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Docosahexaenoic acid prevents dendritic cell maturation, inhibits antigen-specific Th1/Th17 differentiation and suppresses experimental autoimmune encephalomyelitis

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ABSTRACT

Docosahexaenoic acid (DHA), the most abundant essential $n-3$ polyunsaturated fatty acid in the CNS, emerged recently together with eicosapentaenoic acid (EPA) and DHA/EPA metabolic derivatives as a major player in the resolution of inflammation. Protective anti-inflammatory effects of DHA were reported in clinical studies and animal models of colitis, sepsis, and stroke. Here we report for the first time a beneficial effect of dietary $n-3$ fatty acids in experimental autoimmune encephalomyelitis (EAE), a model for human multiple sclerosis. In the present study we investigated the effects of DHA on the function of bone marrow-derived dendritic cells (DC) in $CD4^+$ T cell stimulation and differentiation. Pretreatment of DC with DHA prevented LPS-induced DC maturation, maintaining an immature phenotype characterized by low expression of costimulatory molecules and lack of proinflammatory cytokine production (IL-12p70, IL-6, and IL-23). DHA-treated DC were poor stimulators of antigen-specific T cells in terms of proliferation and Th1/Th17 differentiation. This was associated with an increase in p27(kip1), a cell cycle arresting agent, and with decreases in Tbet, GATA-3, and RORγt, master transcription factors for Th1, Th2, and Th17. In contrast, T cells co-cultured with DC-DHA express higher levels of TGFβ and Foxp3, without exhibiting a functional Treg phenotype. Similar to the in vitro results, the beneficial effect of DHA in EAE was associated with reduced numbers of IFNγ- and IL-17-producing $CD4^+$ T cells in both spleen and CNS.

1. Introduction

In contrast to $n-6$ polyunsaturated fatty acids (PUFA) such as arachidonic acid (AA) which mediate predominantly proinflammatory effects, the $n-3$ docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are mostly anti-inflammatory. The $n-3$ PUFAs were reported to be protective in animal models of Alzheimer's and Parkinson's disease, in the ischemia–reperfusion model of stroke, and more recently in spinal cord and traumatic brain injury models (Bailes and Mills, 2010; Bousquet et al., 2008; Calon et al., 2004; Lopez-Vales et al., 2010; Marcheselli et al., 2003). In human subjects, high intake of dietary DHA and EPA affected the expression of more than 1000 genes, reducing proinflammatory and atherogenic related gene expression (Bouwens et al., 2009). In patients with multiple sclerosis (MS), several studies reported lower levels of inflammatory cytokines and MMP9 and significant improvement in quality of life following 3–6 months of $n-3$ fatty acids supplementation (Gallai et al., 1995; Shinto et al., 2009; Weinstock-Guttman et al., 2005).

Although MS etiology remains elusive, the pathology relies mainly on autoimmune mechanisms that involve activated antigen-specific T cells as major players in addition to macrophages, dendritic cells (DC) and B cells (Frohman et al., 2006; Slavin et al., 2010). Experimental autoimmune encephalomyelitis (EAE) is a widely used animal model for MS. Multiple T cell subsets including $CD4^+$ Th1 and Th17, γδT cells, $CD8^+$ and Treg have been shown to be involved in EAE, with Th1 and Th17 as major pathogenic cells (Slavin et al., 2010). DC play an essential role in antigen presentation and T cell activation in EAE, with CNS perivascular

Abbreviations: AA, arachidonic acid; cDC, conventional dendritic cells; CNS, central nervous system; DC, dendritic cells; DHA, docosahexaenoic acid; EAE, experimental autoimmune encephalomyelitis; EPA, eicosapentaenoic acid; LPS, lipopolysaccharide; MFI, mean fluorescence intensity; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; PCCF, pigeon cytochrome *c* fragment; PTX, pertussis toxin; PMA, phorbol myristate acetate; PI, propidium iodide; PLP, proteolipid protein; PUFA, polyunsaturated fatty acids.

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conventional/myeloid DC (cDC/mDC) activating myelin-specific T cells and inducing differentiation into encephalitogenic Th1/Th17 (Bailey et al., 2007; Slavin et al., 2010).

DC represent an essential cellular link between innate and adaptive immunity. DC are professional antigen presenting cells whose major role is the uptake, processing and presentation of antigens to naïve $CD4^+$ and $CD8^+$ T cells. Following antigen uptake, cDC mature, upregulating MHCII and co-stimulatory molecules, secreting various cytokines and chemokines, and acquiring a new chemokine receptor pattern which enables them to migrate to neighboring lymph nodes. $CD4^+$ T cell activation and differentiation in vivo is mediated through cognate interactions of naïve T cells with antigen-presenting cells, primarily cDC, which provide signaling through MHCII/antigen complexes and costimulatory molecules. Cytokines secreted by mature cDC play an important role in $CD4^+$ T cell differentiation, with IL-12 supporting Th1, and IL-6, TGFβ and IL-23 supporting Th17 differentiation. We and others reported that exposure to DHA maintains cDC stimulated with TLR ligands in an immature state characterized by low MHCII, CD40, CD80, CD86, and CCR7 expression, and lack of inflammatory cytokine production (Kong et al., 2010; Wang et al., 2007; Zapata-Gonzalez et al., 2008; Zeyda et al., 2005). In our previous study (Kong et al., 2010) we did not address the effects of DHA-treated cDC on T cell activation and differentiation. Here we report for the first time that exposure to DHA prevents cDC to induce antigen-specific naïve $CD4^+$ T cells to differentiate into Th17 cells, and that DHA-treated DC arrest T cells in G0/G1 phase through increased expression of p27(kip1). We also report for the first time that dietary DHA reduces EAE disease severity and that this correlates with a significant reduction in both Th1 and Th17 cells in spleen and the CNS.

2. Materials and methods

2.1. Mice

B10.A mice ($I\text{-}E^k$), C57BL/6 mice ($H\text{-}2^b$), TCR-Cyt-5CC7-I/$Rag1^{-/-}$ transgenic (PCCF-specific TCR Tg; $I\text{-}E^k$) and C57BL/6-Tg (Tcra2D2, Tcrb2D2)1Kuch/J (MOG_{35-55} specific TCR) were purchased from Jackson Laboratory (Bar Harbor, ME) and Taconic (Hudson, NY). Transgenic mice were bred and maintained in the Temple University School of Medicine animal facility (Philadelphia, PA) under pathogen-free conditions. All mice used were between 6 and 10 weeks of age. Mice were handled and housed in accordance with the guidelines of the Temple University Animal Care and Use Committee.

2.2. Reagents

Recombinant murine GM-CSF, recombinant murine CCL19, IL-12, IL-6, were purchased from Peprotech Inc. (Rocky Hill, NJ). Docosahexaenoic Acid was purchased from Cayman Chemical (Ann Arbor, MI). Lipopolysaccharide (LPS) (*Escherichia coli* O26:B6), pertussis toxin (PTX), streptavidin-peroxidase, phorbol myristate acetate (PMA) and ionomycin were purchased from Sigma–Aldrich (St. Louis, MO). CD4 and CD11c MicroBeads were purchased from Miltenyi Biotec (Bergish-Gladbach, Germany). Recombinant IL-23, capture and biotinylated anti-mouse IL-23 antibody, PE-conjugated anti-mouse PD-L1 and CD25, FITC-conjugated anti-mouse PD-L2, mouse regulatory T cell staining kit, APC-conjugated anti-mouse INFγ were purchased from eBioscience (San Diego, CA). FITC-conjugated anti-mouse CD80, CD86, CD40, MHCII, CD4, CD44; PerCP-Cy™ 5.5 conjugated anti-mouse CD69, PE-conjugated anti-mouse IL-17, recombinant mouse IL-10, IFNγ and capture and biotinylated anti-mouse IL-2, IL-12p70, IL-6, IL-10, IFNγ; GolgiPlug, annexin V-FITC apoptosis detection kit I, Cytofix/Cytoperm, Perm/Wash buffer, TMB Substrate Reagent Set, and the Cycle TEST PLUS DNA Reagent Kit were purchased from BD PharMingen (San Diego, CA). Pigeon cytochrome *c* fragment (PCCF), myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$, proteolipid protein $(PLP)_{139-151}$, CFSE Cell Proliferation Kit, 1× HBSS and 10× HBSS were purchased from Invitrogen Corporation (Carlsbad, CA). Capture and biotinylated anti-mouse IL-17, recombinant mouse IL-17, recombinant TGFβ, and recombinant mouse IL-2 were purchased from R&D Systems (Minneapolis, MN). DNase I grade II and Liberase TL were purchased from Roche (Indianapolis, IN). Ketamine HCl was purchased from Fort Dodge Animal Health (Fort Dodge, IA). Xylazine was purchased from Butler Animal Health Supply (Dublin, OH). EDTA (0.5 M) was purchased from Promega Corporation (Madison, WI). Percoll was purchased from GE Healthcare (Piscataway, NJ). *Mycobacterium tuberculosis* H37 RA was purchased from Difco (Detroit, MI).

2.3. Generation and purification of DC from bone marrow

DC were generated from bone marrow as described previously (Kong et al., 2010). On day 7, the non-adherent cells were harvested and purified by immunomagnetic sorting with anti-CD11c-coated magnetic beads using the autoMACS system according to the manufacturer's instructions (Miltenyi Biotec). The purity of the sorted cells was determined by FACS analysis (>96% $CD11c^+$ cells).

2.4. Isolation of $CD4^+$ T cells

Purified $CD4^+$ T cells were isolated from the spleen of PCCF-specific TCR-Tg mice or MOG_{35-55} specific TCR-Tg mice by positive immunomagnetic selection using anti-CD4 mAb magnetic beads (Miltenyi Biotec). The purified T cells were 98% $CD4^+$ as determined by FACS analysis.

2.5. FACS analysis

Cells were subjected to FACS analysis in a 3-color FACS Calibur (BD Biosciences, Mountain View, CA). Data were collected for 10,000 cells and analyzed using Cellquest software from BD Biosciences (San Jose, CA). DC or T cells washed with ice cold PBS and incubated for 30 min at 4 °C with various FITC/PE/APC/PerCP conjugated antibodies and were analyzed by flow cytometry. For the detection of Foxp3, cells were first stained with anti-CD4 and anti-CD25, fixed with Cytofix/Cytoperm buffer, incubated with anti-Foxp3, and analyzed by FACS. The specificity of the primary Abs was established with appropriate isotype-matched controls.

2.6. T cell proliferation assay

DC-$CD4^+$ T cell co-cultures or splenocytes were cultured in 96-well flat bottom plates. On day 3 of co-culture, $[^3H]$-thymidine (1 μCi per well) was added and incorporation was measured after 16 h. Cells were harvested on fiberglass filters, and $[^3H]$-thymidine incorporation was measured in a liquid scintillation counter.

Proliferation suppressive assays were performed as follows: 1×10^5 MOG_{35-55} specific $CD4^+$ T cells were activated with MOG-pulsed DC or DC-DHA in the presence or absence of 2 ng/ml TGFβ and 50 U/ml IL-2 for 3 days, rested for 2 days in the presence of IL-2 and re-cultured with CFSE-labeled (5 μM, according to the manufacturer's protocol) naïve MOG Tg-$CD4^+$ T cells (0.1×10^5) and 0.1×10^4 MOG-pulsed DC, in 200 μl medium in 96-well plates. Three days later, proliferation was assessed by CFSE dilution using FACS. The proliferation of naïve $CD4^+$ T cells was based on gated CFSE labeled cells.