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Original Article

Docosahexaenoic acid suppresses pro-inflammatory macrophages and promotes anti-inflammatory/regulatory macrophage polarization through regulation of cytokines

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Abstract: Macrophages are one of the most important immune cells in the innate immune system. Naive monocytes (M0) differentiate into different forms of activated macrophages depending on the cytokines in the microenvironment. Activated macrophages include classic/M1 macrophages and alternative/M2 or regulatory macrophages (Mreg). Docosahexaenoic acid (DHA), a long chain n-3 polyunsaturated fatty acid, is a common dietary supplement used against numerous diseases. The present *in vitro* study pre-treated native THP-1 cells with DHA and induced polarization by phorbol 12-myristate 13-acetate (PMA). In THP-1-derived macrophages, pre-treatment of DHA reduced expression of M1 and increased M2/Mreg cytokines and markers. Oral administration of DHA decreased M1 and increased M2-like macrophages in mice. Moreover, DHA decreased M1 cytokines (IL-12 and IFN-γ) and increased M2/Mreg cytokines (IL-4 and IL-10) in blood. In conclusion, present results showed that DHA inhibited M1 and induced M2/regulatory macrophage polarization through regulation of cytokine in THP-1 cells and mice. These findings suggest that supplementation of DHA may provide a potential way to prevent and improve autoimmune symptoms or diseases.

Keywords: Docosahexaenoic acid, DHA, macrophage, polarization, M1, M2, Mreg

Introduction

Macrophages are one of the most important immune cells in the innate immune system. Naive monocytes (M0) differentiate into different forms of activated macrophages depending on the cytokines in the microenvironment [1]. Macrophage activation includes classic and alternative activation. Monocytes are differentiated into classic/M1 macrophages by IFN-γ or LPS stimulation. M1 macrophages are involved in inflammation, host defense, and promoting the response of T helper 1 cells (Th1) [2]. Alternative activation includes M2 and regulatory macrophages (Mreg). Monocytes are differentiated into M2 macrophages by IL-4 and IL-13 stimulation. M2 macrophages are involved in tissue remodeling and promoting the response of T helper 2 cells (Th2) [3]. Monocytes are differentiated into Mreg upon stimulation by IL-10. They are involved in immune regulation and promotion of the response of regulatory T-cells (Treg) [4-6].

Docosahexaenoic acid (DHA), a long chain n-3 polyunsaturated fatty acid (PUFA), is a common dietary supplement known to have a variety of health benefits against inflammation, obesity, cardiovascular disease, cancer, fatty liver, and autoimmune disease [7-9]. Recent studies have indicated that DHA affects macrophage polarization by inhibiting M1 markers and enhancing M2/Mreg markers in different disease models (including obesity, atherosclerosis, apoptosis, and non-alcoholic fatty liver models) [10-13]. Furthermore, DHA regulates macrophage polarization-related cytokines. DHA inhibits levels of IFN-γ and IL-12 in hypersensitivity or Listeria monocytogenes challenging mice [14, 15] and enhances levels of IL-10 or IL-4 in monocytes,

adipocytes, or intestinal epithelial cells [16-18]. However, the effects of DHA on macrophage polarization and regulation of polarization-cytokines under normal conditions have not been investigated.

Isolation of monocytes/macrophages from tissue or blood is technical and complex. The THP-1 cell line is a human leukemic monocyte cell line derived from an acute monocytic leukemia patient, differentiating into macrophages in the presence of phorbol 12-myristate 13-acetate (PMA) or 1,25-dihydroxyvitamin D_3 [19-21]. A previous study demonstrated that THP-1-derived macrophages express the M2 marker (CD206), implying that THP-1 retains the plasticity of polarization after PMA treatment [21].

The present study examined the effects of DHA on macrophage polarization in the THP-1 cell line and murine model.

Materials and methods

Cell culture

Human monocyte cell line THP-1 (Food Industry Research and Development Institute, Hsinchu, Taiwan) was cultured in RPMI-1640 medium (SH300027; GE Healthcare Life Sciences, Marlborough, MA, USA), with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA), 1% glutamine (GE Healthcare Life Sciences), and 0.05 mM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA). All cells were cultured in humidified 95% air plus 5% CO_2 at 37°C.

DHA treatment and macrophage differentiation

DHA was purchased from Cayman Chemical (90310; Cayman Chemical, Ann Arbor, MI, USA). THP-1 cells were treated with 1% bovine serum albumin (BSA; Gold Biotechnology, St. Louis, MO, USA), conjugated to 100 μM DHA in ethanol or 1% BSA, plus an equal volume of ethanol in the control group, for 24 hours, along with wash out DHA by phosphate buffed saline (PBS) buffer. 4 x 10^5 DHA-treated THP-1 cells differentiated into macrophages after treatment with 100 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) for 48 hours. The conditioned medium was collected for enzyme-linked immunosorbent assay (ELISA). Cell pellets were collected for quantitative polymerase chain reaction (qPCR).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol Reagent (Life Technologies, Grand Island, NY, USA) and cDNA was synthesized using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster, CA, USA). qRT-PCR with DyNAmo Flash SYBR Green qPCR Kit (Thermo Fisher Scientific, Inc.) was performed using a C1000TM Thermal Cycler (Bio-Rad, Hercules, CA, USA). qPCR was performed using SYBR Green, conducted at 95°C for 7 minutes and then 40 cycles of 95°C for 10 seconds and 60°C for 30 seconds. Primer list pairs are noted in Supplementary Table 1.

Experimental animals and DHA administration

All mice were obtained from the National Laboratory Animal Center (Taipei, Taiwan) and animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of National Taiwan University. Male C57BL/6J mice were administrated 36 mg/kg DHA in ethanol or an equal volume of ethanol by oral gavage from 8 weeks of age (eight mice per group, 3 independent experiments). After 6 weeks, whole blood from facial veins was collected into EDTA-coated tubes and centrifuged (2000 g for 30 mins) to separate plasma and blood cells. Plasma was collected for enzyme-linked immunosorbent assay (ELISA). Red blood cells were lysed with Ammonium-Chloride-Potassium (ACK) lysing buffer (Sigma-Aldrich) and leukocyte pellets were suspended in 2% FBS in PBS. Immune cell populations were analyzed by flow cytometry. After 2 weeks, mice were injected with 100 μg lipopolysaccharide (LPS) in 100 μL and blood was collected after 3 hours, 6 hours, and 48 hours. Plasma was obtained and used for ELISA.

Flow cytometry

Leukocytes from mouse blood were stained with mouse-specific antibodies, CD11b-BB515 (564454), CD86-PE-Cy (560582), and CD206-Alexa Fluor 647 (565250) (BD Biosciences, San Jose, CA, USA), for 30 minutes in the dark at 4°C. Cells were then washed by 1% FBS in

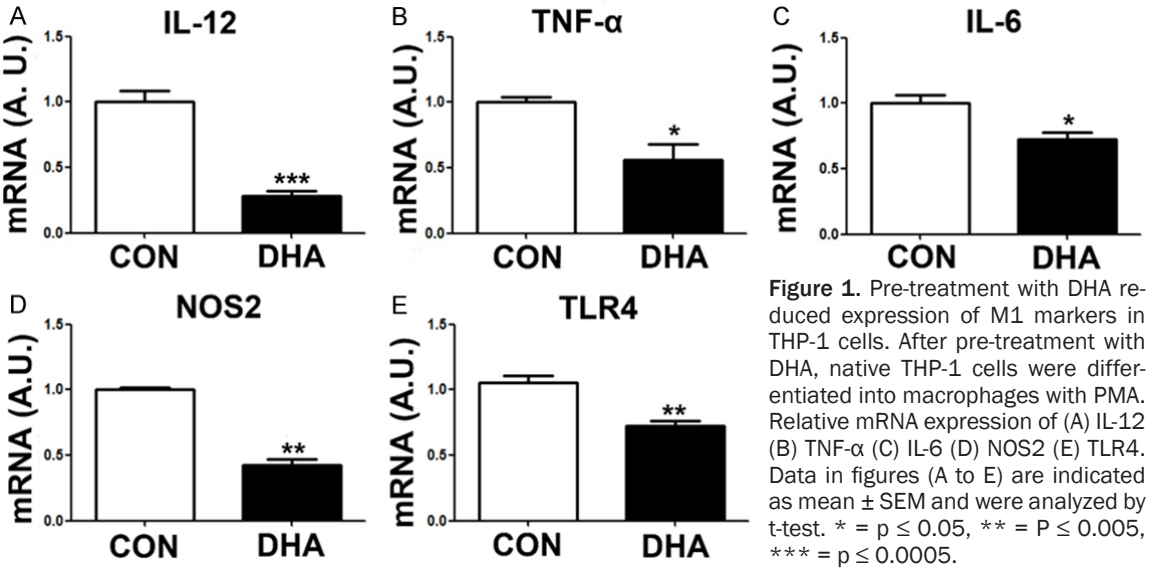

Figure 1. Pre-treatment with DHA reduced expression of M1 markers in THP-1 cells. After pre-treatment with DHA, native THP-1 cells were differentiated into macrophages with PMA. Relative mRNA expression of (A) IL-12 (B) TNF-α (C) IL-6 (D) NOS2 (E) TLR4. Data in figures (A to E) are indicated as mean ± SEM and were analyzed by t-test. * = $p \leq 0.05$, ** = $P \leq 0.005$, *** = $p \leq 0.0005$.

PBS to remove antibodies. Cells were centrifuged at 300 x g for 5 minutes and supernatant fractions were completely removed. Cell samples were re-suspended in 200 μL 1% FBS in PBS and analyzed by flow cytometry, using a BD Accuri™ C6 Cytometer.

Enzyme-linked immunosorbent assay (ELISA)

Plasma was collected from control and DHA groups. Macrophage polarization related to cytokine concentrations (IFN-γ, IL-12, IL-4 and IL-10) was detected, with or without LPS challenge. In the without LPS group, whole blood was collected and levels of IFN-γ, IL-12, IL-4, and IL-10 were collected. For the LPS-challenged, mice were intraperitoneally injected with LPS (100 μg per mice). Blood samples were collected at 3, 6, and 48 hours after the LPS challenge. Whole blood was centrifuged to separated plasma and blood cells. Next, 3-hour plasma samples were used to detect concentrations of IL-10, 6-hour plasma samples were used for IFN-γ and IL-12, and 48-hour plasma samples were used for IL-4. Cytokine concentrations were measured in all plasma samples using mouse-specific enzyme-linked immunosorbent assay (ELISA) kits (eBioseience, San Diego, CA, USA).

Statistical analysis

Data are expressed as mean ± SEM. A paired t-test or one-way ANOVA, followed by Tukey's multiple comparison test, was used for comparisons among groups. Means indicated by different letters are different at $P \leq 0.05$.

Results

Pre-treatment with DHA reduced expression of M1 markers in THP-1 cells

To understand the effects of DHA in macrophage classic polarization (M1), this study pre-treated native THP-1 cells with 100 μM DHA for 24 hours and induced differentiation by 100 nM PMA. After 48 hours, native THP-1 cells differentiated into mature macrophages. DHA pre-treated macrophages expressed lower M1 markers (including IL-12, THF-α, IL-6, NOS2, and TLR4) than the control group (**Figure 1A-E**).

Pre-treatment with DHA increased expression of M2 and Mreg markers in THP-1 cells

To understand the effects of DHA on macrophage alternative polarization (M2 and Mreg), this study pre-treated native THP-1 cells with 100 μM DHA for 24 hours and induced polarization by 100 nM PMA. After 48 hours, native THP-1 cell differentiated into mature macrophages. CD206 (mannose receptor) and CD163 are the cell surface markers of M2 and Mreg [22]. DHA pre-treated macrophages expressed higher CD206 and CD163 than control macrophages (**Figure 2A**, **2B**). DHA pre-treated macrophages expressed higher M2 markers (including IL-4 and Arginase 1) than the control group (**Figure 2C**, **2D**). Moreover, DHA enhanced

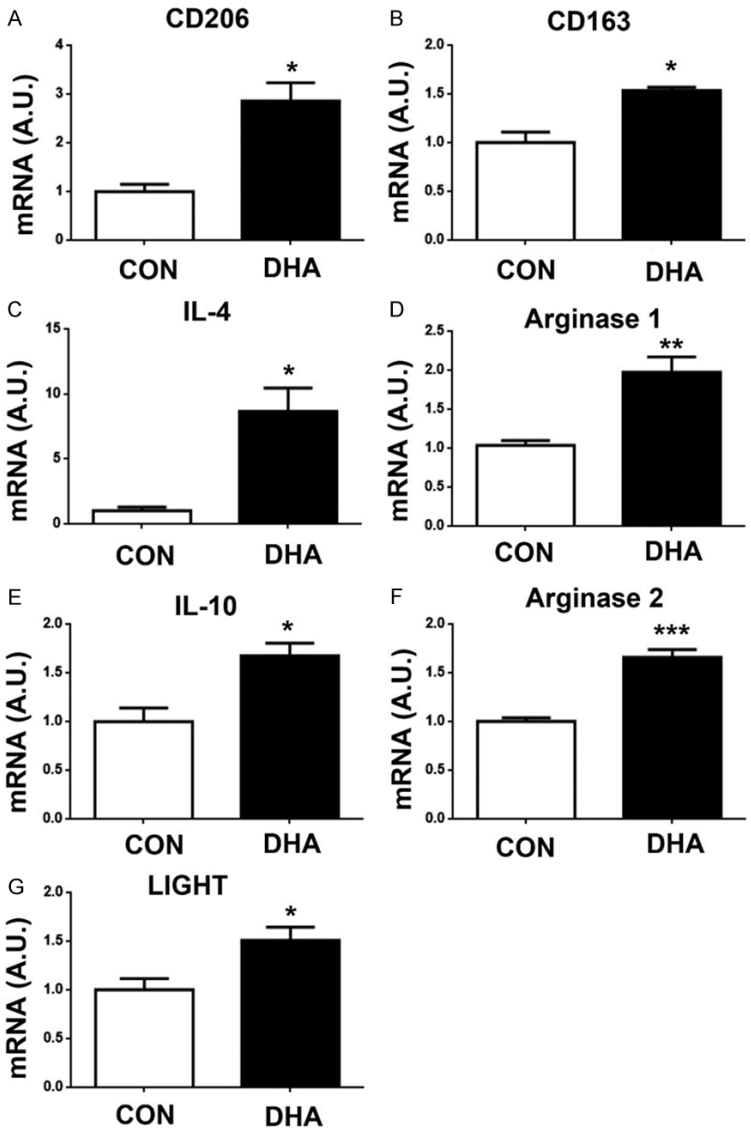

Figure 2. Pre-treatment with DHA increased expression of M2 and Mreg markers in THP-1 cells lines. After pre-treatment with DHA and naive THP-1, cells were differentiated into macrophages with PMA. Relative mRNA expression of (A) CD206 (B) CD163 (C) IL-4 (D) Arginase 1 (E) IL-10 (F) Arginase 2 (G) LIGHT. Data in figures (A to G) are indicated as mean ± SEM and were analyzed by t-test. * = $p \leq 0.05$, ** = $P \leq 0.005$, *** = $p \leq 0.0005$.

expression of Mreg markers (including IL-10, arginase 2, and LIGHT) (**Figure 2E-G**).

DHA decreased M1 and increased M2-like macrophages in mice

To reveal the effects of DHA on macrophage polarization in mice, this study administrated 36 mg/kg DHA to mice by oral gavage for 6 weeks, analyzing classic macrophages or M1 ($CD11b^+/CD86^+$) and alternative macrophages or M2-like ($CD11b^+/CD206^+$). Gate R1 was built separating monocytes from mice leukocytes by cell size (x axis/FSC-A) and granularity (y axis/SSC-A). Gate R1 contained 95.7% CD11b positive cells (**Figure 3A**, **3B**). M1 and M2-like cells were evaluated using the R1 gate (macrophage) and separated based on relative expression levels of CD86 and CD206. $CD11b^+$ $CD86^+$ and $CD11b^+$ $CD206^+$ likely represent M1 and M2-like cells, respectively (**Figure 3C**). Data showed that DHA decreased the $CD11b^+$ $CD86^+$ cells (classic macrophages) and increased $CD11b^+$ $CD206^+$ cells (alternative macrophages) in mice (**Figure 3D**, **3E**).

DHA decreased M1-stimulating cytokines and increased M2/Mreg-stimulating cytokines in mice

Monocytes can differentiate into multiform macrophages by specific cytokine stimulus. The present study detected plasma levels of M1 (IFN-γ and IL-12), M2 (IL-4), and Mreg (IL-10) stimulating cytokines in mice. Without LPS-challenge, IFN-γ and IL-12 could not be detected in the plasma (**Figure 4A**, **4B**). After LPS injection, levels of plasma cytokines increased. Administration of DHA decreased plasma levels of IFN-γ and IL-12 (**Figure 4A**, **4B**). DHA increased plasma levels of IL-4 and IL-10, whether challenged with LPS or not (**Figure 4A-D**). Present data shows that DHA decreased secretion of M1-stimulating cytokines, IFN-γ, and IL-12, while increasing secretion of M2 and Mreg-stimulating cytokines, IL-4 and IL-10.

Discussion

Autoimmune diseases, including type 1 diabetes, rheumatoid arthritis, systemic lupus erythematosus, and inflammatory bowel disease,

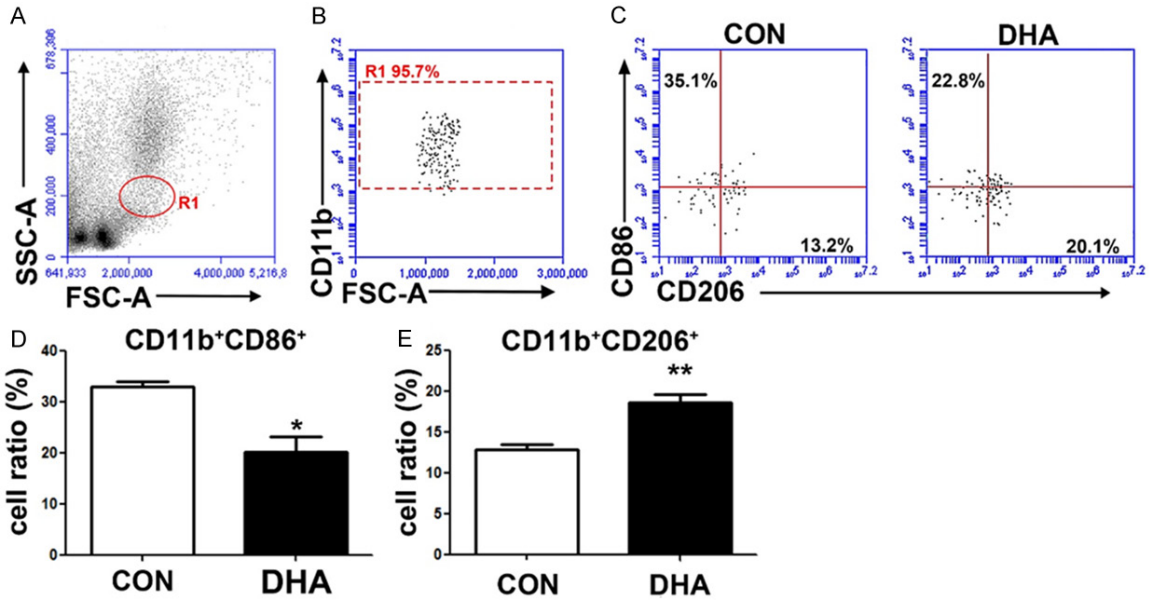

Figure 3. Administration of DHA decreased the percentage of M1 and increased the percentage of M2-like cells in mice. Flow cytometry was used to determine percentages of M1 and M2-like cells. Flow cytometric analysis for surface CD11b, CD86, and CD206 expression. (A) Gate R1 was built separating monocytes from mice leukocyte by cell size (x axis) and granularity (y axis). (B) Gate R1 contained 95.7% CD11b positive cells. (C) M1 and M2-like cells were evaluated using the R1 gate (macrophage) and separated based on relative expression levels of CD86 and CD206. $CD11b^+$ $CD86^+$ and $CD11b^+$ $CD206^+$ likely represent M1 and M2-like cells, respectively. CON were macrophages from control group mice; DNA were macrophages from DHA group mice. (D) percentage of $CD11b^+$ $CD86^+$ cells (E) $CD11b^+$ $CD206^+$ cells. Data in figures (D to E) are expressed as mean ± SEM and were analyzed by t-test. * = $p \leq 0.05$, ** = $p \leq 0.005$.

are caused by abnormal immune responses that attack healthy tissues [23, 24]. More importantly, autoimmune diseases have increased dramatically, worldwide, after World War II. The National Institute of Health (NIH) has estimated that more than 23.5 million people have an autoimmune disease in the United States [23]. Causes of autoimmune disease are complex, but imbalance of macrophages plays an important role [25-27]. M1 macrophages play a critical role in autoimmune disease by producing pro-inflammatory cytokines and NO, which induce cell apoptosis and enhance the chemotaxis of autoantigen-specific T-cells [28]. In contrast, M2 macrophages and Mreg macrophages have the ability of decreasing inflammation and autoimmune disease by increasing anti-inflammatory cytokines, inducing immunosuppressive T-cells (Treg), and enhancing tissue remodeling [25, 28, 29]. Therefore, inhibition of M1 macrophages and induction of M2 and Mreg macrophages may have a potential role in anti-inflammatory and autoimmune disease therapeutics [4].

DHA has a variety of health benefits. Passive studies have shown that administration of DHA or n-3 PUFA improves and retards autoimmune diseases [30-32]. The current study demonstrated that DHA pre-treatment of THP-1-derieved macrophages changed the characterization of these cells (**Figures 1** and **2**). (1) Cell surface markers: DHA increased expression of M2/Mreg cell surface markers, CD206 and CD163, and decreased M1 marker TLR4. (2) Pro-/anti-inflammatory cytokines: DHA decreased expression of pro-inflammatory cytokines (TNF-α and IL-6) and increased expression of anti-inflammatory cytokines (IL-4 and IL-10). (3) Metabolic enzymes: Classic macrophages (M1) produce NO from arginine through NOS2 against pathogens. However, M2/Mreg produces ornithine from arginine through arginase1/2. DHA decreased expression of NOS2 and increased Arginase 1/2. (4) Stimulating cytokines: M1 macrophages secrete IL-12 to affect Th-1 cells, while M2 macrophages secrete IL-4 to affect Th-2 and Mreg macrophages secrete IL-10 to affect Treg. Present data reveals that pre-treated THP-1 decreased expression of

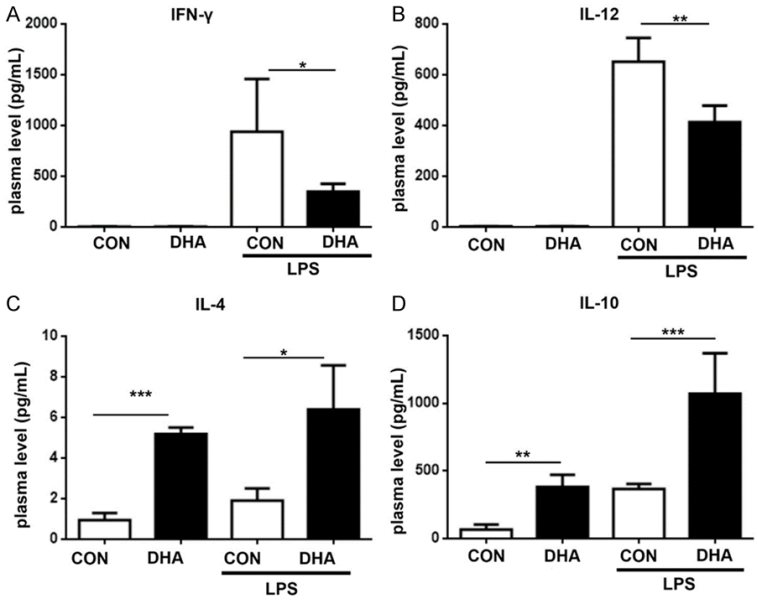

Figure 4. Administration of DHA decreased M1 polarization stimulating cytokine secretion and increased M2 and Mreg polarization stimulating cytokine secretion in mouse plasma. (A) INF-γ (B) IL-12 (C) IL-4 (D) IL-10. Data in figures (A to D) are expressed as mean ± SEM and were analyzed by t-test. * = $p \leq 0.05$, ** = $p \leq 0.005$, *** = $p \leq 0.0005$.

IL-12 and increased expression of IL-4 and IL-10. (5) Mreg markers: LIGHT is a Mreg specific marker, providing co-stimulatory signals for T-cells. DHA increased expression of LIGHT. Moreover, the present murine experiment exhibited that administration of DHA decreased the classic macrophage population and increased the alternative macrophage population in blood (**Figure 3**). Macrophage polarization is regulated by the microenvironment produced cytokines. In the blood of the DHA-treated mice, levels of M1 stimulating cytokines (INF-γ) and Th-1 stimulating cytokines IL-12) were reduced and M2/Th2 stimulating cytokines (IL-4) and Mreg/Treg stimulating cytokines (IL-10) were increased (**Figure 4**).

In conclusion, the present study strengthens the viewpoint that DHA has the ability to modulate the immune system, noting that THP-1 retains plasticity under PMA treatment induced differentiation. In THP-1-derived macrophages, pre-treatment of DHA reduced expression of M1 and increased M2/Mreg markers. Furthermore, the present murine experiment exhibited that administration of DHA decreased M1 macrophages and increased M2-like macrophages, while decreasing M1-related cytokines and increasing M2/Mreg related cytokines in healthy subjects. These findings suggest that supplementation of DHA may provide a potential method of preventing and improving autoimmune symptoms or diseases.

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Disclosure of conflict of interest

None.

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Supplementary Table 1. Primers sets for quantitative real-time PCR. List of human primers used in the present study

Gene	Accession	Forward (5'→3')	Reverse (5'→3')
IL-12	NM_002187	GGCCAGTACACCTGTCACAA	CTGATTGTCGTCAGCCACCA
TNF-α	NM_000594	AGCCTCTTCTCCTTCCTGAT	AAGATGATCTGACTGCCTGG
IL-6	NM_000600	AATGAGGAGACTTGCCTGGTG	CTGGCATTTGTGGTTGGGTC
NOS2	NM_000625	TGAACTACGTCCTGTCCCCT	CTCTTCTCTTGGGTCTCCGC
TLR4	NM_003266	CGTGGAGACTTGGCCCTAAA	GGGAGGTTGTCGGGGATTT
CD206	NM_002438	AACAGTCAGTCAAGCCCAGG	AGGACAGACCAGTACAATTCAG
IL-4	NM_000589	TCTTCCTGCTAGCATGTGCC	TGTTACGGTCAACTCGGTGC
CD163	NM_004244	CGGCTTGCAGTTTCCTCAAGA	GGCCTCCTTTTCCATTCCAGAAA
LIGHT	NM_003807	TGGCGTCTAGGAGAGATGGT	GAGTTGGCCCCTGTGAGATG
IL-10	NM_000572	CTAACCTCATTCCCCAACCA	GTAGAGACGGGGTTTCACCA
Arginase1	NM_000045	ACTTAAAGAACAAGAGTGTGATGTG	CACCAGGCTGATTCTTCCGT
Arginase2	NM_001172	TGCCCAGACCTTTGTGTTGT	GGTGGCCAACTGAGGATTGA
β-actin	NM_001101	GAAGATCAAGATCATTGCTCCTC	CTAAGTCATAGTCCGCCTAGAAG