNCTION OF CD4⁺ T-CELLS IN IMMUNE DISEASES

CD4⁺ helper T (Th) cells are essential regulators of the immune response. Upon activation, naïve Th cells differentiate into effector cells. For example, classical Th1 and Th2 lineages are characterized by their cytokine secretion and immune regulation function [15,16]. IL-2 and IFN- γ are signature cytokines produced by Th1 cells which regulate cell-mediated immune responses, whereas Th2 cells regulate humoral immune responses, in part, by secreting IL-4, IL-5, IL-10 and IL-13 [17].

Most autoimmune and/or chronic inflammatory diseases involve an overactive immune response directed toward a particular tissue, for example, the pancreas in type 1 diabetes, the central nervous system in multiple sclerosis, various joints in rheumatoid arthritis and the small and large intestine in inflammatory bowel diseases (IBD; Crohn's disease and ulcerative colitis). The pathogenesis of immune and inflammatory diseases is made up of a complex network involving a variety of cell types. For example, IBD involves intestinal epithelial cells, lymphocytes, dendritic cells and macrophages [18,19].

Th1 cells are of pivotal importance for the pathogenesis of IBD [20]. A growing body of evidence indicates that IL-23 and TNF- α , as well as Th1-inducing cytokines, drive Th1-induced IBD. Furthermore, an IL-17 producing Th cell lineage activated by IL-23 was recently identified. These cells are classified as distinct pro-inflammatory Th17 (Thi) cells [21–23]. Hence, hyperactivated Th1 cells, as well as Th17 cells, are major players in chronic inflammatory diseases [24–27], and inhibition of the expansion and/or activation of Th1 cells can be one of the mechanisms by which anti-inflammatory agents exert their beneficial effect.

EFFECT OF n-3 PUFA ON CD4⁺ T-CELL FUNCTION

In terms of n-3 PUFA effects on CD4⁺ T-cells, Arrington *et al.* from our laboratory reported that IL-2 secretion by mitogenic anti-CD3/anti-CD28 stimulation of murine splenic T-cells was suppressed by dietary DHA [5]. In a parallel experiment, they further demonstrated that IL-2 production by Jurkat T-cells was suppressed by 50 μ M AA or DHA, compared to LA. Interestingly, DHA treatment suppressed IL-2 secretion compared to AA treatment only when CD28 was engaged in T-cell activation, indicating that n-3 PUFA also affect the function of co-stimulatory CD28 signaling [4].

Since IL-2 production is critical for T-cell proliferation, Switzer *et al.* from our laboratory examined the effect of n-3 PUFA on activation-induced cell death (AICD) of mouse splenic CD4⁺ T-cells [28]. They showed that dietary fish oil (FO) enhanced Th1 polarization and AICD *ex vivo*. They also reported that the n-3 PUFA enriched fatty acid composition of T-cell membranes was maintained in long-term culture in the presence of homologous mouse serum. In contrast, cell culture with fetal bovine serum displaced n-3 PUFA derived from FO feeding, resulting in the loss of the dietary effect [28]. Other investigators have demonstrated that apoptosis is enhanced by DHA in murine CD4⁺ T-cells as well as T-cell lines [29]. The induction of apoptosis by n-3 PUFA might explain why fewer cells entered the cell cycle after long-term culture [30].

Zhang *et al.* from our laboratory also investigated the effect of n-3 PUFA on antigen-induced CD4⁺ T-cell proliferation. They confirmed that n-3 PUFA are capable of suppressing

proliferation of mouse antigen-specific (DO11.10) CD4⁺ T-cells in the presence of homologous mouse serum [30]. The effects of serum on antigen-specific T-cell activation was in accordance with previous observations indicating that homologous mouse serum is required in long-term culture to preserve n-3 PUFA-induced changes in plasma membrane microdomain fatty acyl composition and cell functionality [28]. They further reported that dietary FO modulates the Th1/Th2 balance toward the Th2 pole in the presence of homologous mouse serum. In subsequent experiments, it was demonstrated that Th2 proliferation was not affected by FO, but FO selectively suppressed Th1 clonal expansion *in vivo* [1].

Overall, these findings suggest that n-3 PUFA suppress CD4⁺ T-cell proliferation and function. CD4⁺ T-cells are one of the major immunoregulators and, therefore, a target cell type for immunosuppressive dietary agents including n-3 PUFA.

T-CELL ACTIVATION AND LIPID RAFTS

For T-cell activation to occur, only cognate peptide-major histocompatibility complexes (MHC), among a total pool of peptide-presenting MHC molecules expressed on antigen presenting cells (APC), are recognized by receptors on the T-cell to generate an immune response [31]. At the contact zone between the T-cell and APC (e.g., B cells, macrophages and dendritic cells), the immunological synapse (IS) is formed to facilitate T-cell receptor (TCR) binding to antigen (Ag) peptide. Interestingly, during the formation of the IS, T-cells polarize their cytoskeleton, including actin and microtubule networks. Furthermore, Thauland *et al.* [32] reported that polarized Th1 and Th2 cells form morphologically distinct IS. Specifically, IS formation of Th1 cells is unique in that a compact central cluster of MHC/TCR surrounded by adhesion molecules is formed. In contrast, Th2 polarized cells exhibit a multifocal IS by forming multiple MHC/TCR accumulations at high Ag concentrations.

The plasma membrane is involved in almost all aspects of cell biology, including morphogenesis, proliferation, migration, invasion, transformation, differentiation, secretion and apoptosis. According to the classical concept of the cell membrane, i.e., the Singer-Nicholson fluid mosaic model of the phospholipid bilayer, perturbation of membrane composition had little influence on protein function [33]. However, in the past two decades, a growing body of data indicate that lipids exist in gel, liquid-ordered or liquid-disordered states, depending on the complex environment of the cell. Subsequently, a lipid raft theory has evolved in which liquid-ordered microdomains, i.e., lipid rafts, are floating over the “sea” of bulk membrane, which is in a liquid-disordered state [34]. It is postulated that the preponderance of saturated hydrocarbon chains in sphingolipids allows for tight cholesterol interaction, thereby forming a “packed” liquid-ordered phase. Hence, historically, lipid rafts have been characterized by two operational definitions; the insolubility in cold nonionic detergents, for which lipid rafts are also classified as detergent-resistant membrane fractions, and a loss of function is associated with cholesterol depletion [35].

Among a variety of functions, lipid rafts are now believed to modulate intracellular signaling cascades, endocytosis, protein trafficking and cell-cell communication, in part, by altering the lipid-protein composition of the bulk membrane *via* specialized lipid microdomains [34,36–38]. In terms of immune cell signaling, lipid rafts are known to be involved in signal transduction involving FcεRI receptor [39], T-cell receptor (TCR) [40], B cell receptor (BCR) [41], epidermal growth factor (EGF) receptor [42,43], insulin receptor [44], integrins [45] and endothelial nitric oxide synthase (eNOS) [46]. These data indicate that the composition and formation/dissociation status of lipid rafts is likely critical for T-cell activation.

ALTERATION OF LIPID RAFTS BY n-3 PUFA

Since lipid rafts are thought to regulate many cell functions, a number of studies have examined the effects of fatty acid manipulation on T-cell lipid rafts. Stulnig *et al.* reported the enrichment of n-3 PUFA in plasma membranes of Jurkat T-cells by addition of fatty acids into the culture media [47–52]. These manipulations were associated with a marked displacement of Src family kinases, e.g., Lck and Fyn, from detergent-resistant domains indicating that the alteration of lipid rafts affects the intracellular localization of signaling proteins. Interestingly, the displacement of Lck correlated with diminished calcium signaling indicating the functional role of lipid rafts in T-cell activation [47]. Our laboratory also previously showed that highly purified dietary DHA ethyl ester and FO, in which n-3 PUFA are highly enriched, were incorporated into murine splenic T-cell lipid rafts and soluble membrane phospholipids, resulting in a 30% reduction in raft sphingomyelin content [3,53].

Recently, using the *fat-1* transgenic mouse model, in which the n-3 desaturase gene was cloned from *Caenorhabditis elegans* to endogenously produce n-3 PUFA from n-6 PUFA, and direct visualization of lipid rafts as assessed by fluorescent dye Laurdan labeling, our laboratory demonstrated that lipid raft formation at the IS was enhanced in *fat-1*-containing CD4⁺ T-cells compared to the wild type cells [54]. The alteration of lipid raft formation was subsequently associated with suppressed localization of select signaling proteins, such as F-actin, PLC γ -1, and PKC θ , into the IS as assessed by immunofluorescence labeling as opposed to no effect on LAT and CARMA1 localization. In accordance with the localization data, the phosphorylation status of PLC- γ 1 was selectively down-regulated in *fat-1* transgenic cells, whereas LAT phosphorylation was not altered. The schematic of the effect of n-3 PUFA on lipid rafts and signaling protein localization is shown in Fig. (1). Furthermore, the proximal alteration of lipid rafts and signaling by n-3 PUFA was associated with suppression of CD4⁺ T-cell proliferation. Interestingly, down-regulation in T-cell proliferation was also observed to a lesser extent following stimulation of T-cells by either mitogenic anti-CD3 and anti-CD28 mAbs or pharmacological PMA phorbol ester and Ionomycin calcium ionophore, which bypassed IS formation or TCR engagement, respectively. These results indicate that the effect of n-3 PUFA involves, but not limited to, lipid raft formation.

Alternatively, using immunogold electron microscopy of plasma membrane sheets coupled with spatial point analysis which enabled visualization of morphologically featureless microdomains, our laboratory has shown that DHA altered the size and distribution of lipid rafts in HeLa cells. Specifically, DHA treatment significantly reduced the interparticle distance of lipid raft marker proteins. In contrast, the distance between marker proteins of bulk membrane was not affected by DHA treatment indicating that n-3 PUFA can selectively increase clustering of proteins in cholesterol-dependent microdomains, i.e., lipid rafts [55, 56].

These observations can be explained, in part, by the fact that n-3 PUFA, especially DHA, has a low affinity for cholesterol, providing a lipid-driven mechanism for lateral phase separation of cholesterol/sphingolipid-rich lipid microdomains from the surrounding liquid-disordered (l_d) phase [57–61]. Similarly, Patra *et al.* has demonstrated that enrichment of ceramide, derived from the hydrolysis of sphingomyelin in the outer leaflet of the plasma membrane, resulted in coalescence of lipid raft sub-domains into large-membrane macrodomains, in part, by excluding cholesterol from bulk membrane [62]. In addition, the displacement of cholesterol from lipid rafts suppressed the association of the raft-binding and cholesterol binding proteins. It was reported by Nicolau *et al.* that for a critical and biologically relevant function, the size of lipid rafts must be small (6 to 14 nm in diameter) [63]. These observations may explain, in part, why increased lipid raft formation was associated with n-3 PUFA-mediated suppression of T-cell signaling and function [54].

ALTERNATIVE MECHANISMS OF ANTI-INFLAMMATORY n-3 PUFA ACTION

A summary schematic of following mechanisms is presented in Fig. (2).

Alteration of Cytokine Production

A growing body of data indicates that n-3 PUFA alter cytokine production which, in turn, influences the function of a variety of cell types mediating both the induction and resolution of inflammation. Zhao *et al.* reported that ALA inhibits pro-inflammatory cytokine production, such as IL-2, IL-1 β , and TNF- α , in peripheral blood mononuclear cells from hypercholesterolemic subjects [64]. A reduction in IFN- γ production by FO feeding in multiple sclerosis patients and a rat model has also been reported by several investigators [65–67]. Petursdottir *et al.* demonstrated that dietary FO (18% FO+2% corn oil (CO, contains n-6 PUFA)) decreased the production of both pro-inflammatory TNF- α and anti-inflammatory IL-10 in Concanavalin A-stimulated mouse splenocytes [68], emphasizing the complexity of cytokine biology in inflammation. In addition, Kim *et al.* delineated the effect of n-3 PUFA in inflammation by demonstrating that dietary FO elicited anti-inflammatory effects are mediated *via* modulation of lipid peroxidation and superoxide generation in the murine kidney [69].

Eicosanoids, Protectins and Resolvins

It is well established that dietary intake of AA is correlated with plasma levels of AA-derived pro-inflammatory eicosanoids, prostaglandin (PGE₂) and leukotriene (LTE₄) [70]. This is noteworthy because dietary EPA competes for key metabolic enzymes, i.e., cyclooxygenase (COX)-2 and 5-lipoxygenase (LOX), to be converted to PGE₃ and LTE₅, respectively [71–73]. Furthermore, FO also suppressed the expression of iNOS and COX-2, thereby ameliorating inflammatory markers, e.g., PGE₂ and LTB₄ [69,72,74–76].

Of interest, resolvins (RvE1 and RvE2), docosatrienes and neutroprotectins derived from DHA and EPA were recently classified as anti-inflammatory mediators [77]. For example, it was reported that RvE1 displayed potent ability to regulate leukocyte/dendritic cell trafficking, block IL-12 production, counter-regulate TNF- α induced NF- κ B signaling, and protect against murine colitis [78]. Others have further demonstrated that these n-3 PUFA derived metabolites can counter-regulate and terminate inflammation [79,80], which may explain why the biosynthesis of these metabolites seems to be defective in patients with chronic inflammation [79,81].

With respect to modulation of eicosanoid/docosanoid precursor pools, it is noteworthy that the AA pool size is large and conversion of metabolic precursors to AA is relatively slow, whereas the DHA pool is relatively small and, therefore, susceptible to dietary alteration [82].

Nuclear Receptor Activation

Hontecillas *et al.* reported that the loss of peroxisome proliferator-activated receptor (PPAR)- γ enhanced Ag-specific proliferation and overproduction of pro-inflammatory IFN- γ in response to IL-12 using effector CD4⁺ T-cells from PPAR- γ knockout mice [83], indicating the anti-inflammatory role of PPAR- γ . Furthermore, PPAR- γ knockout resulted in the loss of anti-inflammatory regulatory T-cells (Treg) required to prevent effector T-cell-induced colitis [83]. This is noteworthy, because micromolar levels of n-3 PUFA can activate PPAR- γ [84, 85] which, in turn, is capable of antagonizing a “life signaling” transcription factor, i.e. nuclear factor NF- κ B [86].

Protein Lipidation and Intracellular Targeting

For TCR initiated T-cell activation, it is critical that co-receptors and signaling proteins are palmitoylated. Palmitate, a 16-carbon saturated fatty acid (16:0), is known to be essential for

the targeting of many proteins into lipid rafts. For example, localization of CD4 into lipid rafts requires palmitoylation of CD4 molecules [87]. Src family kinases (SFK), Lck and Fyn, are dual-acylated by myristate (14:0) and palmitate in order to localize into lipid rafts. Interestingly, Webb *et al.* showed that inhibition of palmitoylation resulted not only in dislocation of SFK from rafts but also the failure of TCR signaling and T-cell activation [88,89].

Zeyda *et al.* reported that EPA treated Jurkat T-cells exhibited a displaced linker for activation of T-cell (LAT) from lipid rafts compared to the saturated stearic acid (18:0) treated control group, as assessed by biochemical separation of detergent-resistant domains followed by Western blot of LAT using specific antibodies [52]. In the same experiment, they showed that not only the location of LAT but also the phosphorylation status of LAT and phospholipase C (PLC)- γ 1 was reduced, indicating suppressed activation of signaling proteins by n-3 PUFA [52]. This observation is relevant because LAT is palmitoylated at two cysteine residues, which might drive the migration of LAT into lipid rafts. However, the biological impact of LAT localization into rafts has been recently challenged. Zhu *et al.* demonstrated that mutated LAT, which locates out of lipid rafts, functioned comparably to the wild-type LAT, suggesting that LAT palmitoylation and/or raft targeting is not necessary for LAT function [90]. Therefore, further studies are required to determine the effect of n-3 PUFA on LAT localization with regard to T-cell activation.

CONCLUSION

Inflammation is a complex and inevitable consequence of the immune response which contributes to host defense, although chronic inflammation can be deleterious. Among many factors which affect these processes, dietary components such as n-3 PUFA have been demonstrated to down-modulate chronic inflammation. However, the molecular and/or cellular targets of n-3 PUFA are not fully elucidated to date. Multiple modes of actions have been proposed for n-3 PUFA-mediated suppression of inflammation: including, but not limited to, alteration of cytokine production, antagonism of n-6 PUFA-derived metabolites, transactivation/transrepression of nuclear receptors, modulation of protein acylation for intracellular localization, and altered composition and/or size of lipid rafts. Further studies are needed to dissect the molecular mechanisms of action of n-3 PUFA and to understand how these bioactive dietary constituents can be used rationally for the treatment of chronic inflammatory diseases.

Acknowledgments

This work was supported in part by NIH grants DK-71707, CA59034, CA129444, P30ES09106, USDA 2008-34402-19195, "Designing Foods for Health" through the Vegetable & Fruit Improvement Center. Wooki Kim was a recipient of the American Society of Nutrition Proctor & Gamble Predoctoral Fellowship.

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Fig. (1).

Schematic of the effect of n-3 PUFA on lipid raft formation and localization of signaling proteins at the immunological synapse in CD4⁺ T-cells. The incorporation of n-3 PUFA into plasma membrane increases the formation of lipid rafts at the immunological synapse, which further inhibit the localization and phosphorylation of select signaling proteins. PLC, phospholipase C; PKC, protein kinase C; APC, antigen presenting cells; MHC, major histocompatibility complex; Ag, antigen.

Fig. (2).
Multiple pathways by which n-3 PUFA affect immune cell functions.