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Dietary DHA amplifies LXA$_4$ circuits in tissues and lymph node PMN and is protective in immune-driven dry eye disease

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Abstract

Recently identified regulatory PMN control immune-driven dry eye disease (DED) in females by producing the arachidonic acid (ω−6)-derived specialized pro-resolving mediator (SPM), LXA$_4$, in lymph nodes. Dietary ω−3 docosahexaenoic acid (DHA) is protective in DED but mechanisms of action remain elusive. DHA is converted to ω−3 SPMs by PMN via the same lipoxygenases (LOX) that generate LXA$_4$. We investigated if dietary DHA amplifies SPM formation and affects T-effector cell function and/or regulatory PMN in DED. DED was induced in mice on a DHA-enriched or ω−3 deficient diet. DHA-deficiency amplified DED with marked sex-specific differences. Dietary DHA protection against dry eye disease correlated with increased PMN levels in lymph nodes, ocular tissues and unexpectedly, selective amplification of LXA$_4$ tissue levels. Dietary DHA increased 12/15-LOX and decreased 5-LOX expression in lymph nodes and isolated lymph node-PMN, which correlated with amplified LXA$_4$ formation. Acute DHA treatment rescued DHA-deficient females from exaggerated DED by amplifying lymph node LXA$_4$ formation, increasing T$_{reg}$ and decreasing T$_H$1 and T$_H$17 effector cells. Our results identify DHA regulation of LXA$_4$ producing PMN in ocular tissues and lymph nodes in health and immune disease as novel mechanism and determinant for T cell responses to routine ocular injury or stress signals.

Introduction

Paucity of dietary omega-3 polyunsaturated fatty acids (ϖ−3 PUFA) correlates with higher incidence of diseases with an inflammatory component. Epidemiological, clinical and/or animal studies provide strong evidence that dietary ϖ−3 PUFA are protective in inflammatory and auto-immune diseases[1,2] , which include diseases such as dry eye diseases and age related macular degeneration[3–6] . Docosahexaenoic acid (DHA) is the most abundant and terminal ϖ−3 PUFA and it is highly enriched in the brain, retina and testis. The protective actions of dietary DHA in part have been attributed to formation of specialized pro-resolving lipid mediators (SPM), namely resolvins, protectins and maresins [7–10]. These SPM are generated primarily by the coordinated interaction of 5-lipoxygenase (LOX) and 12/15-LOX, two enzymes that are also essential for the formation of the founding member of the SPM family [11], namely the eicosanoid lipoxin A_4 (LXA_4), which is an arachidonic acid (ϖ−6 PUFA) product.

Increasing evidence indicates that tissue resident SPM circuits are key factors in maintaining health in the eye's immune privileged tissues such as the cornea [7]. The cornea is of particular interest as the corneal epithelium expresses an endogenous LXA_4 circuit that regulates leukocyte function and epithelial wound healing. Treatment with LXA_4 and ϖ−3-derived SPM protects against inflammation and drives epithelial wound healing in the cornea [7,12]. A key enzyme for LXA_4 formation and for many DHA-derived SPM is 12/15-LOX (Alox15) [7,13]. Down-regulation of 15-LOX and LXA_4 formation in the cornea by estrogen has been identified as a factor for sex-specific difference in acute inflammation and wound healing in females [14–16]. Immune driven dry eye diseases (DED), such as Sjögren's syndrome, is a ocular surface diseases with a striking 80% female prevalence. The etiology of immune driven DED is unknown, but it is a chronic autoimmune disease that triggers T-effector cell driven responses against self-antigens in the lacrimal gland, goblet cells, ocular surface and conjunctiva. We recently identified a 15-LOX expressing population of regulatory PMN that reside in draining lymph nodes, lacrimal glands and the corneal limbus that generates LXA_4 [14]. In females this population of LXA_4 producing PMN is selectively regulated compared to males and their depletion causes a female-specific amplified T_H1 and T_H17 response and increased DED.

DHA-derived SPM resolvin D1 is a structural homolog of LXA_4 and both mediated their action via FPR2/ALX in mice and humans. Topical and dietary DHA are protective in mouse models of dry disease [6, 17–19]. A likely mechanism for the protective actions of DHA in the eye and other organs is amplification of endogenous DHA-SPMs. It is unknown if dietary or topical DHA impacts T effector cell function or the recently identified tissue resident regulatory PMN in immune-driven eye diseases. To this end, we placed mice DHA-enriched or matched DHA-deficient diet and investigated the impact of dietary DHA on DED, SPM tissue levels, T effector cells and regulatory PMN. Here we report that dietary DHA sex-specifically inhibits T effector cells and reduces DED. Unexpectedly, dietary and acute treatment with DHA did not markedly amplify DHA-series SPM but predominantly increased tissue levels of the eicosanoid SPM LXA_4 and caused a marked increase in LXA_4 producing PMNs in lymph nodes and expression of 12/15-LOX. Our findings identify a new mechanism of action for dietary DHA, namely amplification of an ϖ−6 SPM circuit.

Results

Dietary DHA is a determinant for the severity of dry eye disease in male and female mice.

Effect of dietary DHA on dry eye pathogenesis was evaluated in an established mouse model of aqueous tear-deficient desiccating stress, which recapitulates key features of human auto-immune dry eye disease. Mice were placed on a balanced open source (AIN-76A, 6.2% fat) DHA-enriched (60% Soybean Oil and 40% DHA) or DHA-deficient (100% Soybean Oil) rodent diet 4 weeks prior to initiating desiccating stress and dry eye pathogenesis. Clinical fluorescence scoring (Fig. 1A, n=10) demonstrated that epithelial defect after 10 days of desiccating stress was significantly amplified in mice on the DHA-deficient diet. Diet induced exacerbation of dry eye pathogenesis (epithelial defect) was significantly greater (p=0.0002) in females (clinical score = 10.2 ± 0.4) than males (clinical score 7.4 ± 0.4). The DHA diet group showed marked reduction in epithelial defect by 50.8 ± 5.9 % (p<0.0001) and 24.9 ± 7.6% (p=0.0041) in females and males respectively, when directly compared to the DHA-deficient diet (Fig. 1A). A key feature of DED is the sex-specific difference that leads to amplified pathogenesis and effector T cell responses in female mice [14]. Hence, it is striking that dietary DHA eliminated sex-specific differences in dry eye induced epithelial defects (Fig. 1A). Tear levels measured by Schirmer's test (n=10) confirmed desiccating stress. More importantly, only female mice in the DHA-enriched diet group (0.7 ± 0.18 mm) showed improved tear production (0.15 ± 0.07 mm) (Figure 1B) after 10 days of desiccating stress. Our standard desiccating stress model uses scopolamine, a cholinergic antagonist, to inhibit lacrimal gland function. Cholinergic regulation of goblet cell associated passages (GAPs) has recently been identified as an important mechanism for maintaining ocular surface immune tolerance [20]. We did not investigate if dietary DHA regulates GAPs.

Dietary DHA regulates tissue levels of the eicosanoid SPM LXA_4 in healthy tissues.

Dietary DHA modulates eicosanoid formation and can amplify $\omega -3$ PUFA SPM pathways [10, 21]. Lipid mediator profiles of plasma (n=4), corneas (n=6), lacrimal glands (n=6) and draining lymph nodes (n=6) were established by LC/MS/MS-based lipidomics in healthy male and female mice on the DHA-deficient or DHA-enriched diet. DHA-SPM pathway markers 17-HDHA and 14-HDHA were significantly increased in all tissues but $\omega -3$ PUFA derived SPM (maresins, resolvins, protectins) were below the limits of quantification for our instrument (AB SCIEX QTRAP 3200). Unexpectedly, dietary DHA significantly increased levels of the SPM eicosanoid LXA_4 in males and females (Fig 2 A-C). LXA_4 was identified by retention time, co-elution with deuterated internal standard, multiple reaction monitoring (MRM) of product ions and quantification of 5 diagnostic transitions [14]. In females, dietary DHA increased LXA_4 levels in plasma from below limits of detection to 325 ± 35 pg/200 μl, by 190 ± 41 % in the cornea, by 150 ± 46 % in the lacrimal gland and by 194 ± 79 % in draining lymph nodes. The effect of dietary DHA on eicosanoid levels was sex-and tissue-specific. LXA_4 levels were significantly higher in male plasma and cornea compared to matched females, while females had significantly higher LXA_4 levels in lacrimal glands and draining lymph nodes. The most striking sex-specific differences in LXA_4 levels were observed in plasma where males had markedly higher levels of LXA_4 both in the DHA-deficient (females = 0 ± 0 pg/200 μl versus males = 617 ± 161 pg/200 μl) and DHA-enriched diet (females = 325 ± 35 pg/200 μl versus males = 1248 ± 242 pg/200 μl). A similar marked

sex-specific difference was observed for the PMN and lymphocyte chemoattractant LTB_4. In both diet groups LTB_4 was only present in the plasma of females but not males. The DHA-deficient diet significantly amplified (p=0.049) female LTB_4 plasma levels by 3.9-fold from 103 ± 26 pg/200 μl to 505 ± 204 pg/200μl (Fig 2A).

Dietary DHA regulates the LXA_4 circuit in resident lymph node PMN

Regulatory PMN are resident in ocular tissues and draining lymph nodes [14]. These regulatory PMN highly express 15-LOX and have a high capacity of generating LXA_4 compared to vascular inflammatory PMN [14]. We isolated lymph node PMN and activated them with calcium ionophore to determine if dietary-DHA regulates their capacity for LXA_4 and LTB_4 generation. Lipidomic analysis established the endogenous formation of LXA_4 and LTB_4 by lymph nodes PMN from healthy male and female mice in both diet groups (Fig 2C). Dietary DHA correlated with a marked increase in LXA_4 formation by lymph node PMN from male and female mice, evidenced by a 2.8 ± 0.9-fold (p=0.0018) and 4.6 ± 1.4 fold (p=0.0027) increase, respectively. By contrast, DHA-deficiency correlated with a marked increase in LTB_4 formation in lymph node PMN in males (345% increase, p=0.0110) and females (124% increase, p=0.0273) compared to mice on the DHA-enriched diet. More importantly, LTB_4 formation in lymph node PMN from females was 161 ± 68 % higher compared to matched males in the DHA-deficient diet groups. LXA_4 was the predominate LOX-derived eicosanoid generated by lymph node PMN in all DHA-diet groups. PMN from females and males produced 567 and 851 pg of LXA_4 in comparison to 73 and 14 pg of LTB_4, respectively.

To assess if dietary DHA had a significant effect on the expression of obligatory enzymes for lipoxin and/or leukotriene formation, we assessed RNA expression of 5-LOX (Alox5) and 12/15-LOX (Alox15) in lymph node PMN from healthy male and female mice in both diet groups (Fig 2D). 5-LOX mRNA expression in PMN was significantly higher in females (108 ± 45 %, p=0.0075) in the DHA-deficient diet group, which correlated with amplified LTB_4 formation (Fig 2C). Consistent with the observed protective effects of dietary DHA, 5-LOX expression was abrogated in lymph node PMN from both male's and female's in the DHA-enriched diet group. 12/15-LOX mRNA expression, an obligatory enzyme for *de novo* LXA_4 formation by PMN, was significantly higher in males compared to females in all diet groups. Dietary DHA reduced PMN 12/15-LOX expression in females by 55 ± 11 % (p=0.0002) and in sharp contrast markedly increased expression in males by 50 ± 20 % (p=0.0144). The results indicate that dietary DHA sex-specifically regulates 5-LOX (Alox5) and 12/15-LOX (Alox15) expression and LXA_4 and LTB_4 formation by lymph node PMN. Specifically, dietary DHA had a greater effect on male PMN in terms of amplifying Alox15 expression and LXA_4 formation.

Sex-specific differences in regulation of tissue PMN levels by dietary DHA in health and dry eye disease

Tissue PMN sex-specifically generate and regulate LXA_4 tissue levels [14]. To assess the impact of dietary DHA on these tissue PMN they were quantified in healthy mice and 10 days after desiccating stress by immunohistochemistry (IHC) and MPO assay. $CD45^+/Ly6g^+$ PMN were manually counted in sections of the limbus region of the cornea (Fig 3A, B). IHC

quantification (n=6) demonstrated higher PMN numbers in the healthy limbus of males (p=0.0074) and females (p<0.0001) in the DHA diet group compared with the DHA-deficient diet group (Fig 3A). PMN numbers in healthy female and male mice were higher by 114 ± 25 % and 50 ± 18 %, respectively, compared with the DHA-deficient diet group (Fig 3A). Dietary DHA did not impact the number of PMN in the limbus of females. However, in male mice dietary DHA correlated with a 11 ± 2-fold increase in PMN in the limbus.

PMN were quantified in the lacrimal glands and draining lymph nodes by measuring MPO activity (n=6). There were marked sex-specific differences in PMN numbers in the lacrimal gland (Fig 3C), which is a primary target for T cells in dry eye disease [22,23]. In healthy females, unlike in males, DHA diet correlated with 2.1 ± 0.6 fold (p<0.0001) increase in PMN in the lacrimal glands compared to the DHA-deficient diet group. Desiccating stress in females eliminated the dietary effect of elevated lacrimal gland PMN as numbers returned to basal level ($77,558 \pm 22,067$ per 10 mg). In males, the DHA-enriched diet did not result in higher PMN number in healthy mice, on the contrary PMN levels were lower compared to the DHA-deficient diet group. However, with desiccating stress lacrimal PMN levels in the DHA diet group increased by 64 ± 18 % and were the same as in the DHA deficient diet group. In draining lymph nodes (Fig 3D) DHA-diet correlated with marked increase in PMN levels in both females and males 110 ± 53 % and 128 ± 70 %, respectively (Fig 3D). Dietary DHA further increased lymph node PMN levels in female mice by 76 ± 39 % with DED. In contrast, in males DED had no impact on numbers of PMN in lymph nodes in either the DHA-deficient or DHA-enriched diet group.

Dietary DHA amplifies cornea and lymph node LXA$_4$ circuits in Dry Eye Disease

A key feature of Dry Eye pathogenesis is epithelial defect in the cornea (Fig 1A). The healthy cornea highly expresses a 12/15-LOX-LXA$_4$ protective circuit that promotes re-epithelialization and controls effector cell function [24–26]. Impact of dietary DHA on expression and activity of the corneal 15-LOX/LXA$_4$ circuit was assessed 10 days after initiating dry eye diseases (Fig 4). Dietary DHA markedly upregulated the primary 12/15-LOX DHA products 14-HDHA and 17-HDHA (Fig 4A). 14-HDHA increased by 125 ± 37 % and 1369 ± 620 % and 17-HDHA increased 225 ± 111% and 105 ± 61% in males and females, respectively. Despite dietary amplification of DHA-series SPM pathway markers (14-HDHA and 17-HDHA), resolvins, protectins or maresins were not consistently detected in single mouse corneas. In health and dry eye disease PMN are not an abundant cell type in the cornea and are largely restricted to the vascular limbus region [14] but the mouse cornea and human corneal epithelial cells can generate LXA$_4$ endogenously [15,24]. Consistent with dietary DHA amplification of LXA$_4$ in healthy mouse corneas, corneas from mice with dry eye disease on the DHA diet also demonstrated a marked upregulation of corneal LXA$_4$ levels (Fig 4A) in both males and females by 109 ± 39 % and 480 ± 158 % (p=0.0038, p=0.0023), respectively. Dietary DHA deficiency correlated with a significant increase in expression of Alox5 and Alox15 in both males and females (Fig 4B). Corneal expression of Alox5 and Alox15 was $1.6 \pm 0.7 – 3.4 \pm 1.2$ and $2.2 \pm 0.5 – 2.7 \pm 1.0$ fold higher (male-female), respectively in the DHA-deficient versus the DHA-enriched diet group.

Regulatory PMN in draining lymph nodes generates significant levels of LXA_4 (Fig 2C). We next assessed the impact of dietary DHA on endogenous formation of lymph node LXA_4 or DHA-derived SPM levels at the peak of DED (Fig 5A). Dietary DHA markedly amplified 14-HDHA and 17-HDHA levels, pathway markers for DHA-derived SPM in both males and females by $6.6 \pm 1.8 – 45.8 \pm 23.0$ (14-HDHA) and $5.3 \pm 0.7 – 6.1 \pm 0.9$ (17-HDHA) fold, respectively. We did not consistently detect and were able to quantify DHA-derived SPM. In contrast male and female mice on the DHA-enriched diet had markedly higher levels of LXA_4 in draining lymph nodes compared to mice on a DHA deficient diet, amplification of lymph node LXA_4 levels was greater in females than in males, 292 ± 121 % versus 105 ± 69 % (p=0.0117, p=0.0559). Consistent with results obtained with draining lymph node PMN, dietary DHA mcorrelated with minimized Alox5 and amplified Alox15 RNA expression in draining lymph nodes during dry eye diseases (Fig 5B). However, lymph node Alox15 expression was higher in males in both diet groups and only in males did dietary DHA significantly amplify lymph nodes Alox15 RNA levels (67 ± 32 %, p=0.0224).

Acute DHA treatment rescues females from amplified immune driven dry eye disease

The protective effect of long-term dietary DHA are multifactorial. We set out to determine if acute treatment with DHA alone would rescue mice on the DHA-deficient diet from exacerbated dry eye disease. Female mice were selected since they have amplified dry eye disease [14], which correlates with a striking female disease prevalence in humans. Female mice were placed on a 4-week DHA-deficient diet prior to initiating desiccating stress. On day 1 of desiccating stress mice were treated with a combined approach of subconjunctival injection (50 ng DHA/5 μl, every 4 days) and intravenous injection (250nmol/kg, *qd*) or sterile saline alone.

Acute DHA treatment rescued mice on the DHA-deficient diet from amplified epithelial defect (Fig. 6A). Epithelial defect was reduced by 57 ± 13% and DHA treatment increased tear production by 140 ± 83% (n=6, p<0.05). A key feature of autoimmune dry eye pathogenesis is the activation and infiltration of $CD4^+$ effector T cells [27,28]. Dietary DHA deficiency markedly decreased PMN in the corneal limbus and draining lymph nodes (Fig 3) and LXA_4 tissue levels (Fig 4, 5). Acute DHA treatment rescued female mice with dietary DHA deficiency from an amplified immune response by increasing PMN levels 150 ± 29 % and decreasing $CD4^+$ T cells by 38 ± 7 % (Fig. 6).

PMN were quantified by flow cytometry in draining lymph nodes (Fig 7A). Acute DHA treatment increased lymph node PMN numbers by 190 ± 105 % (p=0.0116) in the DHA-deficient diet mice. We have established that lymph node PMN have key roles in regulating T-effector and T_{reg} cells in autoimmune dry eye disease [14]. To determine if DHA treatment could rescue mice on the DHA-deficient diet from an amplified effector T cell and impaired T_{reg} response, we quantified $CD4^+$ T_H1 ($IFN\gamma^+$), T_H17 ($IL17^+$) and T_{reg} ($Foxp3^+$) cells in draining lymph nodes by flow cytometry (Fig 7B). DHA treatment caused a marked increase (289 ± 41%, p=0.0002) in T_{reg} and abrogated T_H1 and T_H17 cells in draining lymph nodes as evidenced by 84 ± 1 % (p<0.0001) and 81 ± 2 % (p=0.0004) decrease, respectively. Collectively these finding establish that acute DHA treatment can rescue female mice from amplified effector T cell-driven dry eye diseases.

Acute DHA treatment restores expression and activity of the LXA$_4$ circuit in dietary DHA-deficiency.

We next assessed if acute DHA treatment could restore expression and activity of SPM circuits, specifically LXA$_4$ formation, in mice subjected to long term dietary DHA-deficiency. Lipidomic analysis demonstrated that acute DHA treatment markedly increased LXA$_4$ levels (Fig 7C) in plasma, cornea and draining lymph nodes compared to saline treated mice by 2300 ± 1982 % (p=0.0319), 246 ± 126 % (p=0.0231) and 404 ± 240 % (p=0.0154), respectively. To assess the impact of DHA treatment on expression of the LXA$_4$ pathway, we focused on lymph nodes as they are the main site for regulating effector T cells and initiating adaptive immune responses. The increase in lymph node LXA$_4$ levels caused by acute DHA treatment correlated with a significant decrease in 5-LOX RNA levels and a concurrent increase in 15-LOX RNA levels (Fig 7D). These results provide evidence that acute treatment with DHA can rapidly restore LXA$_4$ circuit expression and activity in lymph nodes and reverse amplified adaptive immune responses caused by dietary DHA deficiency

Discussion

Immune-driven Dry eye disease is characterized by an imbalance in immunoregulatory and proinflammatory pathways. Current treatment options are palliative to restore tear film or are aimed at blocking essential immune pathways. Our experiments demonstrate that depletion of dietary ω−3 PUFA significantly exacerbates effector T cells driven dry eye disease in mice while dietary DHA supplementation is protective. These finding are consistent with a post hoc clinical study, which established that dietary ω −3 PUFAs in women is associated with a decreased incidence of dry eye syndrome [29]. More importantly, our data demonstrates that dietary amplification of dry eye pathogenesis due to ω −3 PUFA depletion was greater in females than males, which supports our previous findings of sex-specific differences in corneal wound healing[15] and immune-driven dry eye disease [14]. There is compelling evidence for significant roles of essential PUFAs in regulating ocular surface immunity and physiology. ω−3 PUFA dietary deficiency in mice reduces tear volume while supplementation stimulate tear production [17] and topical treatment with ω−3 and ω−6 PUFA reduces dry eye pathogenesis and markers of inflammation [6]. DHA is a major PUFA in human tears and we have reported that decreased tear levels of DHA are associated with tear film dysfunction and dry eye disease [30]. DHA is of particular interest as it is the most prevalent ω−3 PUFA in tissues and its tissue levels are highly regulated, especially in the retina, brain and testis. Animal studies have established that topical application of DHA increases nerve density and corneal epithelial cell proliferation and accelerates wound healing after corneal surgery [31, 32].

Beneficial actions of DHA in the ocular surface are multifactorial and include modulation of inflammatory and immune responses. A mechanism for dietary and therapeutic DHA treatment is amplification of DHA-derived resolvins, protectins and maresins. These SPM regulate PMN, macrophage and T effector cell function in many tissues including the cornea and retina [33–36]. Studies using dietary fish oil supplementation or the fat-1 transgenic mouse have demonstrated that increased tissue levels of DHA or EPA can result in increased ω−3 SPM levels or its pathway markers [10, 21, 37–39]. Hence, our findings that dietary DHA

significantly and selectively increased levels of the arachidonic acid derived LXA_4, in lymph nodes, cornea, lacrimal glands at first glance seem counterintuitive. However, LXA_4 is formed by the same enzymes, identical mechanisms and analogous ω−6 intermediates as ω −3 PUFA-derived SPM. It is important to recognize that arachidonic acid is the most abundant substrate for LOX and COX enzymes in most cells and tissues, a fact that is not changed by dietary supplementation with fish oils in humans or mice. Our current findings in mice are in agreement with results from a clinical study where we analyzed serum from human infants that received parenteral nutrition [40]. We analyzed serum from a small cohort of human infants where the intravenous lipid emulsion was switched from soybean to fish oil, unexpectedly LXA_4 serum levels dramatically increased at 1 and/or 4 weeks after switching the patients to the fish oil emulsion.

The molecular mechanism for dietary DHA amplification of endogenous LXA_4 formation in multiple tissue, including draining lymph nodes, remains to be fully defined. Studies have reported dietary DHA regulation of leukocyte function and pro-inflammatory mediator expression [41–44]. This includes a clinical study, which demonstrated that a high DHA and EPA diet changed the expression of more than 1000 genes in monocytes included a marked decrease in pro-inflammatory and induction of anti-inflammatory pathways [45]. We have previously identified a population of regulatory PMN that reside in lymph nodes and the limbus regions of the cornea [14]. These specialized PMN control dry eye disease in females by producing LXA_4, a key feature of these PMN is high expression of 15-LOX and low expression of 5-LOX. Dietary DHA markedly reduced 5-LOX expression in the lymph node PMN population in both males and females, which correlated with a marked increase in LXA_4 formation. More importantly, acute local and systemic treatment with DHA rescued female on a DHA-deficient diet from amplified dry eye disease, abrogated T-effector cell responses and restored the lymph node regulatory PMN population. All of these protective actions of DHA treatment correlated with decreased 5-LOX and increased 15-LOX expression and a marked increase in lymph node LXA_4 levels. Multiple mechanisms have been proposed for direct actions of DHA [1, 2, 43, 44] but how dietary or acute systemic treatment with DHA regulates gene expression or the levels of regulatory PMN in lymph nodes remains to discovered. Several studies [9, 46] have established that DHA-derived SPM regulate 5-LOX activity and expression and induce M2-like phenotypes in macrophages. Hence, even though we did not directly detect DHA-derived SPM their pathways were clearly upregulated in the DHA diet group and it is possible that DHA-derived SPM have a role in amplifying the LXA_4 pathway and regulatory PMN in tissues.

Emerging evidence indicates that SPM such as lipoxins are not just formed during the resolution phase of acute inflammation or that their primary bioaction is regulation of inflammation. Specific cells such as regulatory PMN and astrocytes [14, 47, 48] as part of their homeostatic function maintain a basal tissue tone of LXA_4 in draining lymph nodes, retina, optic nerve head and corneal limbus. This local tissue tone of LXA_4 has important roles in controlling the response of T cells or neurons to routine stress/injury signals in the ocular surface or retina. DHA directly or indirectly regulates the expression of the LXA_4 biosynthetic pathway and increases tissue levels of LXA_4 producing regulatory PMN in the corneal limbus and draining lymph nodes. DHA regulation of 5-LOX and 15-LOX expression and regulatory PMN and LXA_4 levels demonstrated marked and diverse sex- and

tissue-specific difference, which underscores that regulation of routine immune responses and protective roles of DHA is distinct in males and females.

Methods

Animals

Age-matched (6 to 10 weeks old) C57BL/6J male and female mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were maintained on a 12-hrs day/night cycle and received *ad libitum* a standard rodent diet (Rat/Mouse diet LM-485, Harlan Tekland, Madison, WI) prior to initiating the dietary study. All mice were placed on a balanced open source (AIN-76A, 6.2% fat) DHA-enriched (60% Soybean Oil and 40% DHA) or DHA-deficient (100% Soybean Oil) rodent diet (Research Diets, New Brunswick, NJ) for 4 weeks prior to initiating desiccating stress and dry eye pathogenesis. We did not control for estrogen fluctuations due to the normal 4 to 5-day estrous cycle of females. DHA ethyl ester (DHA ethyl ester 71.5% and EPA ethyl ester 12%) for preparing the balanced custom diets was a kind gift from Solutex (Madrid, Spain). All animal studies were approved by the University of California Berkeley Animal Care and Use Committee and follow the NIH Guide for the Care and Use of Laboratory Animals.

Dry eye tear deficient mouse model

Dry eye disease was induced using an established model of desiccating stress as previously described [14]. Briefly, mice were placed in cages with a perforated screen walls and exposed to continuous airflow from fans in a low humidity (20–30%) cubicle. Lacrimal gland function was inhibited by injection with scopolamine hydrobromide (0.1mL of 10mg/mL, formulated in sterile saline; Sigma-Aldrich Corp., St Louis, MO) for 3 or 5 consecutive days, and a reduced dose (0.1 mL of 5 mg/mL) for 10 days, respectively. Scopolamine hydrobromide injections were performed three times per day (9 AM, 2 PM, and 7 PM), subcutaneously into alternating hindquarters of mice. Healthy controls were age- and sex-matched untreated mice on a DHA-enriched or DHA-deficient rodent diet housed in a standard animal facility environment.

Tissue Isolation:

Healthy mice or mice with DED were euthanized. Corneas with complete limbus, lacrimal glands (weighed upon extraction), and cervical draining lymph nodes (diameter controlled 1.8–2.0 mm) were surgically removed using a stainless steel surgical blade. Isolated tissues were washed in ice-cold sterile phosphate-buffered saline under a dissecting microscope and immediately snap frozen for RNA, lipidomic, myeloperoxidase analyses or immediately processed for flow cytometry/immunohistochemistry [14].

PMN Isolation:

PMNs were isolated from cervical draining lymph nodes from male and female mice on the DHA-enriched or DHA-deficient diet. Briefly, lymph nodes were minced and filtered through a 40-mm cell strainer (BD Falcon; BD Biosciences, San Diego, CA). After preparation of single-cell suspension, cells were negatively separated and isolated using PMN isolation kits (Miltenyi Biotec, Bergisch-Gladbach, Germany), according to the

manufacturer's instructions. Cells were stained with trypan blue and counted using light microscopy. The cell suspension was pelleted by centrifugation, followed by washing in RPMI 1640 with 5 % FBS. The cell pellet was resuspended (5×10^5 PMN/ml) in 200 μl RPMI 1640 with 5% FBS and activated with calcium ionophore (37 °C, 15 min, 5 μM). Media was collected to establish endogenous lipid mediator formation by LC/MS/MS-based lipidomics. Unactivated freshly isolated PMN were used to quantify gene expression.

Clinical assessment of dry eye disease:

Epithelial damage was qualitatively assessed by fluorescence microcopy by staining corneas with 0.5 μL of 2.5% Fluorescein Sodium (Bausch & Lomb) before further quantitative assessment was conducted in accordance with the standard NEI scoring system. Using a phenol red thread (Zone-Quick; Showa Yakuhin Kako Co., Tokyo, Japan), the cotton thread test (CTT) measured tear production by placing the thread in the lateral canthus of the conjunctiva fornix of each eye for 30 seconds after excess tears had been removed 30 seconds prior. Light microscopy (Carl Zeiss, Jena, Germany) was used to measure and read the tear fluid distance (in millimeters).

Myeloperoxidase (MPO) Assay

Tissue PMN were quantitated by measuring MPO activity in lacrimal glands and lymph nodes as previously described [14, 24, 25]. Total tissue PMN numbers were established through calibration curves of PMN exudates from zymosan A-induced peritonitis in C57BL/6J mice.

Flow cytometry analysis

PMN and lymphocyte cell populations within lacrimal glands, draining lymph nodes, and corneas were assessed by flow cytometry as previously described [14]. Anti-FcR mAB (BD PharMingen, San Diego, CA) was used to block Fc receptors for 10 minutes before samples were incubated for 30 minutes in titrated amounts of fluorescent-labeled antibodies: FITC-conjugated anti-Ly6g (1A8 clone; BD PharMingen, San Diego, CA, USA) for PMN; PE-conjugated anti-CD45 (MEC 13.3 clone; BD PharMingen) for leukocytes; PE-conjugated anti-CD3 (500A2 clone; BD PharMingen) for T cells; FITC-conjugated anti-CD4 (RM4–5 clone; BD PharMingen) for activated CD4$^+$ T cells; APC-conjugated anti-IFN-γ (XMG1.2; TONBO biosciences); FITC-or APC conjugated anti-IL17 (eBio17B7; ebioscience); APC conjugated anti-Foxp3 (3G3; TONBO biosciences). Live cells were sorted using a high-speed sorter (MoFlo, SX DakoCytomation, Inc., Fort Collins, CO, USA).

qPCR

mRNA from lymph node PMN was isolated using a RNA Easy Mini Kit (Qiagen Sciences, Germantown, MD, USA) and quantified via spectrophotometry. mRNA was then reverse transcribed using a High-Capacity cDNA Kit (Applied Biosystems, Foster City, CA, USA). All samples were run in duplicates using a StepOne Plus qPCR system (Applied Biosystems) β-actin was used as a reference gene and universal mouse reference RNA was used to calculate relative mRNA expression of our samples as previously described [14,15].

Lipid Mediator Lipidomics

Corneas, lacrimal glands, and draining lymph nodes were rapidly homogenized using a refrigerated bead homogenizer in 66% MeOH containing deuterated internal standards (PGE_2-d4, LTB_4-d4, 15-HETE-d8, LXA_4-d5, DHA-d5, AA-d8). Tissue supernatants were extracted using Accubond ODS-C18 cartridges (Agilent Technologies, Santa Clara, CA). Lipid mediators were identified and quantified by LC/MS/MS-based lipidomics using a AB Sciex QTRAP 3200 MS as previously described [14,15]. Analysis was carried out in negative ion mode, and lipid mediators and PUFA were quantitated using scheduled multiple reaction monitoring (MRM) using 4–6 specific transition ions for each analyte. Prominent fragment ions (*m/z*) for identification of LXA_4 (351.2 *m/z*, retention time 10.5 min) included 115.1, 135.1, 189.2, 217.3, 235.3.

Sub-conjunctival and intravenous DHA injections

2 days prior to inducing desiccating stress, female mice on a 4 week DHA-deficient diet received local DHA treatment (50 ng) via a subconjunctival (SC) injection. The local treatment was repeated on day 3 and day 7 of desiccating stress. Control mice received 5 μl PBS SC injection. Subconjunctival injections were carried out under a Zeiss AxioCam microscope with a 10 μl hamilton syringe. Systemic treatment with DHA (250 nmol/kg) was started on day 1 of desiccating stress by tail vein (*IV*) injections. The control group received vehicle alone (20 μl sterile PBS) daily. DHA (Cayman Chemical Company, Ann Arbor, MI) was dried under a stream of nitrogen to remove all EtOH and immediately resuspended in sterile PBS for *IV* or *SC* injections.

Statistical Analysis

One-tailed, unpaired student's *t* test was used to determine differences between males and females. One-way ANOVA with Tukey's post hoc testing was used to determine differences between multiple experimental groups. Overall comparisons were made by significant statistical *P* values of less than 0.05. Unless otherwise noted, all data reported as ± SEM.

Acknowledgments

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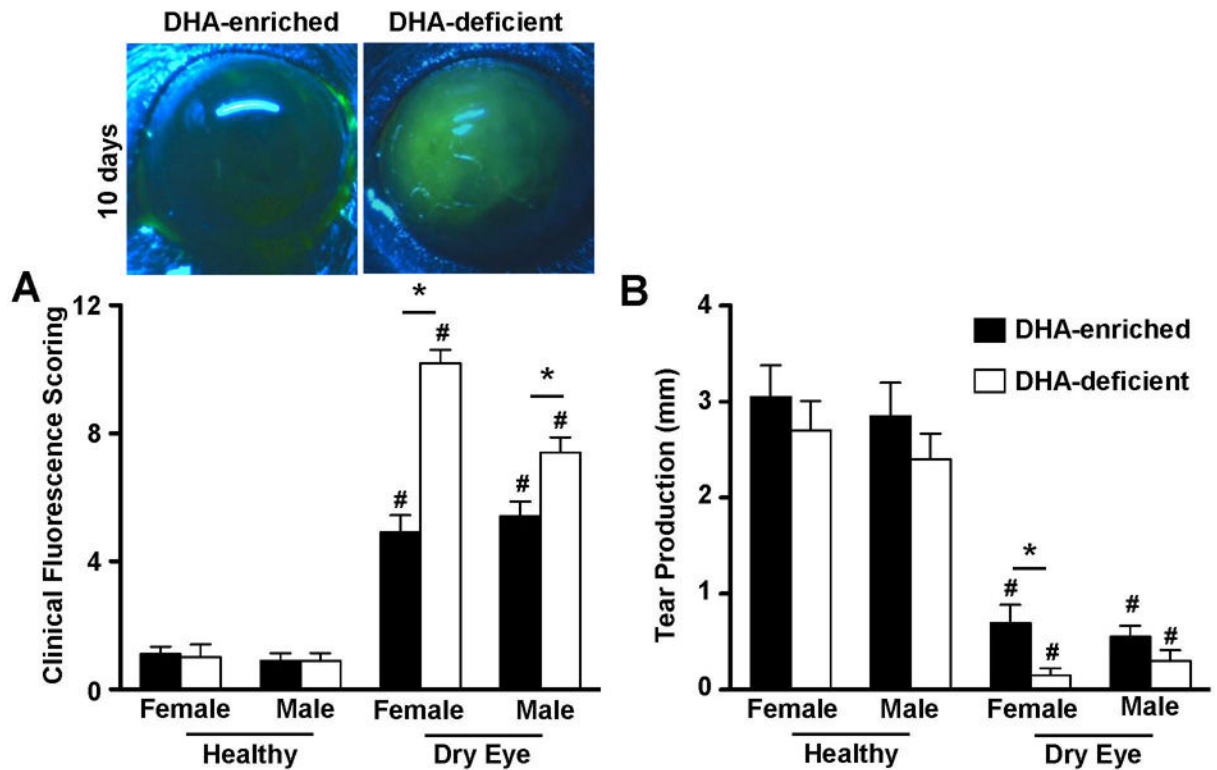
Figure 1. Dietary DHA is a determinant for the severity of dry eye disease in male and female mice.

Age-matched male and female C57BL/6J mice were place on a balanced DHA-enriched or DHA-deficient diet for 4 weeks prior to initiating desiccating stress. Desiccating stress consisted of scopolamine injections to reduce tear flow and housing in low humidity environmental cubical with high airflow for 10 days. Images are representative fluorescein-stained corneas from females in each diet group. A) Corneal epithelial defect, a marker of dry eye diseases severity, was assessed and quantified by clinical fluorescence scoring (n=10) in healthy controls (no desiccating stress) and mice after 10 days of desiccating stress. B) Tear production was measured by Schirmer's test (n=10). [#]$P<0.05$, healthy versus dry eye; *$P<0.05$, DHA-enriched versus DHA-deficient
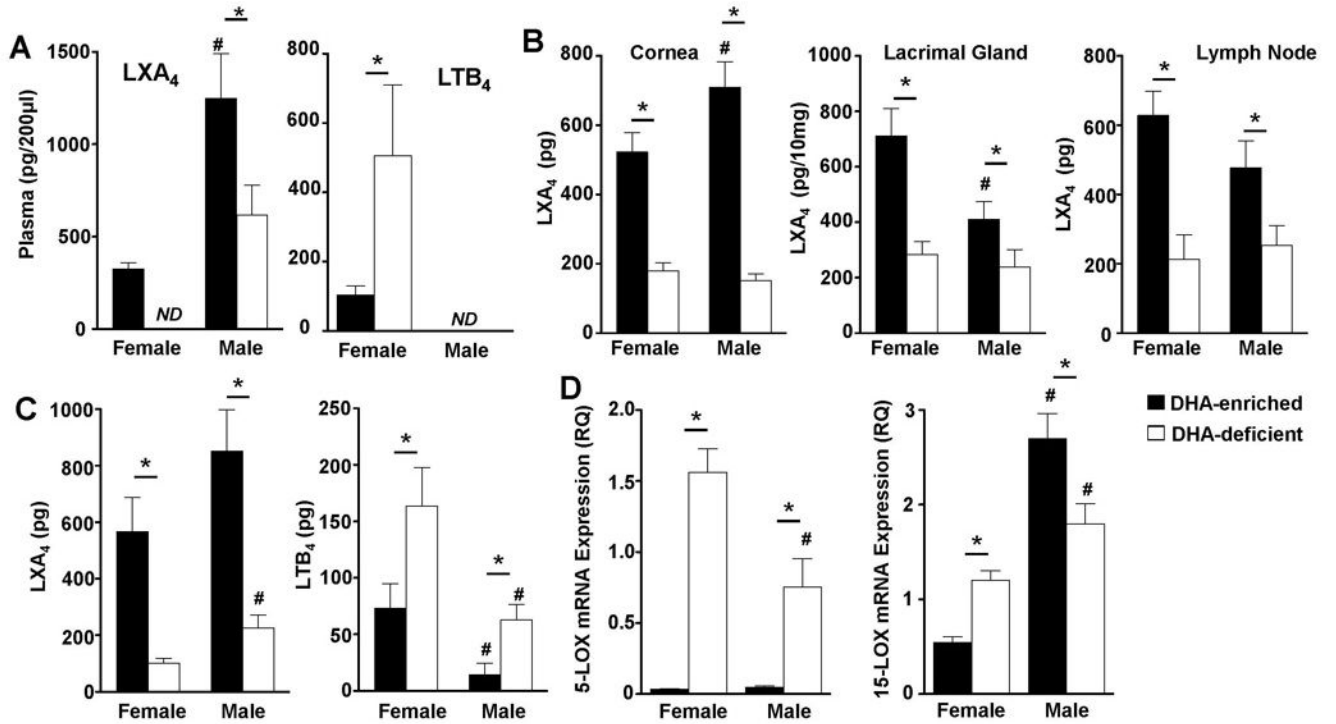
Figure 2. Dietary DHA regulates tissue levels and PMN production of the eicosanoid SPM LXA$_4$ in healthy mice.

Endogenous LXA$_4$ and LTB$_4$ tissue levels in plasma, cornea, lacrimal gland and draining lymph nodes of healthy mice (males & females) on a DHA-enriched or DHA-deficient diet was quantified by LC/MS/MS-based lipidomics. A) LXA$_4$ and LTB$_4$ plasma levels (n=4). B) LXA$_4$ tissue levels (n=6) in corneas, lacrimal glands and draining lymph nodes. C) PMN from draining lymph nodes were isolated from healthy males and females on the DHA-enriched or DHA-deficient diets. PMN were activated with calcium ionophore at 37°C for 15 minutes and endogenous LXA$_4$ and LTB$_4$ formation quantified by LC/MS/MS-based lipidomics (n=5, pg/1×10^5 PMN). D) qPCR analysis of 5-LOX and 15-LOX RNA expression in lymph node PMN (n=4). (A-D) Values represent the mean ± SEM. *P<0.05, DHA-enriched versus DHA-deficient; #P<0.05, female versus male.
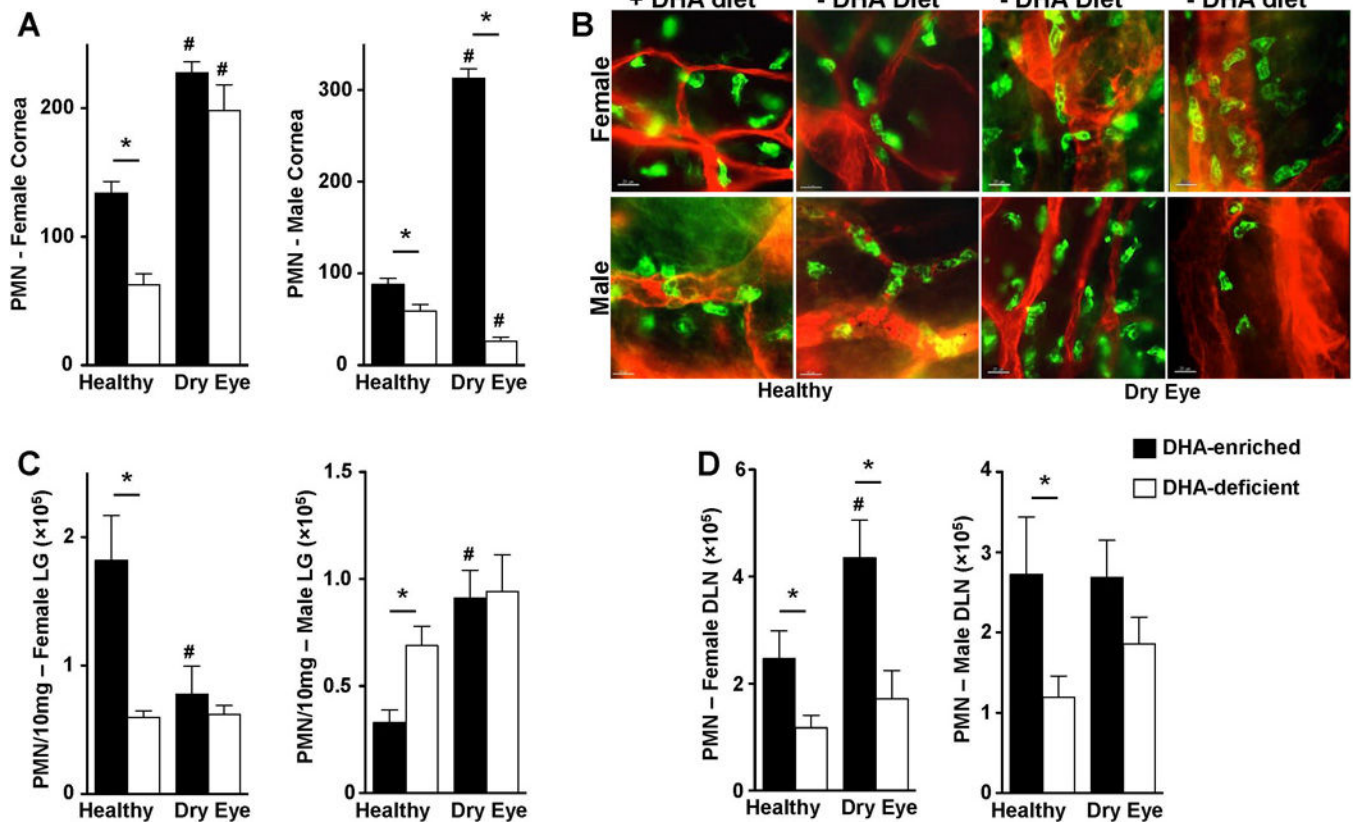
Figure 3. Sex-specific regulation of tissue PMN levels by dietary DHA in health and dry eye disease.

Tissues were collected from males and females on the DHA-enriched or DHA-deficient diet. A) IHC quantification of $CD45^+Ly6g^+$ PMN in flat mounted corneas with limbus (n=6).The total number of corneal PMN was counted in nine, 40x fields of view that comprised the diameter of a cornea (from limbus to limbus). B) Representative images of corneal limbus region measured with scale bars at 20μm. PMN levels quantified by myeloperoxidase (MPO) activity (n=6) in the lacrimal gland (C) and draining lymph nodes (D). *P<0.05, DHA-enriched versus DHA-deficient, unpaired t test; #P<0.05, female versus male.
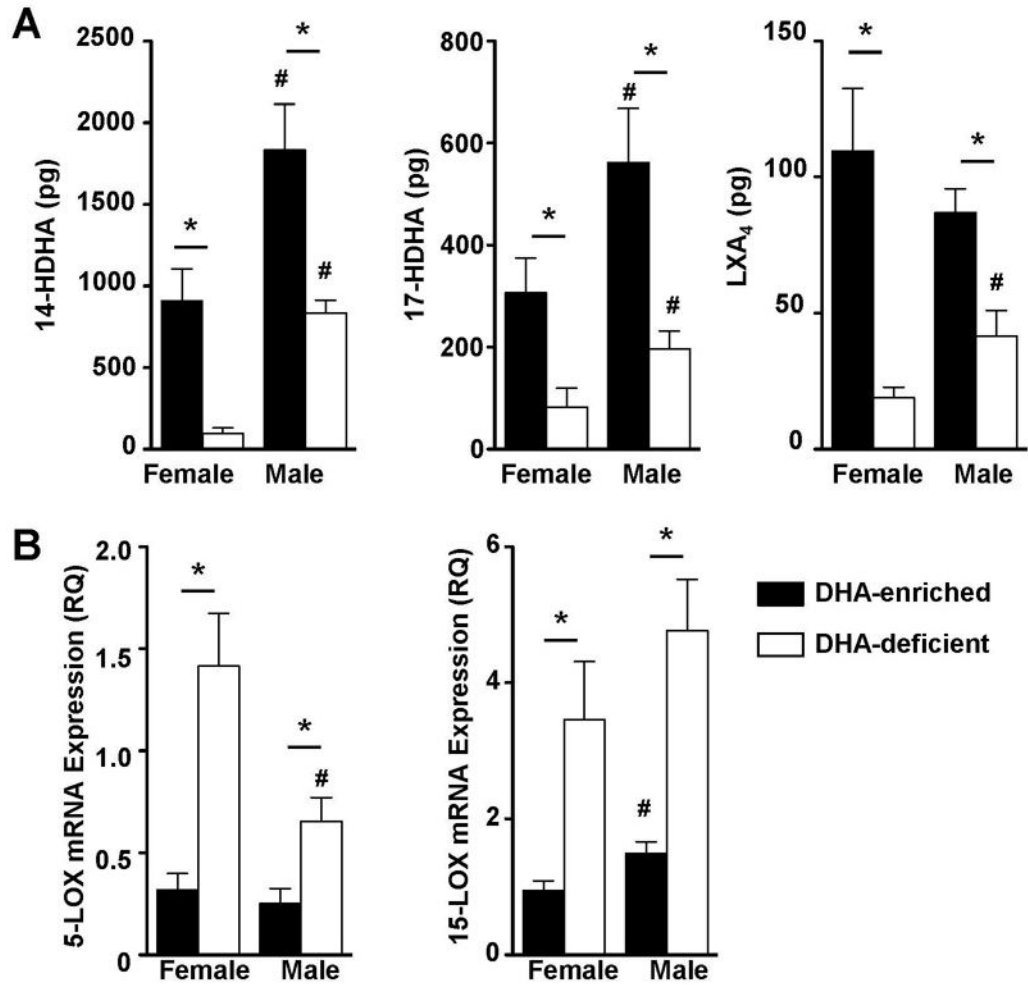
**Figure 4. Dietary DHA amplifies cornea LXA$_4$ circuits in Dry Eye Disease.**
Corneas were isolated from males and females on the DHA-enriched or DHA-deficient diet after 10 days of desiccating stress (n=5). Corneas were processed for lipidomic or qPCR analysis. A) 12/15-LOX DHA-derived products and LXA$_4$ levels (pg/cornea). B) qPCR analysis of 5-LOX and 15-LOX RNA expression in corneas (n=5). Values represent the mean ± SEM. *P<0.05, DHA-enriched versus DHA-deficient, unpaired t test; [#]P<0.05, female versus male.
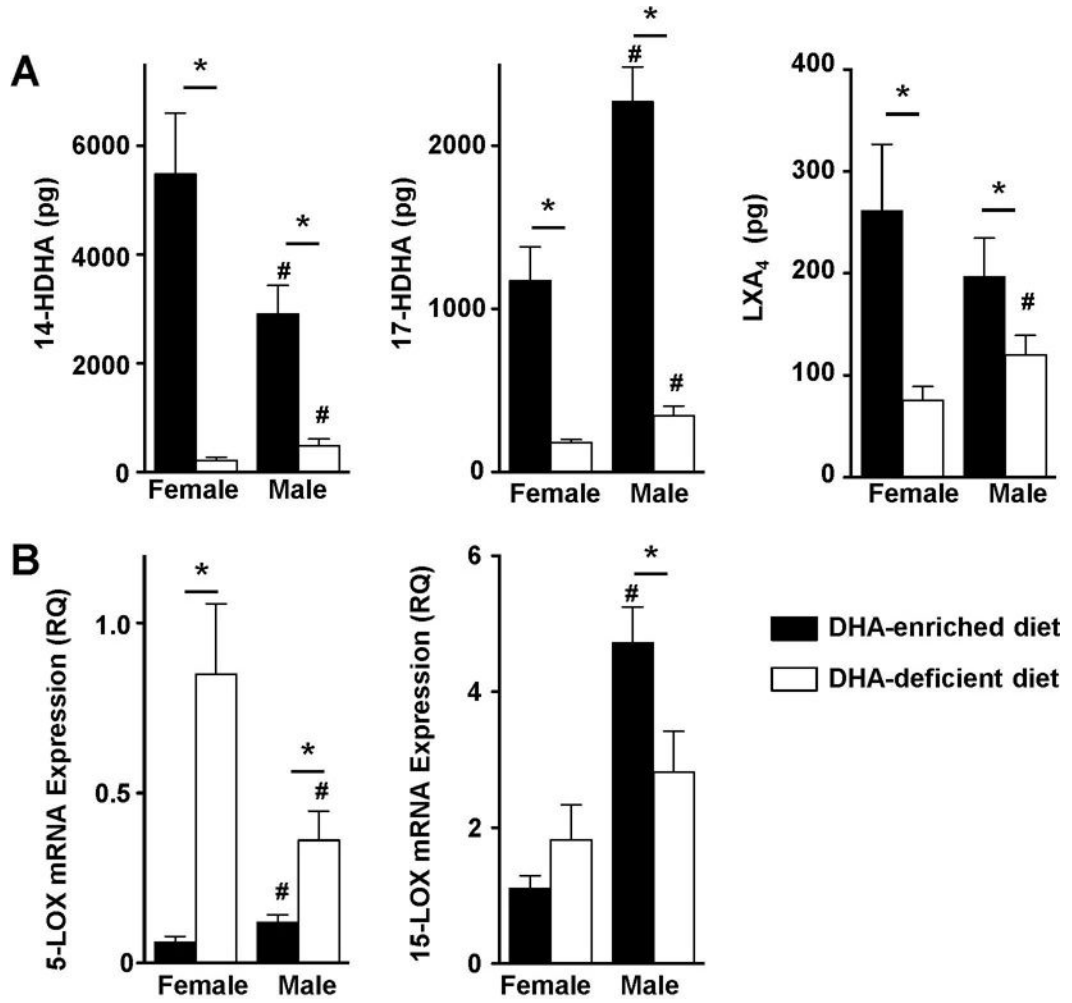
Figure 5. Dietary DHA amplifies lymph node LXA$_4$ circuits in Dry Eye Disease.
Draining lymph nodes were isolated from males and females on the DHA-enriched or DHA-deficient diet after 10 days of desiccating stress (n=5). Pooled lymph nodes from each mouse were processed for lipidomic or qPCR analysis. A) 12/15-LOX DHA-derived products and LXA$_4$ levels (pg/lymph nodes/mouse). B) qPCR analysis of 5-LOX and 15-LOX RNA expression in draining lymph nodes (n=5). (A-B) Values represent the mean ± SEM. *P<0.05, DHA-enriched versus DHA-deficient, unpaired t test; [#]P<0.05, female versus male.
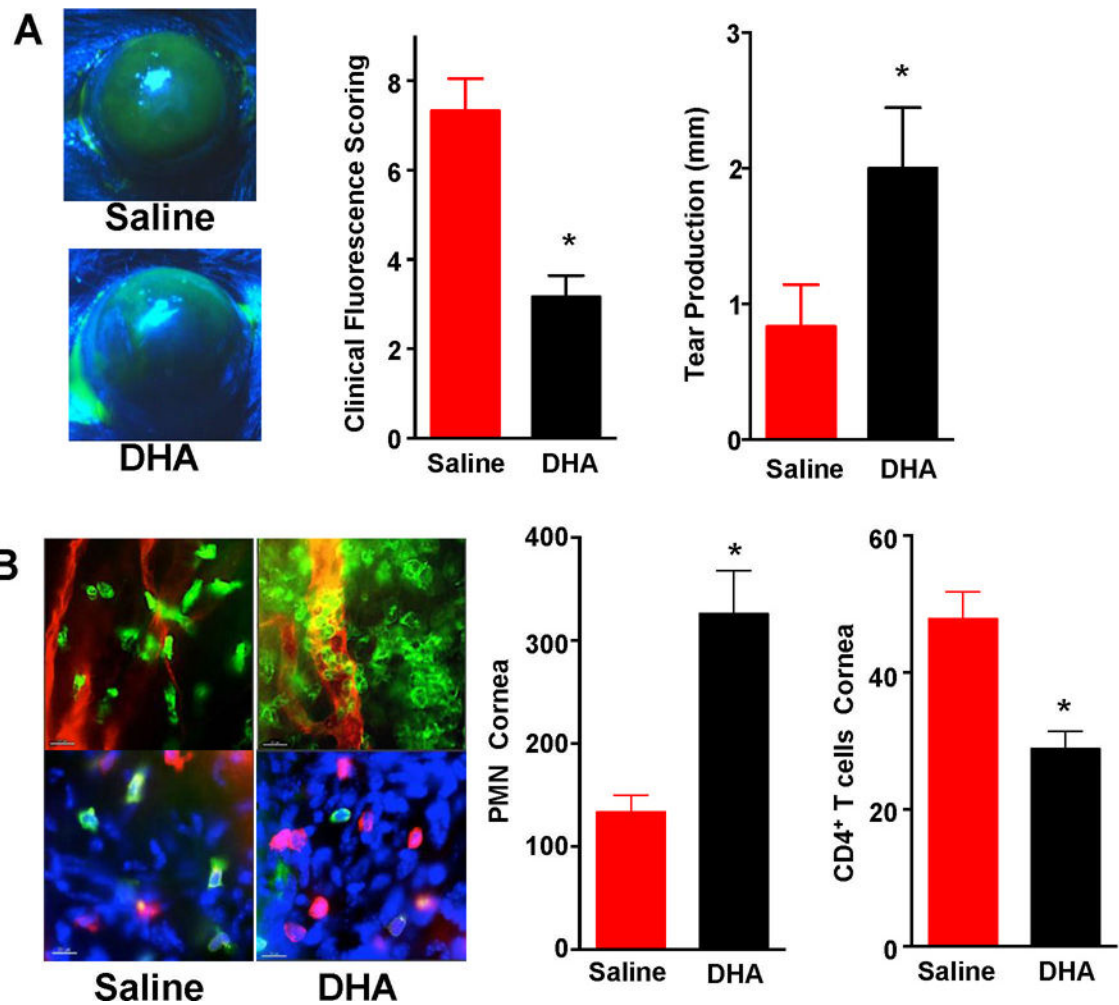
Fig 6. Acute DHA treatment rescues females from amplified immune-driven dry eye disease.
Female mice on the DHA-deficient diet (4 weeks) were treated locally and systemically with DHA or saline alone (vehicle control) starting at day 1 of desiccating stress. After 10 days of desiccating stress dry eyes diseases was assessed and corneas with limbus region isolated for IHC analysis. A) Representative corneal fluorescein staining images. Bar graphs show clinical scoring of epithelial defect and tear production measured by Schirmer's Strip (n=6), B) IHC representation images (left) and quantification (right) of PMN (CD45^{+}, Ly6g^{+}) and CD3^{+}, CD4^{+} T cells in corneas (n=6), measured with scale bars at 20 μm. The total number of corneal PMN and T cells was counted in nine, 40x magnification fields of view that comprised the diameter of a cornea (from limbus to limbus). Values represent the mean ± SEM. *P<0.05, Saline versus DHA.
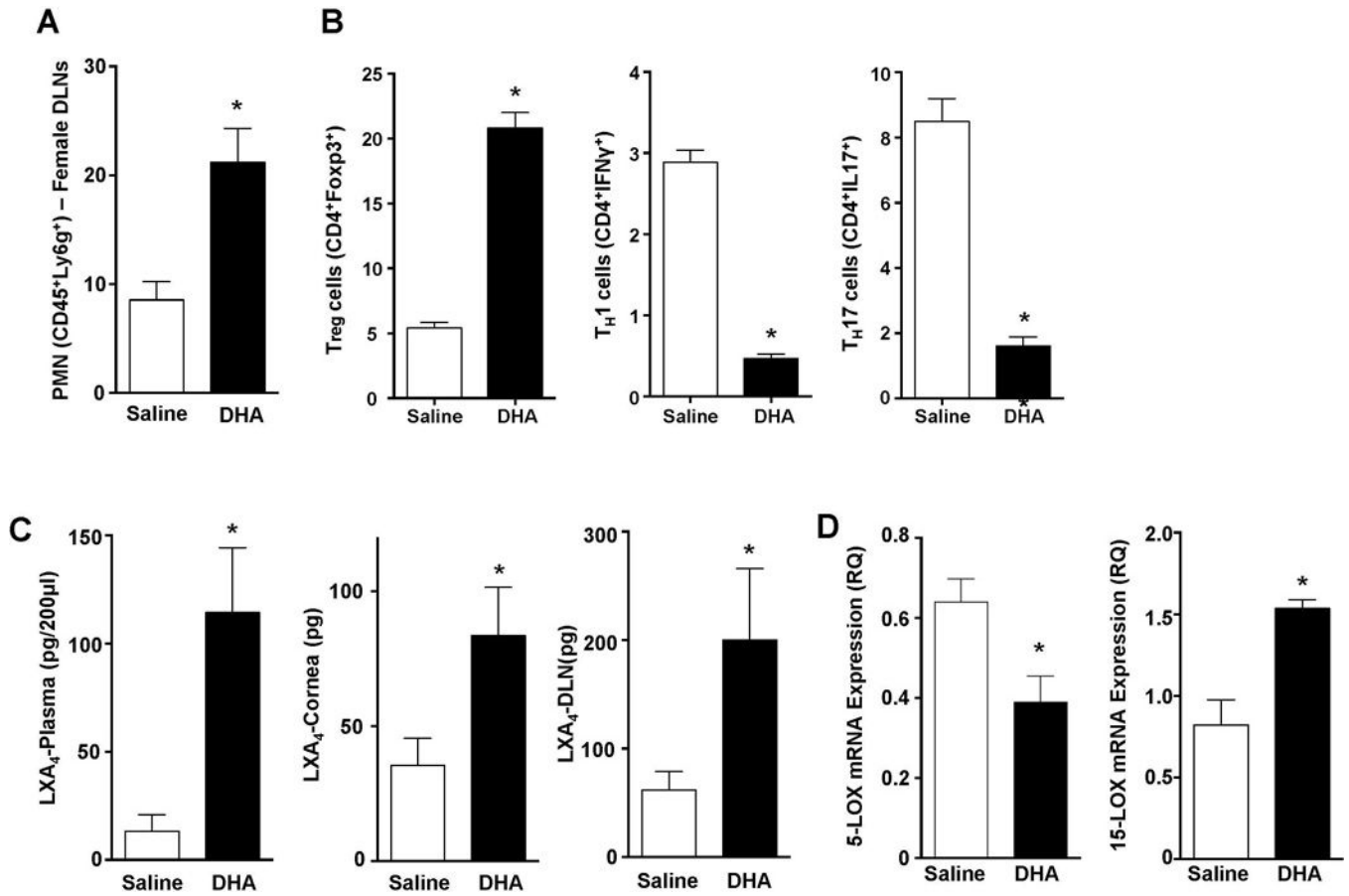
Figure 7. Acute DHA treatment rescues females from amplified T cell responses and amplifies endogenous LXA$_4$ formation in tissues.

Female mice on the DHA-deficient diet (4 weeks) were treated locally and systemically with DHA or saline alone starting at Day 1 of desiccating stress. After 10 days of desiccating stress draining lymph nodes were collected for analysis by flow cytometry. A) Bar graph PMN (CD45^{high}Ly6g^{high}) in draining lymph nodes (n=3, with pooled cervical lymph nodes from 1 mouse for each group). B) Frequencies of T$_{reg}$ cells (CD4$^+$Foxp3$^+$),T$_H$1 cells (CD4$^+$IFNγ^+) and T$_H$17 (CD4$^+$IL17$^+$) cells among absolute number of lymphocytes. C) LXA$_4$ levels were measured by LC/MS/MS-based lipidomics in plasma (n=4), cornea (n=5), lacrimal gland (n=5) and draining lymph nodes (n=5). D) qPCR analysis of 5-LOX, 15-LOX and LXA$_4$ receptor (ALX) RNA expression in draining lymph nodes. Values represent the mean ± SEM. *P<0.05, Saline versus DHA.
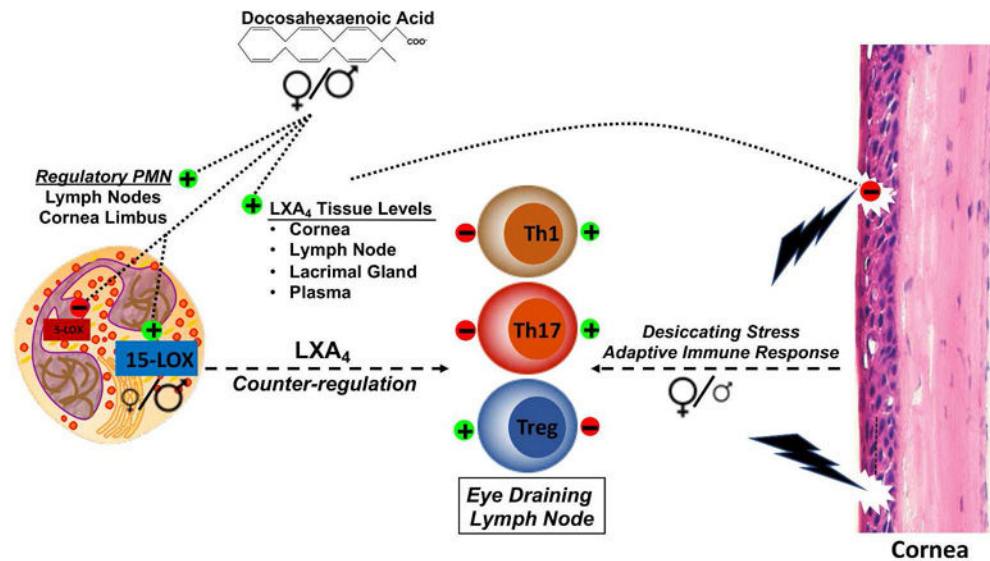
New Figure 8: Summary Scheme

76x47mm (1200 x 1200 DPI)

Figure 8.
Summary scheme illustrating docosahexaenoic acid (DHA) mediated increase in tissue PMN numbers, 15-LOX expression, PMN LXA$_4$ formation and tissue LXA$_4$ levels as a proposed mechanism for inhibiting effector T cell responses (T$_H$1, T$_H$17) and reducing pathogenesis in immune-driven Dry Eye Disease. Male and female symbols and relative symbol size indicate pathways that demonstrated marked sex-specific differences.