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Fish oil disrupts MHC class II lateral organization on the B-cell side of the immunological synapse independent of B-T cell adhesion

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Abstract

Fish oil enriched long chain n-3 polyunsaturated fatty acids disrupt the molecular organization of T-cell proteins in the immunological synapse. The impact of fish oil-derived n-3 fatty acids on antigen presenting cells, particularly at the animal level, is unknown. We previously demonstrated B-cells isolated from mice fed fish oil suppressed naïve CD4⁺ T-cell activation. Therefore, here we determined the mechanistic effects of fish oil on murine B-cell major histocompatibility complex (MHC) class II molecular distribution using a combination of TIRF, FRET, and confocal imaging. Fish oil had no impact on pre-synaptic B-cell MHC II clustering. Upon conjugation with transgenic T cells, fish oil suppressed MHC II accumulation at the immunological synapse. As a consequence, T-cell protein kinase C theta (ζ) recruitment to the synapse was also diminished. The effects were independent of changes in B-T cell adhesion, as measured with microscopy, flow cytometry and static cell adhesion assays with select immune ligands. Given that fish oil can reorganize the membrane by lowering membrane cholesterol levels, we then compared the results with fish oil to cholesterol depletion using methyl-B-cyclodextrin (M CD). M CD treatment of B-cells suppressed MHC II and T-cell PKC ζ recruitment to the immunological synapse, similar to fish oil. Overall, the results reveal commonality in the mechanism by which fish oil manipulates protein lateral organization of B-cells compared to T-cells. Furthermore, the data establish MHC class II lateral organization on the B-cell side of the immunological synapse as a novel molecular target of fish oil.

Keywords

n-3 polyunsaturated fatty acids; plasma membrane; MHC class II; B-cells

INTRODUCTION

Fish oil contains long chain n-3 polyunsaturated fatty acids, which are unique bioactive molecules with emerging clinical applications for treating chronic inflammatory diseases [1]. The immunomodulatory n-3 fatty acids of fish oil are eicosapentaenoic (EPA) and

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docosahexaenoic acid (DHA), which exert their functional effects on immunity at a cellular level through several mechanisms [2]. One central but poorly studied mechanism of EPA and DHA is the modification of plasma membrane lipid and protein molecular organization [2, 3].

Several studies show fish oil derived long chain n-3 fatty acids disrupt lipid and protein lateral organization of CD4⁺ T-cells [4–6]. This leads to suppression of downstream signaling required for T-cell activation. For example, administration of fish oil to mice suppresses recruitment of T-cell protein kinase C theta (PKC θ) into lipid microdomains and subsequent IL-2 secretion upon activation with anti-CD3/28 antibodies [6]. Likewise, *in vitro* treatment of Jurkat T-cells with EPA, or isolation of CD4⁺ T-cells from fat-1 transgenic mice, shows diminished recruitment of signaling proteins to the immunological synapse, the organized junction between antigen presenting cells and T-cells [4, 7]. In contrast, very little is known about the mechanistic effects of fish oil on plasma membrane protein-lipid spatial distribution of B-cells.

B-cells are an emerging cellular target of fish oil (or fish oil-derived metabolites) and remain poorly studied in response to fish oil intervention [8]. B-cells can serve as antigen presenting cells in addition to their canonical role in antibody production [9]. In several disease states, B-cell antigen presentation specifically contributes toward disease pathology [10]. Major histocompatibility complex (MHC) class II proteins on the surface of B-cells can present antigen-derived peptides to cognate CD4⁺ T-cells. MHC II proteins accumulate at the site of the immunological synapse to facilitate recognition of antigen by the T-cell receptor. Our lab has previously shown that B-cells, isolated from mice consuming fish oil (modeling human clinical intake) displayed diminished lipid microdomain clustering accompanied by a decrease in IL-2 secretion from cognate CD4⁺ T-cells [11]. However, we did not address the impact of fish oil on B-cell protein organization, which is regulated by the underlying lipid environment.

In this study, we tested the hypothesis that fish oil could disrupt B-cell MHC II lateral organization. We determined the effects of fish oil on MHC II organization in the absence and presence of the immunological synapse and compared the magnitude of the effect with cholesterol depletion with methyl- β -cyclodextrin (M β CD). The rationale for this approach was that M β CD is a known disrupter of protein lateral organization [12]; more importantly, there is some suggestion that fish oil exerts its effects by lowering membrane cholesterol levels [6, 13]. The data reveal, for the first time, that fish oil can disrupt MHC II accumulation on the B-cell side of the immunological synapse, accompanied by a change in T-cell protein organization.

MATERIALS AND METHODS

Mice and cells

Male C57BL/6 mice were fed a 5% total fat by weight, control or fish oil diet (Harlan-Teklad) for 3 weeks as previously described [11]. The fish oil diet models human intake of approximately 4 grams of fish oil per day, which is a typical dose used in clinical trials and is the current clinical recommendation for treating hypertriglyceridemia. Mice were euthanized using CO₂ inhalation and cervical dislocation. B220⁺ B and CD4⁺ T-cells were respectively isolated from the spleens of C57BL/6 and OT-II transgenic mice [11]. Purity of the cells was confirmed with flow cytometry using a BD LSR II cytometer. All experiments fulfilled guidelines established by the East Carolina University for euthanasia and humane treatment.

Reagents and antibodies

Human VCAM-1 and ICAM-1 were obtained from R&D Systems. Methods for the production and purification the ADAM28 disintegrin fragment were as described previously [14]. Whole human fibronectin (Fn) was purchased from BD Biosciences. M5/114 MHC class II antibody (BioXCell) was conjugated to Cy3 or Cy5 using a fluorophore conjugation kit (GE Healthcare). For some experiments, B-cells were treated for 15 min with 10 mM methyl- β -cyclodextrin (M β CD, Sigma) to deplete cholesterol. -FITC-CD3 (BD Biosciences), goat- α -PKC (Santa Cruz), and -goat Alexa Fluor 633 (Invitrogen) were used for staining the immunological synapse.

Gas chromatography (GC)

Total lipids were extracted from cells and the fatty acids were analyzed as previously described [11]. Only the major fatty acids are reported for simplicity.

Total internal reflection fluorescence (TIRF) microscopy and analysis

6.25×10^5 B-cells were adhered to poly-D-lysine (Sigma) coated coverslips and then fixed with 4% paraformaldehyde. Cells were then stained with anti-MHC II antibody (M1/42.3.9.8-Cy3) and mounted onto slides (Fisher). TIRF images were acquired over a range of penetration depths from 100–150 nanometers. A penetration depth of 100 nanometers was used for cluster analysis. TIRF instrumentation relied on an Olympus IX-71 microscope [11]. Cells were analyzed using NIH ImageJ (<http://rsbweb.nih.gov/ij/>). The average cluster number was determined by creating a region of interest for each cell, as confirmed by DIC. Then a threshold was set to analyze cluster parameters.

Förster resonance energy transfer (FRET) microscopy and analysis

FRET was measured in terms of the efficiency of energy transfer from donor (Cy3) to acceptor (Cy5) fluorophores as previously described [15]. Briefly, B-cells were fixed with 4% paraformaldehyde and stained with separately labeled Cy3 and Cy5 MHC II antibodies. Images were acquired on a Zeiss LSM 510 before and after photobleaching the acceptor fluorophore. All images were analyzed using ImageJ and the FRET calculation plugin for acceptor photobleaching FRET [16]. Positive control images were generated using a single antibody dual labeled with both donor and acceptor. Negative control images were generated to optimize photobleaching time by using only single colors.

Immunological synapse staining and analysis

To form immunological synapses, B-cells were incubated with 10^{-5} M OVA_{323–339} peptide (GenScript) for 1 hour at 37°C. B-cells and CD4⁺ T-cells were then mixed (1:2) and briefly spun down on a bench-top centrifuge. Cell-cell conjugates were subsequently transferred to poly-D-lysine coated coverslips and incubated at 37°C for 30 min. The cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 (Sigma) and blocked overnight in PBS with 2% fatty acid-free BSA at 4°C. Cells were stained for key synapse markers.

Images were acquired on a Zeiss LSM510 or an Olympus IX-81 confocal microscope. Scoring of protein patching at the synapse was performed as described by others [4, 17]. Fluorescence intensity for MHC II and CD3 is reported as the ratio of intensity at the synapse to non-synapse [18]. The percentage of B-cells forming B:T conjugates was also calculated from the same set of images. Select experiments for measuring protein clustering and recruitment of molecules into the synapse were performed blinded to ensure lack of bias.

Static cell adhesion assays and analysis

Recombinant proteins were immobilized at the desired concentration on Immulon-2 HB microtiter wells (Thermo Scientific) in a total volume of 100 μ l of 0.1 M NaHCO₃. Nonspecific adhesion was minimized by blocking wells with 2% (w/v) bovine serum albumin (BSA) in modified HEPES-Tyrodes buffer (5 mM HEPES, pH 7.4, 150 mM NaCl, 12 mM NaHCO₃, 2.6 mM KCl, 1 mg/ml BSA, 1 mM MgCl₂, and 1 mM CaCl₂) at room temperature for 30 minutes. B-cells were washed twice in HEPES-Tyrodes buffer, manually enumerated, and resuspended to the desired concentration. Cells were added to wells (2.5×10^5 /well) in 100 μ l HEPES-Tyrodes and incubated with ligands for 1 hour at 37°C in 5% CO₂. After three consecutive washes with HEPES-Tyrodes, bound cells were disclosed by determining the relative cellular acid phosphatase activity within each well as described [14]. Absorbance values were measured at 405 nm with a plate reader. Adhesion in wells coated exclusively with BSA was used to obtain background values for each dietary condition and were subtracted before reporting final adhesion values.

Statistics

Reported values are means $\pm$ SEM from 3–4 independent experiments. All statistical analyses relied on GraphPad Prism Software. All of the data sets were verified for normality. Significance was determined by a two-tailed t-test. P-values < 0.05 were considered significant.

RESULTS

Fish oil increases B-cell EPA and DHA levels

Prior to initiating studies, it was essential to ensure the experimental fish oil diet increased murine B-cell levels of EPA and DHA (Figure 1). GC analysis revealed fish oil significantly increased 16:1 (n-7) levels by 75% compared to the control. EPA, 20:5 (n-3), and its elongation product 22:5 (n-3) were both increased relative to the control by 760% and 260%, respectively. DHA, 22:6 (n-3), increased by 66% with the fish oil diet compared to the control. This resulted in a 124% increase of total n-3 fatty acid levels with the fish oil diet. Conversely, there was a 36% decrease in 20:4 (n-6) with the fish oil diet compared to the control.

Fish oil lowers MHC class II accumulation at the B:T-cell immunological synapse

MHC II molecules partially cluster on the plasma membrane prior to synapse formation [19–21]. Therefore, we first determined if fish oil could disrupt MHC II clustering prior to synapse formation. We measured micron and nanometer scale MHC II clustering using TIRF and FRET microscopy respectively. Figure 2A shows representative TIRF images of MHC II clustering on the plasma membrane prior to synapse formation. We measured the average B-cell cluster quantity (Figure 2B) and intensity (data not shown) and observed no differences between the diets. Similarly, fish oil had no effect on MHC II distribution measured with FRET (Figure 2C).

We next tested the hypothesis that fish oil could diminish MHC II organization upon B:T-cell synapse formation. We first measured the kinetics of MHC II accumulation in the synapse to determine how long OVA pulsed B-cells and CD4⁺ T-cells would need to be incubated for in order to form stable conjugates. MHC class II showed a significant increase in patching at the B:T-cell synapse at 30 minutes of conjugation (Supplemental Figure 1), as measured by confocal microscopy. Thus, we subsequently determined if fish oil disrupted MHC II accumulation at the B:T-cell synapse after 30 minutes of conjugation. Figure 2D shows representative images of MHC II patching at the B:T-cell interface. B-cells from mice fed fish oil showed a decrease of MHC II patching by 15% when compared to cells isolated

from the control diet (Figure 2E). Fish oil lowered the intensity of MHC II at the synapse relative to non-synaptic regions by 31% (Figure 2F). Moreover, FRET microscopy revealed a trend ($P=0.07$) in lowering FRET efficiency for MHC II molecules at the synapse (Figure 2G).

B-cells from fish oil fed mice decrease cognate CD4⁺ T-cell PKC θ recruitment to the immunological synapse independent of cell-cell adhesion

To address the impact of the changes in MHC II organization on CD4⁺ T-cells, we measured CD3 and PKC recruitment on the T-cell side of the immunological synapse.

Representative images of T-cell CD3 and PKC, conjugated with B-cells from either control or fish oil diets, are shown in Figure 3A. CD3 patching at the synapse was unmodified with fish oil; however, PKC patching decreased by 27% with fish oil relative to the control (Figure 3B). Similar results were observed when CD3 and PKC intensities were measured. Fish oil did not influence CD3 (Figure 3C) localization at the synapse but did lower PKC intensity by 20% (Figure 3D).

In vitro studies show that EPA and/or DHA treatment of cells can suppress B-T cell adhesion, which could mechanistically explain the changes in MHC II and PKC localization in the synapse with fish oil [22, 23]. Thus, we tested if fish oil suppressed B-T cell conjugate formation. There was no difference in the percentage of conjugates formed between B and T-cells between the control and fish oil diets as measured by microscopy (Figure 4A) and confirmed by flow cytometry (data not shown). Furthermore, cell adhesion assays showed the ability of B-cells to bind adhesion proteins ADAM28, VCAM-1, ICAM-1, and the extracellular matrix protein fibronectin was not modified by the fish oil diet (Figure 4B).

Cholesterol depletion of B-cells mimics the effects of fish oil

Given that n-3 fatty acids in fish oil can lower plasma membrane cholesterol levels, we compared the magnitude of the effects of fish oil on B-cells with cholesterol depletion using MCD. Figure 5A shows representative images of B-cells treated with MCD. MHC II patching at the synapse was suppressed with MCD by 23% (Figure 5B). MHC II fluorescent intensity between the synapse and non-synapse regions was lowered with MCD by 35% (Figure 5C).

We then measured the impact of MCD-treated B-cells on T-cell CD3 and PKC clustering at the synapse (Fig. 5D). Similar to fish oil, MCD treatment did not affect conjugate formation between B and T-cells (data not shown). However, MCD treatment reduced PKC patching at the B:T-cell synapse by 26% (Figure 5E). Image intensity analysis revealed no effect of MCD on CD3 localization to the synapse (Figure 5F). PKC accumulation at the synapse was lowered by MCD treatment by 22% (Figure 5G).

DISCUSSION

We show for the first time that fish oil diminishes MHC class II accumulation at the immunological synapse accompanied by a reduction in T-cell PKC recruitment. The study significantly advances the field by establishing MHC II lateral organization as a novel mechanistic target of fish oil. Furthermore, we show that the effects of fish oil on the B-cell side of the immunological synapse are highly similar to cholesterol depletion. Finally, compared to others, the data reveal a commonality in mechanism by which fish oil disrupts the molecular organization of lymphocyte proteins, as discussed below.

Fish oil and MHC class II membrane organization

The rationale for studying MHC II organization was based on our past studies where we demonstrated that B-cells isolated from mice fed a fish oil diet suppressed T-cell IL-2 secretion [11]. Other labs have shown that n-3 fatty acids suppress antigen presentation to T-cells by lowering MHC II surface expression on the surface of monocytes and dendritic cells [24–26]. In this study, B-cell MHC II total surface levels were not lowered with fish oil, as measured with flow cytometry (data not shown). Thus, we focused on the impact of fish oil on MHC II lateral distribution before and after the formation of the immunological synapse.

There are some suggestions in the literature, based largely on studies with immortal B-cells, that MHC II molecules are partially clustered in the absence of an immunological synapse [19, 20]. However, the physiological relevance of these clusters is unclear. Using TIRF and FRET imaging, we measured no effect of fish oil on MHC II clustering prior to the formation of the immunological synapse. This could be attributed to the dynamic nanoscale heterogeneity displayed by membrane microdomains in the absence of a stimulus [27]. In contrast, MHC II accumulation in the synapse was lowered with fish oil after conjugation with the T-cell. These results are highly consistent with our previous studies that focused on the lipid component of the B-cell plasma membrane. We discovered that fish oil diminished lipid microdomain clustering upon cross-linking GM1 molecules with cholera toxin subunit B [11, 15]. Thus, the data suggest that fish oil exerts its effects when lipid or protein molecules are reorganized in response to a stimulus.

Overall, the role of dietary lipids on B-cell membrane organization is poorly studied. The results are consistent with a few other *in vitro* studies testing the role of membrane lipids on disrupting the B-cell side of the synapse [22]. For example, Poloso et al. showed that the B-cells depleted of cholesterol with MCD disrupted MHC II organization at the immunological synapse [28]. Similarly, another study demonstrated that sequestering the signaling lipid phosphatidylinositol 4,5-bisphosphate on the B-cell surface had an impact on CD8⁺ T-cell responses through MHC class I antigen presentation [18]. Shaikh and Edidin showed that DHA treatment of human JY B-cells suppressed conjugate formation with T-cells, consistent with the notion that n-3 fatty acids suppress adhesion *in vitro* [22, 23, 29]. However, as we translated the research to the animal level in this study, we did not find suppressed B-T cell adhesion in response to fish oil using a combination of methods.

The disruption in B-cell MHC II organization was also accompanied by an effect on T-cell PKC recruitment to the synapse. PKC was selected since this protein is an early marker for stable formation of an immunological synapse [7]. The results were in agreement with data from labs to show that fish oil or EPA/DHA treatment of T-cells can displace vital proteins of the immunological synapse [4, 23]. For example, Stulnig et al., showed Jurkat T-cells treated *in vitro* with EPA displaced select proteins from the immunological synapse [23].

Comparison with cholesterol depletion

MHC II molecules cluster in cholesterol-enriched domains [30]. We discovered that cholesterol depletion had an effect on MHC II and PKC in a manner similar to fish oil. We speculate that fish oil may be exerting its effects on the B-cell by targeting cholesterol levels and/or cholesterol lateral distribution. We and others have observed trends in the ability of fish oil to lower murine lymphocyte membrane cholesterol levels [6, 11]. Stronger effects of EPA and/or DHA on membrane cholesterol distribution have been measured *in vitro*. For example, Field and co-workers reported that EPA and DHA treatment lowered the levels of cholesterol in detergent resistant membranes [13]. Similarly, Grimm et al. showed that treatment of SH-SY5Y cells with DHA displaced cholesterol from detergent resistant

membranes to detergent soluble membranes [31]. Furthermore, ^2H NMR measurements in liposomes show DHA, more than EPA, selectively serves to disrupt sphingolipid/cholesterol distribution by presumably targeting cholesterol distribution [32, 33]. Thus, it is entirely plausible that fish oil is lowering cholesterol levels in the B-cell plasma membrane which diminishes the ability of MHC II molecules to cluster and localize in the synapse upon interaction with the T-cell.

Emerging model by which fish oil regulates B and T-cell lipid-protein organization

The effects of fish oil on B-cells are similar to studies with T-cells. For instance, Kim et al. showed that fat-1 mouse CD4^+ T-cells have reduced PKC localization upon CD3/CD28 stimulation (9). The same group later showed that mitochondrial localization and subsequent calcium signaling was also impaired by n-3 fatty acids in the same model system (8). Combined with our previous findings, the data suggest a model in which fish oil exerts similar effects on both lipid and protein molecular organization to suppress the formation of the synapse and thereby T-cell activation (7–9).

Future work should focus on determining the impact of fish oil on other B-cell membrane proteins such as tetraspans and co-stimulatory molecules. Furthermore, since there is some suggestion that fish oil promotes Th2 immunity, it is of interest to know if B-cells specifically promote a Th2 phenotype over time [34]. Finally, it needs to be determined if fish oil exerts similar effects on protein organization of other cell types such as dendritic cells. New studies in these areas will establish a complete mechanistic model by which fish oil targets B and T-cell membranes to exert immunomodulatory effects.

Summary

The data demonstrate that fish oil disrupts MHC class II lateral organization on the B-cell side of the synapse in a manner that is highly similar to *in vitro* cholesterol depletion. We specifically establish that fish oil can disrupt MHC II accumulation in the synapse, which was accompanied by an effect on CD4^+ T-cell PKC organization independent of changes in adhesion. Furthermore, this is the first evidence demonstrating that the molecular organization of the B-cell side of the immunological synapse is sensitive to *in vivo* dietary manipulation. This work significantly expands our understanding of how fish oil, modeling a clinically relevant dose, exerts its pleiotropic effects on B-cell membrane organization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Fatty acid analysis of B-cells isolated from mice consuming a control or fish oil enriched diet
 Fatty acids were extracted and analyzed using gas chromatography. Only the major fatty acids are shown for simplicity. *P<0.05, **P<0.01, ***P<0.001.

Figure 2. Fish oil diminishes B-cell MHC class II accumulation in the immunological synapse
 (A) Representative TIRF images of MHC II molecules on B-cells isolated from mice fed control or fish oil diets. (B) Quantification of the number of MHC II clusters in a region of interest. (C) FRET efficiency for MHC II molecules prior to synapse formation. (D) Representative DIC (inset) and immunofluorescence confocal images of MHC II in the B-T cell immunological synapse. (E) Percentage of conjugated B and T cells displaying patching of MHC II. (F) The ratio of MHC II intensity at the B-T cell contact site relative to non-synaptic areas. (G) FRET efficiency for MHC II in the immunological synapse. Scale bar represents 5 microns. * $P < 0.05$, *** $P < 0.001$.

Figure 3. B-cells from mice fed fish oil suppress T-cell PKC recruitment to the immunological synapse

(A) Representative DIC (inset) and immunofluorescence images of CD3ε and PKCθ on the T-cell side of the immunological synapse. (B) Quantification of the percentage of conjugated B and T-cells displaying patching of CD3ε and PKCθ. (C) The ratio of CD3ε intensity at the B-T cell contact area relative to non-synaptic areas. (D) PKCθ intensity at the immunological synapse. **P<0.01, ***P<0.001.

Figure 4. Fish oil does not diminish B-T cell adhesion

(A) Percentage of B-cells from control and fish oil fed mice conjugating to cognate transgenic CD4⁺ T-cells in the absence and presence of antigen. Conjugates were scored from microscopy images. (B) Adhesion of B-cells from control and fish oil fed mice to select immune ligands. Adhesion was measured in terms of absorbance at 405nm.

Figure 5. Cholesterol-depletion of B-cells suppresses MHC II and T-cell PKC accumulation in the immunological synapse similar to fish oil

(A) Representative DIC (inset) and immunofluorescence images of MHC II at the immunological synapse. (B) Percentage of conjugated B and T-cells displaying patching of MHC II. (C) The ratio of MHC II intensity at the B-T cell contact site relative to non-synaptic areas. (D) Representative DIC (inset) and immunofluorescence images of CD3 ϵ and PKC θ on the T-cell side of the immunological synapse. (E) Quantification of the percentage of conjugated B and T-cells displaying patching of CD3 ϵ and PKC θ . (F) The ratio of CD3 ϵ intensity at the B-T cell contact area relative to non-synaptic areas. (G) PKC θ intensity at the immunological synapse. **P<0.01.