Published in final edited form as:

Chem Phys Lipids. 2011 October ; 164(7): 626–635. doi:10.1016/j.chemphyslip.2011.07.003.

n-3 Fatty acids uniquely affect anti-microbial resistance and immune cell plasma membrane organization

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Abstract

It is now well established that dietary lipids are incorporated into macrophage and T-cell membrane microdomains, altering their structure and function. Within cell membranes, there are specific detergent-resistant domains in which key signal transduction proteins are localized. These regions are classified as “lipid rafts”. Rafts are composed mostly of cholesterol and sphingolipids and therefore do not integrate well into the fluid phospholipid bilayers causing them to form microdomains. Upon cell activation, rafts compartmentalize signal-transducing molecules, thus providing an environment conducive to signal transduction. In this review, we discuss recent novel data describing the effects of n-3 PUFA on alterations in the activation and functions of macrophages and T-cells. We believe that the modifications in these two disparate immune cell types are linked by fundamentally similar changes in membrane lipid composition and transmembrane signaling functions. We conclude that the outcomes of n-3 PUFA-mediated immune cell alterations may be beneficial (e.g., anti-inflammatory) or detrimental (e.g., loss of microbial immunity) depending upon the cell type interrogated.

Keywords

Macrophage; tuberculosis; phagosome maturation; lipid rafts; polyunsaturated fatty acids; fish oil; fat-1 mice

Introduction

A growing number of dietary supplementation studies using healthy human subjects as well as animal disease models have clearly shown that long chain n-3 polyunsaturated fatty acids

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(PUFA) found in fish oil (FO), specifically, eicosapentaenoic acid (EPA; 20:5 Δ 5,8,11,14,17) and docosahexaenoic acid (DHA, 22:6 Δ 4,7,10,13,16,19), are capable of modulating critical determinants which link infectious disease and inflammation [Belluzzi *et al.*, 2000; Davidson *et al.*, 2004; Prescott & Stenson, 2005; Hudert *et al.*, 2006; Jia, 2008; Bouwens *et al.*, 2009; Weaver *et al.*, 2009]. Since EPA and DHA have pleiotropic properties, with respect to molecular mechanisms of action, we will confine our discussion to recent novel findings pertaining to the impact of n-3 PUFA on macrophage and T cell membrane structure and function. Review articles on n-3 PUFA and immunosuppression can be consulted for additional details [Chapkin *et al.*, 2007; Calder, 2009; Kim *et al.*, 2010.]

Effect of n-3 PUFA on membrane-mediated macrophage functions in tuberculosis (TB) pathogenesis and immune control of TB

Mycobacterium tuberculosis (*Mtb*) is a slow-growing acid-fast bacillus transmitted by inhalation of bacterium-containing droplet nuclei, generated when people with active TB cough or sneeze [Glickman & Jacobs, 2001]. Once *Mtb* gains access to alveoli, it is phagocytosed by alveolar macrophages [Rook, 1994]. Phagocytosis occurs through specific cellular receptors, including complement receptors, Fc receptor, scavenger receptor and mannose receptor [Ernst, 1998]. Toll-like Receptors (TLR) also seem to play a role in mycobacterial immune recognition [Reiling *et al.*, 2002]. Mycobacterial components (e.g., lipoarabinomannan and lipoproteins) [Means *et al.*, 1999] interact with TLR2 and TLR4 and trigger intracellular signaling pathways, resulting in secretion of pro-inflammatory cytokines [e.g., interleukin-12 (IL-12) and tumor necrosis factor- α (TNF α), and chemokines (e.g., IL-8, RANTES, MCP-1) [Algood *et al.*, 2003], which subsequently induce activation and migration of additional inflammatory cells, such as T cells, monocytes, dendritic cells, natural killer cells, B cells and neutrophils from the bloodstream to the site of infection [Jo, 2008; Russell, 2007]. Over time, the infiltrating cells organize in a multicellular structure called a tuberculous granuloma, the pathophysiological hallmark of *Mtb* infection [Russell, 2007; Saunders & Cooper, 2000].

The development of a protective immune response to TB depends on both innate and adaptive cellular immune mechanisms, mediated mainly by the coordinated recruitment, activation and interaction of T cells and macrophages [Bhatt & Salgame, 2007; Flynn & Chan, 2001]. Dendritic cells also play an important role as cytokine producers and antigen presenting cells. They internalize and process *Mtb* and then migrate to lymphoid nodes to present mycobacterial antigens to T cells [McShane *et al.*, 2002]. The interaction of T cells with antigen presenting cells leads to activation and clonal expansion of T cells that recognize *Mtb* specifically [Kaufmann & Flesch, 1988]. Resistance to TB is characterized by a CD4⁺ T cell response [Saunders *et al.*, 2002]. Between 2 and 4 weeks post infection, specialized CD4⁺ T cells are activated, recruited to the lung and programmed to secrete protective Type 1 helper cytokines (e.g., IFN γ , TNF α and IL-2) which, in turn, enhance the antimycobacterial functions of macrophages [Flynn & Chan, 2001]. IFN γ induces macrophage activation of antibacterial responses leading to up-regulation of over 200 responsive genes (e.g., inducible nitric oxidase and MHC) [Boehm *et al.*, 1997]. In addition to CD4⁺ T cells, other discrete T-cell subsets, such as CD8⁺, $\gamma\delta$ and CD1-restricted T cells, play a role in the immune response to *Mtb* [Peters & Ernst, 2003].

Macrophages exert their antimycobacterial activity by different mechanisms [Liu & Modlin, 2008]. From a membrane perspective, initially after uptake, bacteria are enclosed in specialized endocytic compartments called phagosomes. Lysosomes are vacuolar organelles of the late endocytic pathway which contain hydrolytic enzymes capable of degrading mycobacteria. Phagosomes can sequentially fuse with early and late endocytic organelles, (e.g., lysosomes) in a process known as phagosome maturation [Vergne *et al.*, 2004]. During

the fusion process, phagosomes acquire proteins essential for membrane-organelle docking, membrane traffic and fusion events [Desjardins, 1995], including Rab GTPases and GTP-binding glycoproteins, resulting in bacterial killing. Mycobacterial phagosomes retain Rab5, a GTPase that mediates the interaction with early endocytic compartments [Desjardins *et al.*, 1994], but exclude Rab7 [Press *et al.*, 1998] and other lysosomal markers, which regulates late endosomal membrane trafficking. As a result, mycobacterial phagosomes cannot mature to phagolysosomes [Press *et al.*, 1998; Via *et al.*, 1997]. Second, activated macrophages generate reactive oxygen and nitrogen species which have antimycobacterial activity [Nathan & Shiloh, 2000; Shiloh & Nathan, 2000]. As mentioned earlier, immune stimulation of macrophages, via T cells, TLR agonists, or cytokines such as IFN γ , TNF α and GM-CSF, enhances their activation and antimycobacterial capacity; improving their ability to control the infection [Crowle *et al.*, 1987; Rook *et al.*, 1986]. However, *Mtb* has the ability to avoid destruction within macrophages [Houben *et al.*, 2006], in fact, bacteria can survive and grow within these cells. This pathogen prevents maturation of macrophages and dendritic cells [Hanekom *et al.*, 2003], impairing the activation of a wide range of antimycobacterial responses.

Cytokines induce effective macrophage killing and can influence the polarization of the adaptive immune response and the subsequent development of resistance or susceptibility to mycobacterial infection [Sharma & Bose, 2001]. *Mtb* induces secretion of IL-12 [Ladel *et al.*, 1997], IL-6 [Zhang *et al.*, 1995] and TNF α [Flynn *et al.*, 1995] by murine macrophages and mouse dendritic cells. These pro-inflammatory cytokines are required for a protective response to TB [Flynn *et al.*, 1995]. For example, IL-12 is critical for activation and migration of murine dendritic cells [Cooper *et al.*, 1995] and TNF α is required for granuloma formation in mice [Flynn *et al.*, 1995]. On the other hand, TNF α is also associated with the histopathological damage during TB, mediating necrosis and tissue dysfunction [Rook & al Attiyah, 1991].

Impact of dietary lipids on the immune response to TB and other infections

Lipids serve as structural membrane components, a source of energy and regulators of the host immune response. For example, the incorporation of cholesterol into host cell membranes has a detrimental impact on the response against TB. This lipid seems to be required for *Mtb* internalization, phagosomal maturation prevention and mycobacterial survival [Gatfield & Pieters, 2000; de Chastellier & Thilo, 2006]. Conversely, other lipids have been shown to promote phagosomal maturation. For example, arachidonic acid (AA, 20:4n-6), ceramide and sphingomyelin induce actin assembly, acidification and lysosome fusion in *Mtb*-containing phagosomes, favoring more effective mycobacterial control in mouse macrophages [Anes *et al.*, 2003].

Docosahexaenoic acid (DHA; 22:6n-3) and eicosapentaenoic acid (EPA; 20:5n-3) are long chain n-3 PUFA synthesized by sequential desaturation-elongation reactions from α -linolenic acid (ALA, 18:3n-3), a dietarily essential fatty acid. Diets rich in these n-3 PUFA down-regulate immune cell functions by being precursors of anti-inflammatory eicosanoids (e.g., prostaglandin-3 thromboxane-3, leukotriene-5 series and resolvins) or by inducing changes in membrane composition and fluidity, gene transcription, signal transduction and cell activation [Calder, 2006; Fritsche, 2006; Gil, 2002; Yaqoob & Calder, 1995]. Many epidemiological and clinical studies have demonstrated that the anti-inflammatory properties of n-3 PUFA in healthy humans [Bouwens *et al.*, 2009; Farzaneh-Far *et al.*, 2009; Vedin *et al.*, 2008; Weaver *et al.*, 2009]. In addition, n-3 PUFA may attenuate a wide range of inflammatory diseases, including rheumatoid arthritis [Cleland *et al.*, 2003], cardiovascular disease [Sanderson & Calder, 1998; de Roos *et al.*, 2009], inflammatory bowel disease [Cleland *et al.*, 2003; Uchiyama, 2010] and cancer [Chapkin *et al.*, 2008]. However, the

same anti-inflammatory properties which are so beneficial in chronic inflammatory diseases may be detrimental in the context of anti-microbial immunity. Indeed, n-3 PUFA feeding increases mouse susceptibility to intracellular pathogens, such as *Listeria monocytogenes* [Fritsche *et al.*, 1997, *Salmonella typhimurium* [Chang *et al.*, 1992 and *Paracoccidioides brasiliensis* [Oarada *et al.*, 2003]. Guinea pigs infected with virulent *M. tuberculosis* fed with n-3 PUFA, and transgenic *fat-1* mice with endogenous enrichment of n-3 PUFA, showed increased bacterial loads and pronounced progression of the disease [Bonilla *et al.*, 2010a; McFarland *et al.*, 2008; Paul *et al.*, 1997]. These experimental findings suggest that increased tissue levels of n-3 PUFA can impair resistance to TB.

Effect of n-3 PUFA on macrophage activation and antimicrobial capacity

It has been shown that n-3 PUFA exert a deactivating effect on macrophages (Table I). As suggested earlier, macrophage activation is indispensable for clearance of mycobacteria and other intracellular microbial pathogens. Macrophage activation leads to induction of a variety of cellular processes and allows control of intracellular pathogens in a more effective way. n-3 PUFA decrease macrophage activation in response to LPS, IFN γ , or TLR ligands [Lee *et al.*, 2003; Lu *et al.*, 1998]. This deactivating effect of n-3 PUFA leads to diminished production of pro-inflammatory eicosanoids and cytokines, as well as expression of costimulatory and adhesion molecules. It has been proven that these effects of n-3 PUFA are mediated by modulation of the activation of transcription factors, such as PPARs [Krey *et al.*, 1997], CREB [Jolly *et al.*, 1997] and NF- κ B [Novak *et al.*, 2003] and interference with IFN signaling [Irons & Fritsche, 2005] or TLR activation [Weatherill *et al.*, 2005]. Generation of pro-inflammatory cytokines, such as TNF α [Yaqoob & Calder, 1995; Zeyda *et al.*, 2005], IL-12 [Zhang & Fritsche, 2004], IL-1 β [Yaqoob & Calder, 1995] and IL-6 [Bagga *et al.*, 2003; Yaqoob & Calder, 1995] is also impaired by n-3 PUFA supplementation in mouse macrophages. As mentioned above, production of IFN γ [Cooper *et al.*, 1993; Flynn *et al.*, 1993], IL-12 [Cooper *et al.*, 1997] and TNF α [Flynn *et al.*, 1995] are essential for the development of resistance to TB. Additionally, n-3 PUFA enhance production of anti-inflammatory cytokines, including TGF β [Venkatraman *et al.*, 1994], IL-4 [Petursdottir & Hardardottir, 2008] and IL-10 [Bradley *et al.*, 2008]. The n-3 PUFA effect on cytokine production is mediated by down-regulation of the activation of transcription factors, such as NF κ B [Denys *et al.*, 2005; Fan *et al.*, 2004; Weber *et al.*, 1995 and AP-1 [Fan *et al.*, 2004] in murine macrophages.

Preliminary findings suggest that DHA and EPA modify lipid composition of endosomal membranes [Sturgill-Koszycki *et al.*, 1994 and alter actin assembly in *Mtb*-containing phagosomes [Anes *et al.*, 2003], impairing phagolysosome formation in J774 macrophages. In addition, lipid membrane composition, integrity and permeability can be modified by dietary intake of n-3 PUFA. In contrast, the n-6 PUFA arachidonic acid increases phagosomal maturation and bacterial clearance in *Mtb*-infected J774A.1 cells [Anes *et al.*, 2003]. Reactive oxygen species (ROS) have anti-mycobacterial activity and their production is negatively regulated by antioxidant enzymes, including superoxidase dismutase and catalase. In addition, ROS have been demonstrated to induce activation of transcription factors [Janssen-Heininger *et al.*, 2000] which regulate the production of cytokines and other immune mediators. It has been established that n-3 PUFA dietary supplementation reduces superoxide production in monocytes [Fisher *et al.*, 1990; Luostarinen & Saldeen, 1996], macrophages [Wang *et al.*, 2004] and neutrophils [Thompson *et al.*, 1991] by down-regulating NADPH oxidase activity. Decreased production of ROS is accompanied by increased activity of antioxidant catalase, glutathione peroxidase and superoxide dismutase in both DHA-treated macrophages [Wang *et al.*, 2004] and animals supplemented with fish oil [Bas *et al.*, 2007]. Nitric oxide (NO), an antibacterial compound generated by the inducible nitric oxide synthase (iNOS), is decreased by DHA, both *in vitro* [Khair-el-Din *et*

et al., 1996; Komatsu *et al.*, 2003; Wang *et al.*, 2003] and *in vivo* [Bas *et al.*, 2007; Sarsilmaz M, 2003].

Macrophages are both host and effector cells for *M. tuberculosis*, being responsible for mycobacterial killing via enzymatic degradation within mature phagolysosomes and through generation of oxygen and nitrogen reactive species. *M. tuberculosis* is able to evade the macrophage response and the effectiveness of those antimycobacterial mechanisms depends on an appropriate level of macrophage activation [Deretic *et al.*, 1997; Vergne *et al.*, 2004. IFN γ activates macrophage maturation [Via *et al.*, 1998], inducing upregulation of proinflammatory cytokines and surface markers. However, n-3 PUFA have been found to impair IFN γ -induced activation, thereby reducing the ability to kill some pathogens and to stimulate an acquired immune response [Clouva-Molyvdas *et al.*, 1992; Fritsche *et al.*, 1997; Lu *et al.*, 1998]. Different processes important for killing activity are affected by DHA, including the respiratory burst [Khair-el-Din *et al.*, 1996], chemotaxis [Schmidt *et al.*, 1992], antigen presentation [Arrington *et al.*, 2001], the expression of adhesion molecules and MHC class II and class I [Hughes & Pinder, 1997]. It has been also established that DHA-treated cells produce less pro-inflammatory eicosanoids [Bagga *et al.*, 2003] and cytokines [Endres *et al.*, 1989], such as IL-1 β , IL-12p70 and TNF α , which are known to be associated with a protective response to TB [Flynn *et al.*, 1995; Peres *et al.*, 2007]. Furthermore, lipids have been found to influence phagolysosome maturation which is critical for mycobacterial clearance by macrophages. Arachidonic acid induced actin assembly, acidification and lysosome fusion in phagosomes from *Mtb*-infected macrophages, leading to more effective mycobacterial control [Anes *et al.*, 2003]. In contrast, DHA inhibited actin assembly and favored mycobacterial survival. This inhibitory effect on phagosome maturation might also be mediated by modification of the endosomal membrane lipid composition [Russell, 2003]. Together, these studies suggest that n-3 PUFA could impair host resistance to *Mtb* infection by suppressing macrophage activation and killing capacity.

Based upon these considerations, we conducted a series of experiments to elucidate the mechanisms whereby n-3 PUFA alter the host response to TB. The central hypothesis was that n-3 PUFA incorporation would alter macrophage membrane composition and function, thereby suppressing the antimycobacterial activity of macrophages. Two different macrophage model systems were used in these studies. The first approach utilized the mouse macrophage cell line J774A.1, which was treated with exogenous n-3 PUFA *in vitro* and then activated and/or infected with *M. tuberculosis*. The second approach employed macrophages isolated and studied *ex vivo* from *fat-1* transgenic mice which produce n-3 PUFA endogenously under the control of the desaturase enzyme transgene from *C. elegans* [Kang *et al.*, 2004]. In both models, the readouts included alterations in the lipid composition of the macrophage membranes, receptor protein expression and co-localization with lipid rafts, membrane-associated post-phagocytic events, and intracellular anti-microbial effector functions.

Influence of n-3 PUFA on membrane composition in J774 and *fat-1* macrophages

Mouse macrophage-like J774.A1 cell (ATCC TIB-67) monolayers were incubated for 24 hours with 50 μ M conjugated BSA-DHA (docosahexaenoic acid; 22:6n-3), BSA-AA (arachidonic acid) or BSA-PA (palmitic acid; 16:0). Fatty acids (FA) were added at a FA/BSA 3:1 mole ratio according to Arrington et al [Arrington *et al.*, 2001. AA, an n-6 PUFA, was included to discriminate between an n-3 PUFA effect and a general polyunsaturated fatty acid effect. PA, a saturated fatty acid, was used to rule out a general unsaturated fatty

acid effect. IFN γ treatment was used to induce activation and macrophages primed with 10 ng/ml LPS were used as positive controls.

For *ex vivo* experiments, thioglycollate-elicited peritoneal macrophages were isolated from *fat-1* mice and plated in 96-well flat bottom plates (2×10^5 cells/well) or onto coverslips and allowed to adhere. After 2 hours, non adherent cells were removed and adherent cells were activated by infection with virulent *M. tuberculosis*. Virulent *M. tuberculosis* H37Rv (ATCC 27294) was handled as described previously [Grover *et al.*, 1967]. For infection of either J774 cells or *fat-1* peritoneal macrophages, bacteria were thawed, sonicated, vortexed, and passed through a 28G needle fifteen times, as we have described previously [Lasco *et al.*, 2005]. Adherent cells were infected at a multiplicity of infection (MOI) of 10 or 20, according to the type of experiment. At specific time points after infection, cell monolayers were washed with PBS and incubated with 50 μ g/ml gentamicin to kill extracellular bacteria [Elsinghorst, 1994].

Total lipids were extracted from fatty acid-treated J774A.1 cells or *fat-1* peritoneal macrophages by the method of Folch [Folch, 1957]. Phospholipids were separated by TLC, and fatty acid methyl esters were analyzed by capillary gas chromatography as previously described [Chapkin *et al.*, 1991]. J774A.1 cells efficiently incorporated fatty acids provided in the culture medium into their cell membranes under the conditions used in this study. DHA-treated cells significantly ($p < 0.05$) increased the DHA content into their membrane phospholipids. For example, the amount of DHA in DHA-treated cells was 18.24 ± 0.52 mol %, compared to $1.70 \pm 0.04\%$ or $2.15 \pm 0.50\%$ in cells incubated with AA or medium alone, respectively. Conversely, AA-treated cells had increased incorporation of AA ($20.96 \pm 0.72\%$), compared to other treatments (DHA $5.01 \pm 0.06\%$, PA $5.12 \pm 0.04\%$, Control $5.74 \pm 0.59\%$). Cells treated with either DHA or AA had a reduced percentage of oleic acid ($18.1 \pm 0.9\%$, $14.07 \pm 0.22\%$ and $13.36 \pm 0.15\%$ respectively, compared to Control ($20.93 \pm 0.32\%$) or PA-treated cells ($21.36 \pm 0.65\%$). In thioglycollate-elicited *fat-1* peritoneal macrophages used in *ex vivo* experiments, the ratio of n-6 to n-3 was significantly lower in *fat-1* cells (4.25 ± 2.05), compared to WT (24.93 ± 1.65) ($p < 0.001$). In *fat-1* macrophages, we observed that AA levels were maintained even though n-3 PUFA were being produced endogenously [Bonilla *et al.*, 2010b].

Monolayers of lipid-treated J774 cells were resuspended and labeled with rat anti-mouse antibodies to CD14, CD40 and CD86. Cells were fixed in 2% paraformaldehyde in PBS and analyzed by flow cytometry. DHA significantly suppressed the upregulation of both CD40 and CD86 in infected J774 cells primed with IFN γ . No significant changes in CD14 expression were observed.

Effect of n-3 PUFA on membrane-mediated functions in J774 and *fat-1* macrophages

Either J774 cells or *fat-1* peritoneal exudates macrophages were infected with GFP-expressing bacteria and incubated with 50 nM LysoTracker Red DND99 for 2 hours before and during the infection. Alternatively, J774 cells were fixed with formaldehyde, permeabilized, and incubated with primary antibodies against LAMP-1, Rab5, Rab7 and LAMP-3 [Vergne *et al.*, 2004]. Cells were analyzed using spinning disk scanning confocal microscopy. The quantitative scoring of colocalization was performed by determining the percentage of lysosomal marker-positive mycobacterial phagosomes. Serial sections were taken with Z-stack spanning to confirm the colocalization. We observed reduced lysoTracker colocalization in IFN γ -primed J774 cells treated with DHA, demonstrating that the ability of IFN γ to drive phagosome-lysosome fusion was significantly suppressed in infected cells by DHA. DHA induced a 62% reduction in lysoTracker-positive mycobacterial phagosomes

over 1 hour after infection. We also demonstrated the reduced recruitment of LAMP1, LAMP-3, Rab-5, and Rab-7 to mycobacterial phagosomes in DHA-treated cells [Bonilla *et al.*, 2010b] LAMP-1, LAMP-3 and Rab-7 are markers of late endosomes indicating maturation into a phagolysosome within which mycobacterial replication would be controlled.

Similar results were observed in *fat-1* peritoneal exudate macrophages. At 1 hour post infection, the quantitative scoring of colocalization showed a significantly reduced percentage of LysoTracker positive *M. tuberculosis*-containing phagosomes in *fat-1* cells ($29.83 \pm 2.69\%$ versus $44.82 \pm 2.24\%$ in WT, $p < 0.001$), suggesting a defective phagolysosome maturation. GFP-*M. tuberculosis* rapidly colocalized with LysoTracker in WT cells while, in *fat-1* macrophages, the probe was excluded from mycobacterial phagosomes [Bonilla *et al.*, 2010a. The colocalization of the other late-endosomal markers mentioned above was not addressed in *fat-1* macrophages.

To better understand the underlying mechanisms by which DHA negatively affects IFN γ -mediated macrophage activation, the surface expression of IFN γ receptors R1 and R2 was quantified by fluorescent staining on J774.1 cells treated with DHA and activated with IFN γ . DHA treatment alone did not affect the cell surface expression levels of IFN γ R1 or IFN γ R2 as assessed by mean fluorescent intensity. Subsequent activation with IFN γ slightly enhanced the surface expression of IFN γ R1, but not IFN γ R2. Therefore, the impaired activation status in DHA-treated, IFN γ -activated J774 cells cannot be explained by a loss of IFN γ receptor expression [Bonilla *et al.*, 2010b].

To determine whether IFN γ -induced cell signaling was affected by DHA treatment, J774 cells were treated with DHA and activated with IFN γ at various time points. The lysates were immunoblotted with specific antibodies to total and phospho-STAT1 to determine the activation status of these cells. DHA treatment had no effect on total STAT1 protein expression, and the expression of phosphorylated STAT1 peaked at 60 min post-activation in both DHA-treated and untreated cells. However, quantitative densitometry analysis indicated that DHA treatment significantly suppressed the phosphorylation status of STAT1 protein at both 30 min and 1 hour post-activation with IFN γ . These data imply that DHA may impair downstream cell signaling events of the IFN γ receptor and, thus, suppress cell function [Bonilla *et al.*, 2010b].

The observation that DHA treatment did not affect total IFN γ R expression, but did impair downstream signaling via STAT1, suggested that IFN γ R function might be altered by DHA. To determine whether DHA affected recruitment of the IFN γ R to lipid rafts during cell activation, DHA-treated, non-infected macrophages were activated with IFN γ and fluorescently stained with anti-IFN γ R1 antibody and anti-GM1, a lipid raft marker. Based upon confocal analysis, the presence of DHA dramatically interrupted the interaction between IFN γ R1 and the lipid raft marker by ~80% at 5 min post-activation. Taken together, these data demonstrate that while DHA does not directly affect the surface expression levels of the IFN γ receptors, it does impair cell activation by disrupting the localization of the IFN γ R1 to lipid rafts and thus, phosphorylation of key signaling molecules critical for macrophage activation [Bonilla *et al.*, 2010b].

Impact of n-3 PUFA on anti-mycobacterial effector functions in J774 and *fat-1* macrophages

Macrophage monolayers were infected with a green fluorescent protein (GFP)-expressing virulent strain (H37Rv) of *Mycobacterium tuberculosis*. Discrimination between intracellular and extracellular bacteria was accomplished by using a polyclonal antibody

against whole mycobacterial cell lysate. Serial sections across entire cells were taken with a Z-stack and 3D reconstruction was performed to confirm the intracellular bacterial localization. The percentage of cells with internalized bacteria was determined using scanning confocal microscopy. The percentage of infected cells was also calculated by flow cytometry. After infection, monolayers were lysed and the colony forming unit (CFU) counts were determined after 3–4 weeks of incubation as described previously [Lasco *et al.*, 2005].

In order to determine if DHA incorporation impaired the ability of IFN γ -treated J774 cells to control *M. tuberculosis*, we measured intracellular bacterial accumulation by flow cytometry and fluorescent microscopy and bacterial survival by the CFU assay. The presence of DHA significantly increased the percentage of infected J774 cells compared to the other fatty acids after 30 and 90 min of infection. Moreover, DHA facilitated bacterial survival within macrophages, as a greater number of viable mycobacteria were recovered from DHA-treated cells at all intervals post-infection (30 min, 1 hour, 1, 3 and 7 d) [Bonilla *et al.*, 2010b].

Culture supernatants from IFN γ -activated, infected J774 cells were harvested and assessed for IL-6, IL-12p70, TNF α , MCP-1, IL-10 and IFN γ by flow cytometry. The IFN γ -induced activation of infected cells as assessed by the production of pro-inflammatory cytokines was impaired significantly by DHA. Infected J774 cells enriched in DHA produced significantly less TNF α protein levels at 3, 24 and 48 hour. In addition, DHA significantly reduced IL-6 levels in infected cells over 3, 6 and 48 hour. We observed a similar suppressive effect of DHA on TNF α and IL-6 levels after LPS treatment, demonstrating that the deactivating effects of DHA was not stimulus specific [Bonilla *et al.*, 2010b].

J774 cells were infected and intracellular ROS levels were measured by staining with oxidative fluorescent dyes 10 μ M 5-(and 6-)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (DCFDA) (Invitrogen) and 5 μ M Dihydroethidium (DHE) (Invitrogen), according to a published protocol [Carter *et al.*, 1994]. DHE becomes a fluorescent compound (Ethidium bromide, EB) after oxidation by reactive species. In the presence of DHA, IFN γ -treated *M. tuberculosis*-infected J774 cells exhibited significantly ($p < 0.01$) reduced oxidative activity as measured by EB fluorescence (DHA $24.4 \pm 3.7\%$ vs Control $51.2 \pm 4.2\%$, $p < 0.01$). An increase in oxidative burst was observed after infection, however, the response was blunted in DHA-treated cells. Neither AA nor PA had a significant effect on oxidative activity in infected J774 cells. The suppressive effect was also seen following LPS treatment. Overall, these data suggest that the increased bacterial loads found in DHA-treated cells might be explained by an impaired oxidative burst [Bonilla *et al.*, 2010b].

A similar series of experiments were conducted in peritoneal exudates macrophages from *fat-1* mice which were infected immediately *ex vivo* with virulent *M. tuberculosis*. *fat-1* macrophages demonstrated increased susceptibility to infection and sustained higher bacterial loads over 7 days of infection. At 1 hour post infection, *fat-1* cells exhibited a significantly ($p < 0.001$) increased percentage of infection ($44.82 \pm 2.24\%$) compared to WT macrophages ($29.83 \pm 2.69\%$). The n-3 PUFA levels in the *fat-1* and WT macrophages were positively correlated with increased CFU values ($R = 0.54$ $p < 0.001$). Exponential bacterial growth was observed between 1 hour and 3 days post infection, after which infection was controlled in both cell types. However, a greater number of viable mycobacteria were recovered from *fat-1* cells at all intervals evaluated (1 hour, 1, 3, 5 and 7 days) [Bonilla *et al.*, 2010a].

In order to determine whether the increased susceptibility to *M. tuberculosis* infection in *fat-1* macrophages was explained by n-3 PUFA-induced deactivation, we measured levels of

pro-inflammatory cytokines as markers of cell activation. In infected WT cells, significant upregulation of TNF α , IL-6, IL-1 β , and MCP-1 was observed in WT cells. However, *fat-1* macrophages produced only 15% of the TNF α protein levels (264 ± 22 pg/ml) produced by macrophages from WT animals (1619 ± 298 pg/ml) at 6 hour post infection ($p < 0.05$). Furthermore, levels of IL-6, IL-1 β , and MCP-1 were also significantly down-regulated following mycobacterial infection in *fat-1* cells. For example, at 12 hour post infection, the percentages of cytokine reduction in *fat-1* macrophages were 72% for TNF α , 86% for IL-6, 82% for IL-1 β and 67% for MCP-1, compared to WT. We observed a similar suppressive effect in *fat-1* cells stimulated with LPS [Bonilla *et al.*, 2010a].

The ability of infected *fat-1* macrophages to generate anti-mycobacterial effector molecules was determined by measuring levels of reactive oxygen intermediates (ROS) by DHE oxidation and nitric oxide (NO) by the Griess reaction. One hour after infection, cells from *fat-1* mice had significantly ($p < 0.01$) lower levels of ROS (20.78 ± 3.12 EB %) than those from WT animals (38.77 ± 4.09 EB %). A trend toward reduced NO levels in *fat-1* macrophages also was observed after 24 and 48 hour post infection. Overall, these data suggest that the increased bacterial loads found in *fat-1* cells might be explained by an impaired anti-mycobacterial effector activity, as demonstrated by impaired phagosomal maturation and reduced oxidative burst [Bonilla *et al.*, 2010a].

Macrophage summary and conclusions

Taken together, the results of the published work reviewed here lead to the inescapable conclusion that dietary fat, including n-3 PUFA, and cholesterol exert a detrimental impact on the host immune response against *M. tuberculosis* infection, both *in vivo* in infected mouse and guinea pig models, *ex vivo* in macrophages enriched in n-3 PUFA by diet or genetic manipulation (e.g., *fat-1*), and *in vitro* in a mouse macrophage cell line treated during culture with n-3 PUFA [Anes *et al.*, 2003; Bonilla *et al.*, 2010a; Jordao *et al.*, 2008; Kaul *et al.*, 2004; McFarland *et al.*, 2008; Pandey & Sasseti, 2008; Paul *et al.*, 1997; Pieters, 2008.]

Using J774A.1 cells and *fat-1* macrophages, we observed that n-3 PUFA favored mycobacterial survival and reduced activation of macrophage antibacterial responses in both cell types. DHA treatment of infected J774A.1 cells reduced IFN γ -induced macrophage activation as measured by lower levels of cytokines produced (TNF α , IL-6 and MCP-1) and costimulatory molecules expressed (CD40 and CD86). DHA-treated J774A.1 cells were more permissive for infection with virulent *M. tuberculosis*, exhibited impaired phagolysosomal fusion, and generated a dramatically impaired oxidative burst. Although surface expression of IFN γ receptors was not altered by DHA treatment, the co-localization of IFN γ R1 with lipid rafts was impaired, leading to downstream deficits in the phosphorylation of STAT-1, a critical promoter of macrophage activation. Figure 1 summarizes the results obtained *in vitro* using J774A.1 macrophages [Bonilla *et al.*, 2010b].

Remarkably similar n-3 PUFA induced defects in macrophage activation and anti-mycobacterial effector functions were observed in peritoneal exudate macrophages freshly isolated from *fat-1* mice. In *fat-1* primary macrophages, as in J774A.1 cells, we observed increased uptake and growth of *M. tuberculosis*, accompanied by impaired phagosome maturation, blunted pro-inflammatory cytokine production, and a reduced respiratory burst [Bonilla *et al.*, 2010a]. The striking similarities in the results obtained with these two very different macrophage models engender great confidence in the biological relevance and application of the observations.

Dynamic organization of the plasma membrane: Lessons from dietary studies in T lymphocytes

Many of the defects in macrophage activation reviewed above could be linked mechanistically to the obvious alterations in the n-3 PUFA content of macrophage membranes. The data from many published reports support a direct effect of n-3 PUFA on membrane structure and biological function as a likely explanation for the loss of critical anti-microbial functions in macrophages [Calder, 2001; Calder *et al.*, 1990; Chapkin *et al.*, 2008; Halvorsen *et al.*, 1997; Kim *et al.*, 2008; Lee *et al.*, 2004; Stillwell & Wassall, 2003; Wiesenfeld *et al.*, 2001]. We support the membrane “hypothesis” to explain many of the observations reported in this review, and we believe that the underlying mechanisms which link n-3 PUFA enrichment and alteration in cell function will turn out to be shared by both macrophages and T lymphocytes. For that reason, we have pursued highly mechanistic studies as to how dietary n-3 PUFA alter T-cell activation and function by incorporation into both plasma and intracellular membranes [Calder, 2009].

Generally, increasing the dietary intake of n-3 PUFA from marine/algae oil sources results in the increased incorporation of DHA and EPA into T-cells, typically at the expense of n-6 PUFAs, especially linoleic (LA, 18:2n-6) and AA [Chapkin, 2007; Calder, 2009]. In summary, the cellular level of esterified fatty acids is readily influenced by diet. Further, time-course studies indicate that the incorporation of EPA and DHA into human immune cells reaches its peak within four weeks post initiation of dietary intake in a dose-response manner [Calder, 2009]. The sub-cellular incorporation of n-3 PUFA is localized primarily within plasma and intracellular membrane phospholipids at the *sn*-2 position [Anderson & Sperling, 1971; Stillwell & Wassall, 2003]. Of biological relevance, dietary n-3 PUFA have been shown to specifically alter plasma membrane micro-organization (lipid rafts) at the immunological synapse (IS) between T-cells and antigen-presenting cells ultimately suppressing signal transduction and nuclear translocation/activation of transcription factors [Fan *et al.*, 2004; Kim *et al.*, 2008, 2010; Yog *et al.*, 2010]. Interestingly DHA and EPA enrichment has been shown to occur in both membrane lipid raft and non-raft membrane fractions isolated from CD4⁺ T cells [Switzer *et al.*, 2004]. This is noteworthy, because the presence of long chain n-3 PUFA in the membrane imparts unique physiochemical properties to cellular phospholipids, and DHA-induced alterations in membrane structure and function have been proposed to underlie the beneficial anti-inflammatory effects of n-3 PUFA [Stillwell & Wassall, 2003; Ma *et al.*, 2004; Seo *et al.*, 2006; Chapkin *et al.*, 2007].

Modulation of T cell signaling by n-3 PUFA

Lipid rafts are enriched in the immunological synapse, the site of T-cell and antigen presenting cell (APC) contact for antigen recognition. This is noteworthy, because sites of contact serve as platforms for the migration of T-cell receptors, co-activating receptors and cell signaling mediators [Friedl *et al.*, 2005]. Of interest, the addition of fatty acids in culture or diet modulated the formation of the immunological synapses and targeting of signaling proteins into lipid rafts. It has been demonstrated that purified PUFA, i.e. LA, ALA, AA, EPA and DHA suppressed CD3-induced migration of Src family kinases, i.e. Lck and Fyn, into detergent-resistant membrane (lipid raft) fractions compared to the saturated stearic acid (18:0) or monounsaturated oleic acid (18:1 n-9) treated control human Jurkat CD4⁺ T-cells [Geyeregger *et al.*, 2005]. In contrast, the intracellular localization of ganglioside GM1 was not affected by PUFA treatments. We have also observed that, following IS formation by anti-CD3 (clone 145-2C11) expressing hybridoma cells, GM1 localization in CD4⁺ T-cells from endogenous n-3 PUFA producing *fat-1* transgenic mice was not altered, as compared to wild type control T-cells [Kim *et al.*, 2008]. Since GM-1 is localized to the outer (exofacial) leaflet of the plasma membrane, these data imply that PUFA treatment

exclusively altered the fatty acid composition in the inner leaflet (cytofacial) of the plasma membrane.

Recent findings from our laboratory [Fan et al., 2003, 2004] demonstrated that a diet containing fish oil suppressed the colocalization of PKC θ into GM-1 specific lipid rafts in mitogen-stimulated murine CD4⁺ T-cells. Further examination indicated that n-3 PUFA down-modulated the migration and activation status of PKC θ and PLC γ -1, as assessed by immunostaining of pan- or phospho-proteins in fixed cells, respectively [Kim *et al.*, 2008]. These data suggest that following the formation of an immunological synapse between CD4⁺ T-cells and antigen presenting cells, dietary n-3 PUFA alters the temporal location of signaling proteins at the immunological synapse by affecting the formation of lipid rafts. Since a synergistic interaction exists between the actin cytoskeleton and membrane rafts [Chichili & Rodgers, 2009], and n-3 PUFA can displace F-actin from the IS [Kim *et al.*, 2008], where T cells and APC form a conjunction, we also examined actin-dependent mitochondrial translocation. Data from [Yog *et al* 2010] demonstrate that n-3 PUFA suppressed mitochondrial translocation to the IS and perturb Ca²⁺ signaling, which is critical to T cell activation. These results provide a critical new paradigm in understanding the molecular mechanisms through which n-3 PUFA dampen T-cell activation. Additional studies are needed to further elucidate the effect of dietary n-3 PUFA on T cell mitochondrial motility.

PUFA - lipid raft paradox

It is not known precisely how dietary lipids modulate lipid rafts due to plasma membrane heterogeneity and the limitations of experimental approaches [Kaiser et al., 2009; Otahal et al., 2010]. To determine the effect of n-3 PUFA on the formation of lipid rafts, Kim *et al.* demonstrated that n-3 PUFA increased the formation of lipid rafts in endogenous n-3 PUFA enriched CD4⁺ T-cells [Kim *et al.*, 2008]. Similarly, it has been reported that lipid raft size was increased by DHA treatment in EL-4 B-cell cultures [Shaikh *et al.*, 2009]. Collectively, these data are consistent with the fact that the unique hairpin structure of DHA is incompatible with cholesterol and subsequently forms DHA-rich microdomains, which may explain the above observations. We have also analyzed the effects of dietary lipids on cholesterol-dependent and cholesterol-independent nanoclusters within the inner plasma membrane leaflet of cells using immuno-gold electron microscopy of plasma membrane sheets coupled with spatial point analysis of validated microdomain markers [Chapkin *et al.*, 2008; Kim *et al.*, 2010]. Overall, DHA significantly increased lipid raft clustering. In contrast, in cell line experiments using human Jurkat CD4⁺ T-cells, EPA incubation at physiologically relevant concentrations impaired lipid raft formation following co-culture with anti-CD3 coated beads as assessed by Laurdan (6-dodecanoyl-2-dimethyl aminonaphthalene) labeling [Zech *et al.*, 2009]. In order to address this apparent conundrum, we analyzed lipid raft formation in Laurdan labeled Jurkat T-cells at the IS following co-culture with superantigen Staphylococcal Enterotoxin E-pulsed human Raji B-cells. Similar to the findings of Zech *et al* (2009), general polarization values at the IS were significantly suppressed in DHA treated cells, as compared to control cells [Kim *et al.*, 2010]. These data suggest that the accumulation of lipid rafts in cell lines may be fundamentally distinct from primary cells with respect to the dynamic organization of the plasma membrane, and therefore may not serve as a suitable model to probe fatty acid effects on lipid raft-dependent signaling. To summarize, despite a significant amount of research, due to the complexity of different membrane domains, experimental model systems and cell types, activation methodologies, and fatty acids doses, many questions remain regarding the nutritional and clinical significance of lipid rafts in immune cell types.

Summary

In this review, we have attempted to link n-3 PUFA-induced changes in biological activation state and function to alterations in cell membrane structure and transmembrane signaling in two disparate immune cell types, namely, T-cells and macrophages. The mechanistic link between n-3 PUFA (especially DHA) enrichment in membranes, alteration in lipid rafts, and loss of pro-inflammatory function is very strong in T-cells. The therapeutic effects of n-3 PUFA in several inflammatory diseases can be explained, at least in part, by membrane-mediated loss of a pro-inflammatory T-cell phenotype. On the other hand, the effects of n-3 PUFA enrichment on membrane-mediated functions in macrophages may actually result in a detrimental outcome, i.e., loss of antimicrobial resistance. We provide compelling evidence that murine macrophages lose the ability to control intracellular *M. tuberculosis* following n-3 PUFA enrichment by dietary fish oil, in vitro culture, or genetic (i.e., *fat-1*) intervention. The role of membrane alteration in the macrophage phenotype is cogent, since many of the functional deficits are associated with events at either the cell membrane (e.g., uptake of mycobacteria, association of IFN γ R1 with lipid rafts, blunted STAT-1 signaling), or the phagosome membrane (e.g., endosome maturation). Additional research is required to determine the optimal dose and duration of n-3 PUFA in the diet in order to maintain physiologic control of the functional status of a healthy immune system.

Highlights: (1) Dietary fatty acids alter membrane-mediated macrophage functions and promote the pathogenesis and immune control of infectious diseases; (2) n-3 PUFA uniquely affect membrane composition in J774 and *fat-1* macrophages; (3) PUFA alter the dynamic organization of the lymphocyte plasma membrane and modulate downstream cell signaling; (4) the accumulation of lipid rafts in cell lines may be fundamentally distinct from primary cells.

Acknowledgments

This work was supported by grant DK07107 and CA59034 from the National Institutes of Health (NIH) and the U.S. Department of Agriculture CSREES Special Grant, "Designing Foods for Health 2010-34402-20875.

Abbreviations

AA	arachidonic acid
ALA	alpha-linolenic acid
APC	antigen presenting cell
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
FO	fish oil
LA	linoleic acid
LPS	lipopolysaccharide
Mtb	mycobacterium tuberculosis
PA	palmitic acid
PUFA	polyunsaturated fatty acids

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Figure 1. Effect of DHA on IFN γ -treated, *Mtb*-infected J774A.1 cells

DHA treatment induced defective cell activation in J774A.1 macrophages, interfering with the IFN γ -induced up-regulation of pro-inflammatory cytokines and costimulatory molecules, compared to cells treated with AA, PA or media alone (Control). Under those conditions, DHA-treated cells were less able to control *Mtb* infection. Defects in bacterial control were accompanied by reduced generation of reactive oxygen species and impaired maturation.

Symbols on the macrophage surface signify CD40 (+) and CD86 (V) co-stimulatory receptors.

Table I

Deactivating effects of n-3 PUFA on macrophage activation and antimicrobial responses

Macrophage Function	n-3 PUFA Impact on Macrophage Function		
	Effect induced	Species used	Reference
- Activation in response to:			
IFN γ	↓	Mouse	[Lu <i>et al.</i> , 1998]; [Irons & Fritsche, 2005]
LPS	↓	Mouse	Lu <i>et al.</i> , 1998
TNF α	↓	Human	[Weber <i>et al.</i> , 1995]
TLR ligands	↓	Mouse	[Lee <i>et al.</i> , 2003], [Wong <i>et al.</i> , 2009]
- Production of pro-inflammatory cytokines:			
TNF α	↓	Mouse	[Yaqoob & Calder, 1995]
IL-12	↓	Mouse	[Zhang & Fritsche, 2004]
IL-1 β	↓	Mouse	[Yaqoob & Calder, 1995]
IL-6	↓	Mouse	[Yaqoob & Calder, 1995] [Bagga <i>et al.</i> , 2003]
- Production of anti-inflammatory cytokines:			
TGF β	↑	Mouse	[Venkatraman <i>et al.</i> , 1994]
IL-10	↑	Mouse	[Petursdottir & Hardardottir, 2008]
IL-4	↑	Mouse	[Bradley <i>et al.</i> , 2008]
- Generation of reactive species:			
ROS	↓	Human	[Fisher <i>et al.</i> , 1990]
RNS	↓	Mouse	[Komatsu <i>et al.</i> , 2003]
- Migration	↓	Human	[Schmidt <i>et al.</i> , 1992] [Weber <i>et al.</i> , 1995]
- Phagosome maturation	↓	Mouse	[Anes <i>et al.</i> , 2003]
- Phagocytosis	↓↑	Mouse	[Guimaraes <i>et al.</i> , 1991] [Chapkin <i>et al.</i> , 1992]
- Antigen presentation	↓	Human	[Hughes & Pinder, 1997]

IFN γ , gamma interferon; IL,
interleukin; LPS,
lipopolysaccharide; RNS, reactive
nitrogen species; ROS, reactive
oxygen species; TNF α , tumor
necrosis factor α