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Session 3: Fatty acids and the immune system
Mechanisms underlying the immunomodulatory effects of *n*-3 PUFA

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The enrichment of immune cell membranes with *n*-3 PUFA is associated with modulation of immune function. The degree of incorporation of *n*-3 PUFA (and therefore the impact of dietary *n*-3 PUFA on immune function) appears to depend on a number of factors including species and age. The mechanisms involved are still largely unclear, but recent work has focused on two areas; lipid rafts and eicosanoids. *In vitro* studies suggest that lipid rafts could play a role in the immunomodulatory effects of *n*-3 PUFA, but there is still little information regarding the extent to which membrane microdomains in human lymphocytes are modulated by dietary supplementation. The enrichment of cell membranes with *n*-3 PUFA also modulates the production of eicosanoids, the full extent of which has not yet been realized; this represents a key area for future research.

Fatty acid: Fish oil: Eicosanoid: Lipid raft: T-cell: Lymphocyte: Inflammation

Fatty acids play diverse roles in all cells. They are important as a source of energy, as structural components of cell membranes which influence the physical and functional properties of membranes and as signalling molecules and regulators of gene expression[1]. In addition, some PUFA including dihomo-γ-linolenic acid (20:3*n*-6), arachidonic acid (AA), EPA and DHA can serve as precursors for the synthesis of bioactive lipid mediators, such as PG, leukotrienes, lipoxins and resolvins[2–6]. Since the 1980s, a large body of evidence has evolved that suggests that fatty acids are capable of modulating immune function[7]. Initially, many of the effects were demonstrated in animals, but studies are now increasingly being conducted in either healthy human subjects or in patients suffering from specific immune-related diseases. However, human studies investigating the relationship between dietary fatty acids and the immune response have been disappointingly inconsistent. There are likely to be several reasons for this. First, the doses of fatty acids tested in human studies, even when administered at levels many-fold higher than normal dietary intakes, do not compare with the very high levels employed in most animal studies. Second, in studies investigating the effects of fish oil on immune function, preparations of fish oil have varied considerably in their relative contents of EPA and DHA, which might have resulted in different effects. Third, the majority of human studies have been insufficiently powered to take into account the enormous variation in the parameters of immune function, for example, *ex vivo* cytokine production, which we now recognize to be influenced by genotypic variation[8]. The issue of dose is clearly an important one in view of potential public health policy and recommendations. The considerable inconsistency in the previous reports on the effects of *n*-3 PUFA on *ex vivo* production of inflammatory cytokines was thought to be due to differences in administered doses[9]. However, this does not fully account for the inconsistency, since some studies employing high doses of *n*-3 PUFA showed no effect on cytokine production, whereas others using low doses reported inhibition. Mantzioris *et al.*[10] adopted the approach of setting target tissue concentrations of EPA, rather than target dietary intakes; they aimed to increase the mononuclear cell EPA content to 1·5% total fatty acids by 2 weeks of dietary modification. Although this resulted in

Abbreviations: AA, arachidonic acid; PE, phosphatidylethanolamine.

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individual subjects consuming different quantities of *n*-3 PUFA, the strategy was based on the observation by Caughey *et al.*[11] that the EPA content of mononuclear cells is strongly associated with *ex vivo* production of IL-1β and TNFα and that 1·5% EPA results in maximum suppression of cytokine synthesis. However, Thies *et al.*[12] reported that fish oil supplementation, providing 0·72 g EPA/d and 0·28 g DHA/d, failed to inhibit *ex vivo* cytokine production in healthy subjects, even though the mononuclear cell EPA content reached 1·5% of total fatty acids. Furthermore, a study using a much higher dose of 2·1 g EPA/d plus 1·1 g DHA/d also showed no effect of fish oil supplementation on *ex vivo* production of cytokines, despite achieving mononuclear cell EPA levels of 2·5% total fatty acids after 4 weeks and 3·3% at 12 weeks[13]. It is likely that many of the studies described above lacked sufficient power and may therefore have missed the subtle effects of *n*-3 PUFA.

Is there a relationship between fatty acid composition and function of immune cells?

In vitro studies have clearly demonstrated that alterations in the phagocyte membrane fatty acid composition are associated with altered phagocytic capacity[14]. Dietary studies have tended to be contradictory, but it has been demonstrated that the phagocytic activity of neutrophils and monocytes is positively related to the membrane content of *n*-3 PUFA[15] and that supplementation with *n*-3 PUFA enhances phagocytic activity[16]. For other functional outcomes, the picture is less clear. Fritsche[17] recently conducted a cross-study meta-regression dose–response analysis of the effect of dietary *n*-3 PUFA on the AA content of human, murine and rat immune cells, citing the accumulation of AA in tissues as a key factor in the biosynthesis of eicosanoids with pro-inflammatory activity. Baseline fatty acid profiles of human peripheral blood mononuclear cells and rat splenocytes appear to be remarkably similar in terms of AA content, while *n*-3 PUFA content is more variable. Rodent immune cells typically contain 15–20% AA as a proportion of total fatty acids, and very little EPA or DHA. Human immune cell phospholipids, on the other hand contain about 1% EPA and 2·5% DHA in addition to 20% AA[14,17]. As the long chain *n*-3 PUFA content of the diet increases, lymphocyte AA decreases in a curvilinear fashion[17]. In human studies, dietary *n*-3 PUFA never exceeded 3%, whereas in the animal studies the intake was considerably higher, but even when this is taken into account, it is apparent that rodent lymphocytes are much more responsive to the impact of *n*-3 PUFA on AA content than are human lymphocytes. *n*-3 PUFA do not appear to lower AA or enrich cell membranes with *n*-3 PUFA in human blood monocytes as much as in tissue macrophages from either rats or mice[17]. Furthermore, murine lymphocytes, which already contain more DHA than rat or human lymphocytes, accumulate DHA more readily in response to dietary enrichment than these species[17]. Fritsche[17] suggests that this may help explain the discrepancies that exist between animal and human studies investigating the immunomodulatory effects of *n*-3 PUFA. However, it remains unclear how much *n*-3 PUFA is required for biological effects in human subjects.

It is apparent from the literature that fish oil has a greater impact on immune function in the elderly compared with young subjects[12,18,19]. The mechanistic basis for this is not understood, but it is interesting to note that older subjects appear to incorporate EPA into plasma and peripheral blood mononuclear cells more readily than younger subjects[20]. This was associated with a dose-dependent decrease in the neutrophil respiratory burst in older, but not younger subjects[20]. However, PGE_2 production by peripheral blood mononuclear cells was decreased in both groups and phagocytosis and cytokine production was not affected in either group[20]. This highlights the fact that age is likely to be an important factor when considering the impact of *n*-3 PUFA on immunity, not only because of the influence of immunosenescence but also because immune cells from older subjects appear to be more responsive to the availability of *n*-3 PUFA. Recent work suggests that the cholesterol content of T-lymphocytes from healthy elderly subjects is higher than that of young subjects and that membrane fluidity is subsequently decreased[21]. Furthermore, the coalescence of lipid rafts at the site of T-cell receptor engagement was impaired in elderly subjects[21,22]. The impact of ageing on lipid raft composition and function was most evident in the $CD4^+$ T-cell population and affected cytokine signalling[22,23]. Thus, enriching T-cell membranes of older subjects with *n*-3 PUFA could modulate the immune function via effects on the lipid raft structure which are distinct from those in younger subjects.

Are lipid rafts responsible for the immunomodulatory effects of *n*-3 PUFA?

The lipid raft hypothesis suggests that there is a degree of self-organization within the cell membranes such that dynamic microenvironments are created within the exoplasmic leaflets of the phospholipid bilayer of plasma membranes to preferentially group transmembrane proteins according to their function[24]. These rafts have been proposed to serve as platforms to facilitate apical sorting, the association of signalling molecules and interactions between cell types[24,25]. Despite a large body of work, some doubts still persist regarding the existence and nature of lipid rafts[26–29]. These doubts have arisen mainly due to limitations in the interpretation of the methods available to study rafts. The most widely used technique is the preparation of detergent-resistant membranes which are suggested to represent raft domains, since they contain the glycosylphosphatidylinositol-anchored proteins, cholesterol and sphingolipids characteristic of lipid rafts. In support of this idea, the liquid-ordered phases rich in cholesterol and sphingolipids in artificial membranes are resistant to detergent extraction[30]. However, there is a possibility that detergent solubilization could induce non-physiological rearrangements in the bilayer structure, and in particular that the detergent could induce the formation of holes in the membrane, which allow mixing of the inner and outer leaflet and the appearance of cell-signalling proteins in

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detergent-resistant membrane fractions[26,27]. Thus, while *in vitro* studies using the detergent extraction procedure for isolation of lipid rafts have demonstrated that treatment with EPA at a concentration of 50 μM results in marked enrichment of both EPA and docosapentaenoic acid in lipids isolated from rafts and the subsequent displacement of acylated proteins[31]. If raft formation and stability are based on interactions between saturated acyl chains and cholesterol, then the observations that PUFA incorporated into these detergent-resistant fractions is somewhat contrary to expectations. Although a limited number of animal studies support the role of lipid rafts in mediating the effects of PUFA on immune function, it is still largely unclear whether the displacement of key signalling proteins from putative lipid rafts and the down-regulation of signalling pathways by *n*-3 PUFA are physiological phenomena that could explain the immunomodulatory properties of fish oil in human subjects[32]. Clearly, direct visualization of rafts would resolve uncertainties about their existence and structure, but fluorescence microscopy studies have tended to produce mixed results[27] and it is argued that rafts are too small to be resolved by conventional microscopy techniques[29]. The size, stability and functionality of putative membrane microdomains including rafts are therefore still very much in debate. It has been suggested that although some domains are macroscopic and stable for extended periods, others are tiny and unstable, existing only momentarily and as a result are very poorly understood[33,34]. Kenworthy[35] suggests that mechanistic models linking the microdomain structure and function are required to systematically evaluate how the structural and dynamic features of lipid rafts influence protein diffusion and reaction kinetics.

Both linoleic acid and DHA increased the clustering of a lipid raft probe compared with oleic acid and untreated cells, demonstrating that PUFA appear to specifically increase the clustering of proteins in cholesterol-dependent microdomains[36]. The authors suggest that the poor affinity of long-chain PUFA for cholesterol provides a lipid-driven mechanism for lateral phase separation of cholesterol-rich microdomains and alters the dynamic partitioning of acylated proteins[36]. Similarly, Shaikh and Edidin[37,38] suggest that phospholipids containing highly disordered polyunsaturated acyl chains that exhibit low affinity to cholesterol would be expected to phase separate from rafts. They further demonstrated that an oleic acid-containing phosphatidylethanolamine (PE) and a DHA-containing PE phase separated differently from the lipid raft molecules, sphingomyelin and cholesterol in monolayer and bilayer membranes[39,40]. The interactions between DHA-containing PE and cholesterol were less favourable, and as a result, these PE species were less likely to be found in detergent-resistant membrane fractions than oleic acid-containing PE[40].

Membrane microdomains have been studied extensively with respect to T-lymphocyte responses to activation[24,25,41]. There is visual evidence of clustering of signalling components at T-lymphocyte synapses using the non-toxic B subunit of cholera toxin, which binds the glycosphingolipid, GM1, a putative raft reporter[25]. A novel approach whereby anti-T-cell receptor-coated microbeads were attached to T-cells and then stripped away, along with patches of membrane, has demonstrated the presence of the T-cell receptor and associated signalling molecules including linker for activation of T-cells, but there did not appear to be an increase in cholesterol, as might be expected on the basis of its presence in putative lipid rafts[42]. The authors concluded that protein–protein interactions, rather than protein–lipid interactions, and subsequent clustering are the key features of signalling assemblies[43]. This view is supported by Douglas and Vale[44], who used sophisticated imaging techniques to track individual fluorescent proteins involved in T-cell receptor signalling to show that linker of activated T-cells mutants lacking residues specifically required for protein–protein interactions did not cluster. Other studies by the Harder group have used fluorescence techniques to show that condensation of the plasma membrane occurs at the T-cell receptor activation site, suggesting the formation of ordered lipid phases[45,46]. These studies used a dye, Laurdan, which is incorporated into the membrane and undergoes changes in its emission spectrum, corresponding to the level of lipid ordering, allowing quantification of the condensation[45,46]. *In vitro* treatment of Jurkat T-cells with EPA impaired membrane condensation of T-cell receptor activation sites, which was directly related to perturbation of the fatty acid composition of phospholipid species in the immuno-isolated membrane fractions[47]. This is the first study relating to PUFA modification of membrane domains which is not based on detergent extraction. Recently, *fat-1* transgenic mice have been used to gain further insight into the impact of *n*-3 PUFA on lipid rafts in T-cells. These mice bear the *Caenorhabditis elegans* desaturase gene capable of converting *n*-6 PUFA into *n*-3 PUFA, resulting in substantially elevated levels of *n*-3 PUFA in tissues including T-cells. Kim *et al.*[48] investigated the effect of this *n*-3 PUFA enrichment of lipid raft formation at the immunological synapse by co-culturing Laurdan-labelled $CD4^+$ T-cells with anti-CD3 hybridoma cells (serving as antigen-presenting cells). They reported that raft formation was enhanced in the fat-1 cells compared to wild-type, but the relocalization of several signalling molecules into the immunological synapse and cell proliferation were suppressed[48]. The enhanced raft formation can be explained by the low affinity of DHA for cholesterol which effectively causes the coalescence of cholesterol-rich domains, consistent with immunogold data showing increased lipid raft clustering in response to DHA enrichment[48,49]. There is no information regarding the extent to which membrane microdomains in human lymphocytes are modulated by dietary supplementation with *n*-3 PUFA, although lymphocyte lipids (in a whole-cell extract) are readily modified by fish oil supplementation[50] and murine T-lymphocyte rafts have been shown to be responsive to dietary fish oil[51,52]. Interestingly, peripheral blood T-cells from patients with systemic lupus erythamatosus, an autoimmune disorder, contain higher levels of the ganglioside, GM1 and cholesterol, which may alter membrane organization and potentially create an impact on signalling homeostasis[53]. Higher levels of CD45 in lipid rafts in autoimmune lymphocytes have also been documented[53]. In an experimental model of colitis, *n*-3 PUFA prevented inflammation-induced exit of tight junction proteins from lipid rafts and decreased disruption of tight junctions in the intestinal mucosa[54]. This suggests that the reported

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beneficial effects of *n*-3 PUFA in inflammatory bowel disease (and perhaps other inflammatory disorders), may be mediated, at least in part, through the modulation of membrane microdomains. Clearly, there is some compelling evidence from *in vitro* and animal experiments indicating that *n*-3 PUFA modulate the immune function by effects on membrane microdomains, yet direct evidence that this is physiologically relevant in human subjects is still lacking and should be considered for future research.

Fatty acid-derived mediators

Immune cell membranes typically have a high content of AA, which acts as a precursor for the synthesis of eicosanoids, the exact nature of which depends upon the cell type. It is well documented that the enrichment of cell membranes with EPA and DHA decreases the production of AA-derived eicosanoids, such as PGE_2, in a dose-dependent fashion[20]. Incorporation of *n*-3 PUFA also results in the generation of a wider range of eicosanoids, since these fatty acids can also act as precursors for cyclooxygenase and lipoxygenase enzymes. These *n*-3 PUFA-derived eicosanoids are often (but not always) biologically less active than those derived from AA[14]. The transgenic *fat-1* mice, which have tissue greatly enriched with *n*-3 PUFA, generate large amounts of PGE_3 in the colonic tissue after the induction of inflammation by dextran sodium sulphate[55]. The full extent of the number and nature of eicosanoids has not yet been realized, as evidenced by the increasing number of EPA- and DHA-derived eicosanoids which have been shown to be produced under physiological or pathological conditions and to have anti-inflammatory activities[56]. These include the emerging family of lipoxins, resolvins, protectins and most recently maresins, which appear to have important roles in the resolution of inflammation and return to homeostasis[57]. A recent study demonstrated that deuterium-labelled or radioactively labelled *n*-3 PUFA could be identified in inflammatory exudates in a murine model of peritonitis within 2 h of induction[58]. However, since DHA-derived resolvin D1, but not DHA itself, inhibited neutrophil movement and protected lung tissue from excessive leucocyte infiltration, the authors concluded that the resolution of inflammation required conversion to resolvins[58].

Conclusion

Recent studies have focused on the central role of fatty acids in immune cell regulation, highlighting the fact that their location and organization within cellular lipids has a direct influence on the behaviour of a number of proteins involved in immune cell activation and on fatty acid-derived inflammatory mediator production. Key areas for future work include further characterization of the impact of *n*-3 PUFA on lipid raft structure and composition, extrapolation of *in vitro* lipid raft data to human dietary studies and further characterization of novel eicosanoid pathways involved in the resolution of inflammatory responses.

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