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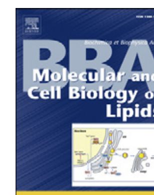

Docosahexaenoyl serotonin, an endogenously formed n-3 fatty acid-serotonin conjugate has anti-inflammatory properties by attenuating IL-23–IL-17 signaling in macrophages

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ABSTRACT

Conjugates of fatty acids and amines, including endocannabinoids, are known to play important roles as endogenous signaling molecules. Among these, the ethanolamine conjugate of the n-3 poly unsaturated long chain fatty acid (PUFA) docosahexaenoic acid (22:6n-3) (DHA) was shown to possess strong anti-inflammatory properties.

Previously, we identified the serotonin conjugate of DHA, docosahexaenoyl serotonin (DHA-5-HT), in intestinal tissues and showed that its levels are markedly influenced by intake of n-3 PUFAs. However, its biological roles remain to be elucidated. Here, we show that DHA-5-HT possesses potent anti-inflammatory properties by attenuating the IL-23-IL-17 signaling cascade in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages. Transcriptome analysis revealed that DHA-5-HT down-regulates LPS-induced genes, particularly those involved in generating a CD4+ Th17 response. Hence, levels of PGE2, IL-6, IL-1β, and IL-23, all pivotal macrophage-produced mediators driving the activation of pathogenic Th17 cells in a concerted way, were found to be significantly suppressed by concentrations as low as 100–500 nM DHA-5-HT. Furthermore, DHA-5-HT inhibited the ability of RAW264.7 cells to migrate and downregulated chemokines like MCP-1, CCL-20, and gene-expression of CCL-22 and of several metalloproteinases. Gene set enrichment analysis (GSEA) suggested negative overlap with gene sets linked to inflammatory bowel disease (IBD) and positive overlap with gene sets related to the Nrf2 pathway. The specific formation of DHA-5-HT in the gut, combined with increasing data underlining the importance of the IL-23-IL-17 signaling pathway in the etiology of many chronic inflammatory diseases merits further investigation into its potential as therapeutic compound in *e.g.* IBD or intestinal tumorigenesis.

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1. Introduction

Conjugates of fatty acids with ethanolamine, mono-amine neurotransmitters or amino acids are found in many living organisms. Well-known examples are the endocannabinoid anandamide (arachidonoyl ethanolamine; AEA) [1] and related ethanolamine conjugates, including palmitoyl ethanolamine (PEA) [2] and oleoyl ethanolamine (OEA) [3]. In contrast to AEA, much less is known about the physiological roles of most other, 'endocannabinoid-like', fatty acid conjugates. This holds particularly true for congeners of the n-3 long-chain PUFA-derived subclass of fatty acid amides (FAAs) [4,5]. However, emerging evidence indicates that several members of this subclass can act as endogenous immune-modulatory mediators. Previously we demonstrated that docosahexaenoyl ethanolamine (DHEA), a n-3 structural analogue of AEA, displays marked anti-inflammatory properties by modulating the activity of cyclooxygenase-2 (COX-2), the key enzyme in the synthesis of pro-inflammatory prostaglandins [6]. DHEA has been identified in many tissues, including brain, intestine, fat, liver and plasma, and its

Abbreviations: AA-5-HT, arachidonoyl serotonin; AEA, arachidonoyl ethanolamide (anandamide); COX-2, cyclooxygenase 2; DHA, docosahexaenoic acid (22:6n-3); DHA-5-HT, docosahexaenoyl serotonin; DHEA, docosahexaenoyl ethanolamine; EPA-5-HT, eicosapentaenoyl serotonin; FAA, fatty acid amides; FAAH, fatty acid amide hydrolase; FDR, false discovery rate; GSEA, gene-set enrichment analysis; IL, Interleukin; n-3 LC PUFA, (n-3) long chain polyunsaturated fatty acid; LPS, lipopolysaccharide; NO, nitric oxide; NOS2, nitric oxide synthase 2; NES, normalized Enrichment Score; OA-5-HT, oleoyl serotonin; OEA, oleoyl ethanolamine; PA-5-HT, palmitoyl serotonin; TRPV1, transient receptor potential channel type V1.

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release has shown to be induced during inflammatory conditions [7–14]. Docosahexaenoic acid (DHA; 22:6[n-3]), being an important n-3 PUFA in the human diet [15,16], can also be metabolized to endocannabinoid congeners other than DHEA with potential, but so far undiscovered physiological bioactivities. One of these putative bioactive FAA mediators is the serotonin conjugate of DHA (Fig. 1).

Starting from the fact that most of the body's serotonin resides in the gut, our group was the first to demonstrate the presence and formation of serotonin conjugates from 6 different fatty acids in intestinal tissues of pigs and mice [17]. We also found that intestinal levels of DHA-serotonin (DHA-5-HT) and EPA-serotonin (EPA-5-HT) were increased in mice fed a fish oil rich diet, in line with the idea that 'cellular combinatorial chemistry' appears to be an important mechanism to explain the biology of endocannabinoids and their congeners [18]. Notwithstanding the presence of these conjugates in gut tissues hardly anything is known so far on their biological effects or endogenous role(s). The only N-acyl serotonin studied in more detail is arachidonoyl serotonin (AA-5-HT) which was found to act as a TRVP1 (transient receptor potential channel type V1) antagonist and FAAH inhibitor with potential analgesic and anxiolytic properties [19–22]. Based on the anti-inflammatory properties found for related ethanolamine conjugates, in particular DHEA [6,23,24] we decided to investigate the effects of the DHA conjugate of serotonin, DHA-5-HT, on innate inflammatory processes in stimulated macrophages. In the present study, we show this compound to be a potent anti-inflammatory compound with profound effects on several key mediators, among which NO, COX-2, and IL-6. By using transcriptome analyses we elucidated the effect of DHA-5-HT on LPS-induced gene expression of cytokines and co-stimulatory molecules. Altogether, our data show that DHA-5-HT modulates inflammation in macrophages by reducing levels of key mediators all involved in the establishment of the IL-23-IL-17 signaling pathway. Interestingly, this signaling pathway has recently received a lot of attention due to its pathogenic role in several chronic disorders like IBD, psoriasis, arthritis, but also colon cancer.

2. Materials and methods

2.1. Chemicals and materials

DMEM, fetal bovine serum (FBS), streptomycin and penicillin were acquired from Lonza (Verviers SPRL, Belgium). LPS, 5-HT, TMB, docosahexaenoic acid, paraformaldehyde and Hoechst were obtained from Sigma-Aldrich (Schnelldorf, Germany). Docosahexaenoyl serotonin (DHA-5-HT), PGE2 EIA kits, Griess reagents and nitrite standard were purchased from Cayman Chemical (Ann Arbor, MI, USA). ELISAs (IL-6, MCP-1, IL-23 and CCL-20) were performed using R&D Systems kits (Abingdon, U.K.). LDH Cytotoxicity Detection Kit was obtained from Roche Applied Science (Almere, The Netherlands). BD™ CBA IL-1β Flex Set was purchased from BD Biosciences (Breda, The Netherlands). Purecol was obtained from advanced Biomatrix (San Diego, USA). Transwell systems were acquired from BD Lifesciences (San Jose, USA). For the microarray experiments, the RNeasy micro kit was obtained from Qiagen (Venlo, The Netherlands), while Whole Transcript cDNA synthesis kits and Affymetrix GeneChip Mouse Gene 1.1 ST arrays were purchased from Affymetrix, (Santa Clara, USA).

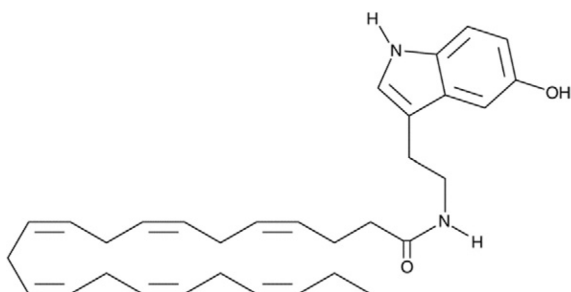

Fig. 1. Structural formula of docosahexaenoyl serotonin (DHA-5-HT). (https://pubchem.ncbi.nlm.nih.gov/compound/52224409#section=Top).

2.2. Cell culture

Experiments were performed with RAW264.7 macrophages (mouse origin) purchased from the American Type Culture Collection (Teddington, UK). Macrophages were grown and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% streptomycin and penicillin at 37 °C in a 5% CO_2 humidified air atmosphere.

To determine CCL-20, IL-6, IL-23, IL-1β and PGE2 production and to perform nitrite and cytotoxicity analysis (LDH), cells were seeded in 48-wells plates ($2.5 \cdot 10^5$ cells.mL^{-1}, 300 μL per well) or in 6-wells plates ($2.5 \cdot 10^5$ cells.mL^{-1}, 2 ml per well) to perform gene expression analysis and assess MCP-1 protein expression.

2.3. Effects of DHA-5-HT on nitric oxide and cytokine release

RAW264.7 macrophages were incubated with different concentrations of DHA-5-HT in combination with 1 μg/mL LPS. Ethanol was used as solvent (final solvent concentration never exceeded 0.1% v/v). After 24 h incubation, the amount of nitrite in the culture medium was determined as an indicator of nitric oxide levels using the Griess method. After stimulation of RAW264.7 cells with LPS (1 μg/mL) applying a concentration range of DHA-5-HT for 24 h, release of IL-6, IL-23, MCP-1, CCL-20 and PGE2 in the culture medium was measured by performing enzyme-linked immunosorbent assays (ELISAs) and an enzyme immunoassay (EIA) in the case of PGE2. Released levels of IL-1β after 24 h stimulation with LPS (1 μg/mL) and DHA-5-HT were determined by flow cytometry using a BD™ Cytometric bead array (CBA) IL-1β Flex Set according to manufacturer's instruction. Data were analyzed with FCAP array software.

2.4. Migration assay

Transwell systems consisting of a 24-well upper chamber with a cell culture insert having a 5-μm pore size membrane were used to perform migration experiments. Before use, membranes were coated for one hour with 50 μg/mL bovine collagen I solution (purecol). After coating, 5×10^5 RAW264.7 cells were seeded in the upper chamber and 600 μL of DMEM containing 0.1 μg/mL LPS was added to the lower chamber. To test effects of DHA-5-HT, 600 μL of DMEM containing both DHA-5-HT and 0.1 μg/mL LPS was added to the lower chamber and DHA-5-HT was added to the upper chamber. After 4 h migration, the cells were fixed with 4% paraformaldehyde in DMEM for 10 min and subsequently washed and stained with Hoechst for 1 min. Cells attached to the upper part of the membrane were removed with a cotton swap. In four randomly selected fields, the cells that migrated to the reverse surface of the membrane were counted by fluorescence microscopy at 200× magnification.

2.5. Cell cytotoxicity

Effects of test compounds on the cytotoxicity of the RAW264.7 cells were assessed using a Cytotoxicity Detection Kit according to the manufacturer's instructions. Briefly, cells were first incubated with the test compound(s) and LPS (1 μg/mL) for 24 h. Thereafter, LDH release from damaged cells into the cell culture medium was measured as an indicator for cell death. Conditions were considered toxic if the measured LDH release was decreased or increased by >20%. As a control Triton ×100 was added to the cells, yielding total cell lysis.

2.6. RNA purification and quantitative reverse-transcription real-time PCR

Total RNA was extracted using TrizolR (Invitrogen, Breda, The Netherlands). RNA (1 μg per sample) was reverse transcribed to give complementary DNA (cDNA) using the reverse-transcription system from Promega (Leiden, The Netherlands). cDNA was amplified by PCR using the master-mix Sensimix SYBR (Bioline Reagents Ltd., London, U.K.) on a CFX Real Time System apparatus (Bio-Rad, Veenendaal, The Netherlands). The following primer pairs were used for amplification of COX-2: 5′-TGAGCAACTATTCCAAACCAGC-3′ (forward) and 5′-GCAC GTAGTCTTCGATCACTATC-3′ (reverse), IL-6: 5′-CTTCCATCCAGTTGCCTT CTTG-3′ (forward) and 5′-AATTAAGCCTCCGACTTGTGAAG-3′ (reverse), NOS2: 5′-GTTCTCAGCCCAACAATACAAGA-3′ (forward) and 5′-GTGGAC GGGTCGATGTCAC-3′ (reverse). Samples were analyzed in duplicate and mRNA expression levels of the different genes were normalized to Rps27a2. Primer pairs for Rps27a2 were: 5′-GGTTGAACCCTCGGACAC TA-3′ (forward) and 5′-GCCATCTTCCAGCTGCTTAC-3′ (reverse). Primer pairs used for the amplification belonging to the genes shown in Fig. S2 of SA1 were as follows: IL-12p40: 5′-CAGAGACGCCATTCCACATGT-3′ (forward) and 5′-AAGACCCTGACCATCACTGTC-3′ (reverse), CD80: 5′-GCAAGGCAGCAATACCTTA-3′ (forward) and 5′-CTCTTTGTGCTGCT GATTCG-3′ (reverse), CD86: 5′-TCTCCACGGAAACAGCATCT-3′ (forward) and 5′-CTTACGGAAGCACCCATGAT-3′ (reverse), MMP-13: 5′-GCAACAAAGTAGATGCTGTCTATGAGA-3′ (forward) and 5′-ATGCGA TTACTCCAGATACTGTATTCAA-3′ (reverse) and CCL-7: 5′-GCTGCT TTCAGCATCCAAGTG-3′ (forward) and 5′-CCAGGGACACCGACTACTG-3′ (reverse).

2.7. Transcriptome analysis

2.7.1. RNA isolation and microarray processing

RAW264.7 macrophages were incubated for 24 h with 1 μM DHA-5-HT in combination with 1 μg/mL LPS. RNA of the RAW264.7 cells was purified using the RNeasy micro kit. RNA yield was determined with the Nanodrop ND-1000 Spectrophotometer and the quality of the RNA samples was verified with an Agilent 2100 Bioanalyzer (Agilent Technologies, Amstelveen, Netherlands). One hundred nanograms of RNA was used for Whole Transcript cDNA synthesis after which hybridization, washing and scanning of Affymetrix GeneChip Mouse Gene 1.1 ST arrays was carried out on an Affymetrix GeneTitan instrument (Affymetrix, Inc., Santa Clara, USA) according to standard Affymetrix protocols.

2.7.2. Microarray analysis

Arrays were normalized using the Robust Multi-array Average method [25,26]. Probe sets were assigned to unique gene identifiers, in this case Entrez IDs. The probes on the arrays represent 21115 Entrez IDs [27]. Array data were analyzed using MADMAX pipeline for statistical analysis of microarray data [28] including gene set enrichment analysis (GSEA; www.broadinstitute.org/gsea; [29]). To asses differential expression of specific genes a filter was applied (log2 intensity value >4.32 and log2 interquartile range >0.2) and Intensity-Based Moderated T-statistics (IBMT) were performed [30]. Microarray data have been submitted to the Gene Expression Omnibus (GEO), under accession number GSE87369.

2.8. Data analysis

All experiments in RAW264.7 macrophages were performed in duplicate and repeated at least three times in independent experiments. Data from experiments are generally expressed as a percentage of the LPS-treated controls (set at 100%). Data are presented as means with SEM (see legends to the figures). Statistical analyses were performed on the data as depicted in the figures, where differences between treatments and controls were evaluated by one-way ANOVA followed by *post hoc* test (Dunnett's *t*-test). A P value <0.05 was considered as statistically significant. P values were assigned at three different levels, namely $P < 0.05$, $P < 0.01$ and $P < 0.001$. Experiments with RAW264.7 macrophages performed for transcriptome analysis were done in duplicate and repeated three times in independent experiments.

3. Results

3.1. DHA-5-HT strongly inhibits inflammatory mediators

As DHA-5-HT was not cytotoxic (See Fig. S1 of SI) for LPS-activated RAW264.7 macrophages at the concentrations studied, DHA-5-HT was tested for its ability to inhibit NO production. Fig. 2A shows that DHA-5-HT reduced nitrite production following 24 h of incubation. Next, it was assessed whether the compound was able to modulate the release of IL-6 and the COX-2-derived metabolite PGE2, a pro-inflammatory cytokine and a pro-inflammatory prostaglandin, respectively. After 24 h of incubation, DHA-5-HT concentration-dependently inhibited the release of IL-6 to about 75% at 2.5 μM (Fig. 2C) and of PGE2 to almost complete inhibition at 2.5 μM (Fig. 2B). For IL-6, a significant effect (62%) was found with a concentration as low as 500 nM. Both DHA and serotonin (5-HT), the precursors of DHA-5-HT, did not affect nitrite, IL-6 and PGE2 production at 2.5 μM (Fig. 2A-C). To establish whether these reductions were regulated at the gene-expression level NOS2, COX-2, and IL-6 gene expression were measured by quantitative RT-PCR. DHA-5-HT significantly suppressed mRNA levels for all three mediators (with an effective concentration as low as 100 nM for COX-2 and IL-6), confirming that DHA-5-HT acts at a transcriptional level (Fig. 3A–C).

3.2. DHA-5-HT reduces gene expression of mediators involved in the induction of inflammatory T cells

To further investigate which inflammatory molecules and mediators are involved in the immune-modulatory effects of DHA-5-HT, micro-array studies were performed (from three independent experiments). Tables 1A–B and 2A–C show the expression of immune-related genes that were changed by LPS treatment and found to be highest downregulated and/or upregulated, respectively (cut off value 1.2) in response to 1 μM DHA-5-HT. In general, a significant downregulation was found for interleukins (Table 1A), co-stimulatory molecules (Table 1B) and chemokines (Table 2B, C) that are involved in mediating the adaptive immune response. Among the highest downregulated genes were IL-12b, IL-23a, IL-1a, IL-27, IL-1f6, IL-6 and IL-1β of which the respective expression was downregulated −3.52, −2.27, −1.89, −1.83, −1.67, −1.58 and −1.41-fold. Additionally, the well-known co-stimulatory molecules CD80 (−1.57), CD40 (−1.24) and CD86 (−1.23) were down-regulated, as well as other so far less known molecules like CD300e (−2.18) and CD300lf (−1.50). Cd1d1 (1.74) and Cd28 (1.50) were upregulated. Furthermore, the expression of several genes of the CXC chemokine family (CXCL) (Table 2C), the interferon inducible proteins family (see Table S1 of SA1) as well as suppressors of cytokine (SOCS) 1 and 3 (see Table S2 of SA1) were found to be predominantly downregulated. From the tumor necrosis factors family (TNF), TL1A (TNFSF15) was down-regulated (−1.94-fold) (see Table S4 of SA1) while the 3.5-fold LPS-induced TNFα expression was not changed by DHA-5-HT. IL-12A which encodes for IL-12p35 and which forms one of the two subunits of the heterodimeric cytokine IL-12 (known to induce Th1 cells), was not expressed.

For a selected number of genes found to be altered by DHA-5-HT in the micro-array experiments control quantitative RT-PCR experiments were performed to determine possible effects of its precursors DHA and serotonin (5-HT). Fig. S2 of SA1 shows that both DHA and serotonin (5-HT) did not reduce gene-expression of the interleukin IL-12p40, the co-stimulatory molecules CD80 and CD86, the matrix metallopeptidase MMP13 and the chemokine CCL-7, while DHA-5-HT significantly reduced expression of all these selected genes.

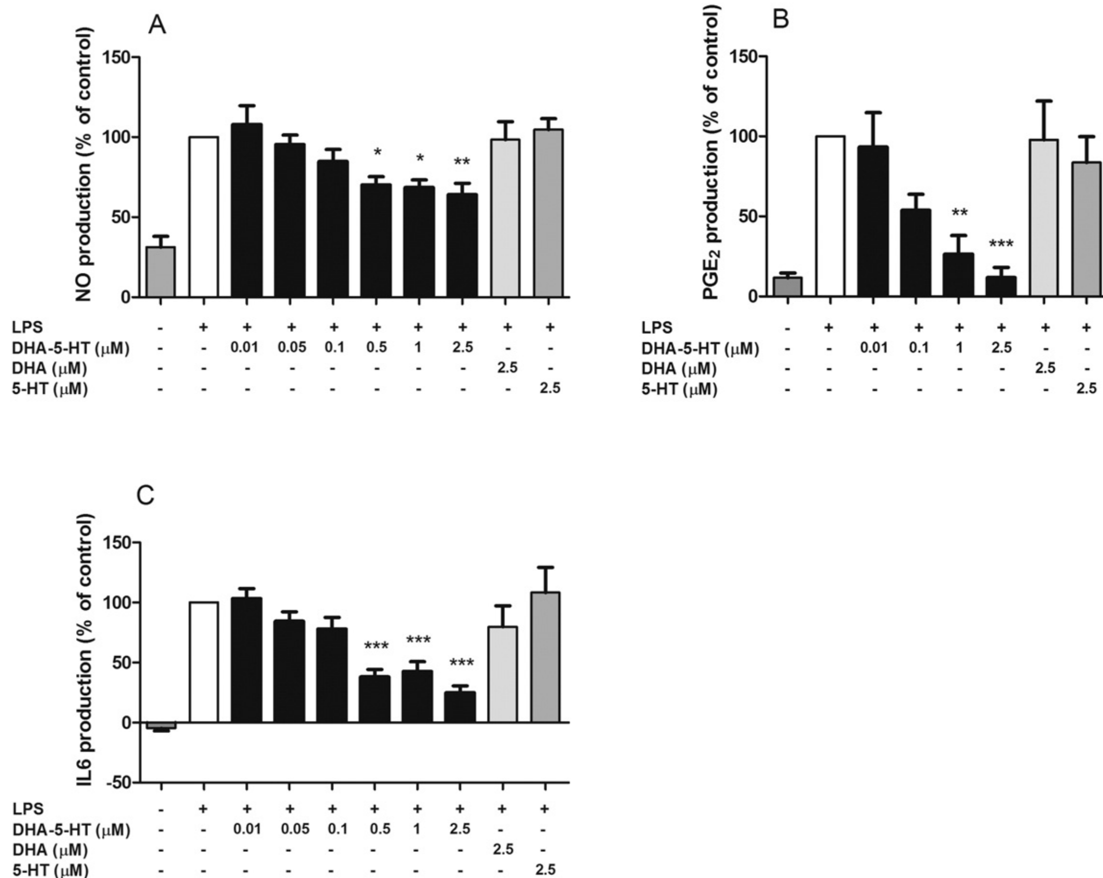

Fig. 2. Inhibitory effects of DHA-5-HT on levels of NO, PGE2 and IL-6. Concentration-response effects of 24 h of incubation with DHA-5-HT (concentration range DHA-5-HT from 0.01 to 2.5 μM); DHA (2.5 μM) and 5-HT (2.5 μM) for NO (A), PGE2 (B) and IL-6 (C) in RAW264.7 cells stimulated with 1 μg/mL LPS (+ LPS). Figures depict means ±SEM (n = 3 independent experiments) presented as percentages of the + LPS control (− LPS; is control without LPS). Levels were as follows: Nitrite (μM) ± SEM - LPS: 3 ± 0.4; + LPS (1 μg/mL): 11 ± 1.2; + LPS (1 μg/mL) with 2.5 μM DHA-5-HT: 7 ± 1.5; PGE2 (pg/ml) ± SEM; − LPS: 177 ± 25; + LPS (1 μg/mL): 1740 ± 317; + LPS (1 μg/mL) with 2.5 μM DHA-5-HT: 161 ± 52; IL-6 (ng/ml) ± SEM; - LPS:0; + LPS (1 μg/mL): 34 ± 4; + LPS (1 μg/mL) with 2.5 μM DHA-5-HT: 8 ± 2.

3.3. DHA-5-HT suppresses the release of the cytokine IL-23 and IL-1β by macrophages

Next, it was assessed whether the downregulated gene expression for IL-12b and IL-23a (together coding for the two subunits, IL-12p40 and IL-23p19, respectively, of the heterodimeric cytokine IL-23) results in suppressed production of IL-23 protein. Fig. 4A shows that the release of IL-23 is concentration-dependently inhibited by DHA-5-HT in LPS-stimulated macrophages. In response to 0.5 μM and 1 μM DHA-5-HT a significant decrease by 58% and 72% was found, respectively. Also, the release of IL-1β was significantly reduced in a concentration-dependent manner by DHA-5-HT (Fig. 4B). Both parent DHA and serotonin did not affect levels of IL-23 and IL-1β in LPS-stimulated macrophages.

3.4. DHA-5-HT inhibits migration and reduces chemokine release and MMP expression in macrophages

From the microarray analyses it was found that DHA-5-HT reduced gene-expression of the metalloproteinases MMP13 (−2.86), MMP8 (−1.78), MMP25 (−1.54) (Table 2A) and the chemokines CCL-22 (−2.20), CCL-7 (−1.63), CCL-6 (−1.34), CCL-17 (−1.22), CXCL11 (−1.80), CXCL3 (−1.78), CXCL10 (−1.61), CXCL2 (−1.27) (Table 2B, C), which are known for their role in migration. Therefore, we tested whether DHA-5-HT could affect the migration of RAW264.7 towards LPS. Fig. 5A shows that the presence of 1 μM DHA-5-HT and 2 μM DHA-5-HT indeed reduced the migration of the macrophages towards 0.1 μg LPS by 52% and 77% respectively. Next, it was assessed whether DHA-5-HT inhibited the release of the chemokines MCP-1 and CCL-20. LPS-induced production of both MCP-1 (Fig. 5B) and CCL-20 (Fig. 5C) was inhibited in a concentration-dependent manner by DHA-5-HT.

3.5. GSEA (gene-set-enrichment-analysis) indicates overlap with gene sets associated with TGF beta signaling, IBD and Nrf2 signaling

Following our observations that in LPS-activated macrophages, DHA-5-HT-induced gene-expression as well as altered protein levels indicated a suppression of the IL-23-mediated pathway, GSEA was performed. With GSEA analysis it can be assessed whether a defined gene set (in this case the gene set that is altered by DHA-5-HT in LPS-stimulated RAW264.7) shows significant overlap with reported gene sets altered in biological processes or disorders. The downregulated gene-set-enrichment-profile of DHA-5-HT showed highest overlap with that of the TGF Beta signaling pathway (−2.24) and Inflammatory bowel disease (−2.22) gene sets, indicating that DHA-5-HT suppresses gene sets that are found to be altered in the TGF beta signaling pathway and altered with IBD. The upregulated gene set-enrichment-profile showed most overlap with the Nrf2 targets (2.92) suggesting that DHA-5-HT upregulates gene-sets that overlap with gene sets altered with Nrf2 targets. The Nrf2 (nuclear factor erythroid-2-related factor 2) pathway is known for its antioxidant and anti-inflammatory activity. Table 3A displays the 10 highest negative overlapping gene sets and Table 3B the 10 highest positive overlapping gene sets. A full list of all gene sets is given in the supplemental data (see Table S6, S7 of SA2 and SA3, respectively).

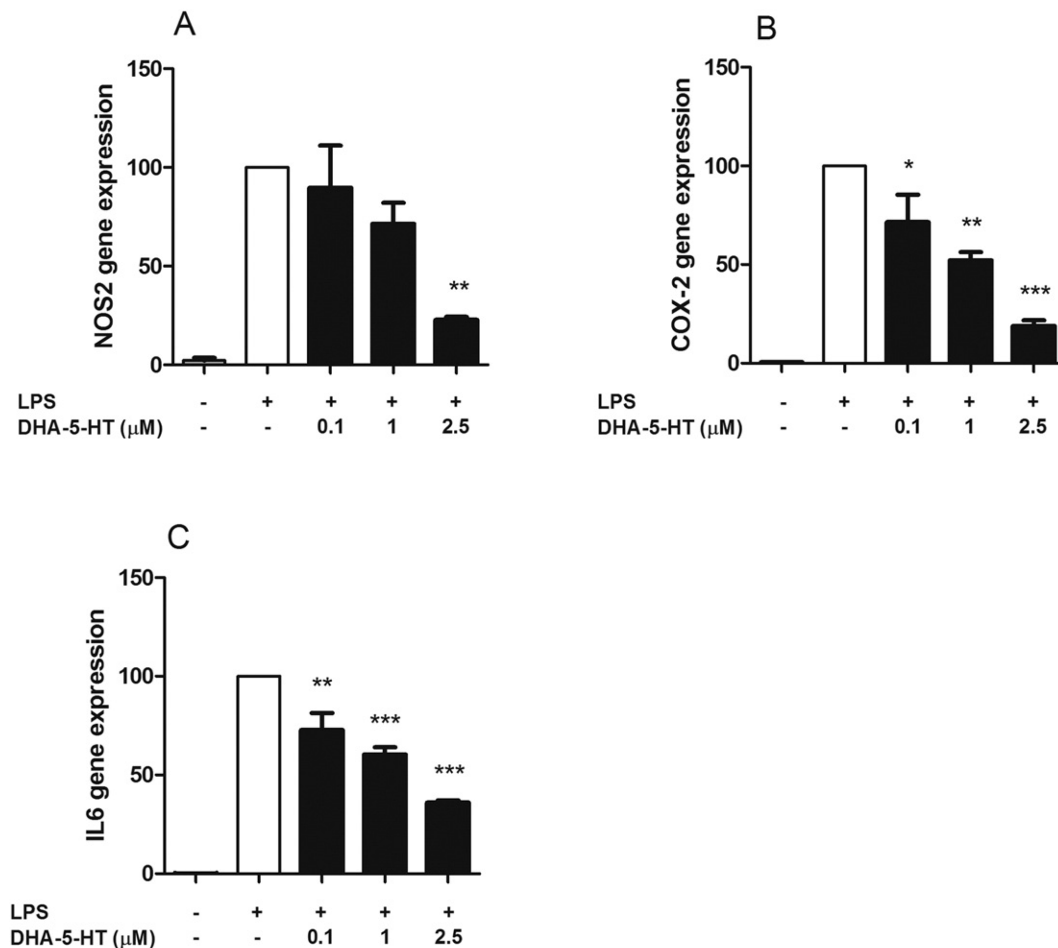

Fig. 3. Inhibitory effects of DHA-5-HT on mRNA expression levels of NOS2, COX-2 and IL-6. Effects of 24 h of incubation with DHA-5-HT (0.1, 1 and 2.5 μM) for NOS2 (A), COX-2 (B) and IL-6 (C) in RAW264.7 cells stimulated with 1 μg/mL LPS (+ LPS). Figures depict means ± SEM (n = 3 independent experiments) presented as percentages of the + LPS control (− LPS; control without LPS).

4. Discussion

In an earlier study we demonstrated for the first time the presence of several acyl serotonins in the gastro-intestinal tract of pigs and mice, in particular in the jejunum and ileum. We found that the pattern of serotonin conjugates formed *in vivo* in mice was shown to be dependent on the fatty acid composition of the diets and results with pig intestinal tissue cultures suggested that their formation is also dependent on the available serotonin [17].

To the best of our knowledge, the only N-acyl serotonin investigated so far in more detail is AA-5-HT. This compound has been identified as a FAAH inhibitor, a TRVP1 ligand, a potential suppressor of allergic response and was shown to have analgesic properties in animal models when injected [21,31–33]. AA-5-HT was also identified in human brain [34] and studies in *Drosophila* showed that one of the biosynthetic routes for AA-5-HT, PA-5-HT and OA-5-HT, involved the enzyme AANATL2 (arylalkylamine *N*-acyltransferase) [35]. More recently, the serotonin conjugate of palmitic acid, PA-5-HT, has been found to ameliorate deficits of memory and learning in mice [36].

The present study has identified DHA-5-HT as a potential novel anti-inflammatory member of the DHA-derived subclass of fatty acid amides. Our data revealed that DHA-5-HT predominantly suppressed the release and gene expression of prominent key players of the

Table 1A
Gene expression of interleukins changed after 24 h DHA-5-HT treatment in LPS-stimulated RAW264.7.

Gene name	Description	Gene array fold change	*P*-value
IL-12b	Interleukin 12b	−3.52	2.57E-10
IL-23a	Interleukin 23, alpha subunit p19	−2.27	1.37E-09
IL-1a	Interleukin 1 alpha	−1.89	0.000119
IL-27	Interleukin 27	−1.83	0.000123
IL-1f6	Interleukin 1 family, member 6	−1.67	0.000146
IL-6	Interleukin 6	−1.58	7.68E-06
IL-1β	Interleukin 1 beta	−1.41	0.003204

Table 1B
Gene expression of co-stimulatory molecules changed after 24 h DHA-5-HT treatment in LPS-stimulated RAW264.7.

Gene name	Description	Gene array fold change	*P*-value
Cd300e	CD300e antigen	−2.18	7.76E-06
Cd80	CD80 antigen	−1.57	1.99E-06
Cd300lf	CD300 antigen like family member F	−1.50	0.000137
Cd69	CD69 antigen	−1.43	0.000799
Cd274	CD274 antigen	−1.30	0.000173
Cd40	CD40 antigen	−1.24	0.000798
Cd86	CD86 antigen	−1.23	9.74E-05
Cd28	CD28 antigen	1.50	0.000965
Cd1d1	CD1d1 antigen	1.74	0.000219

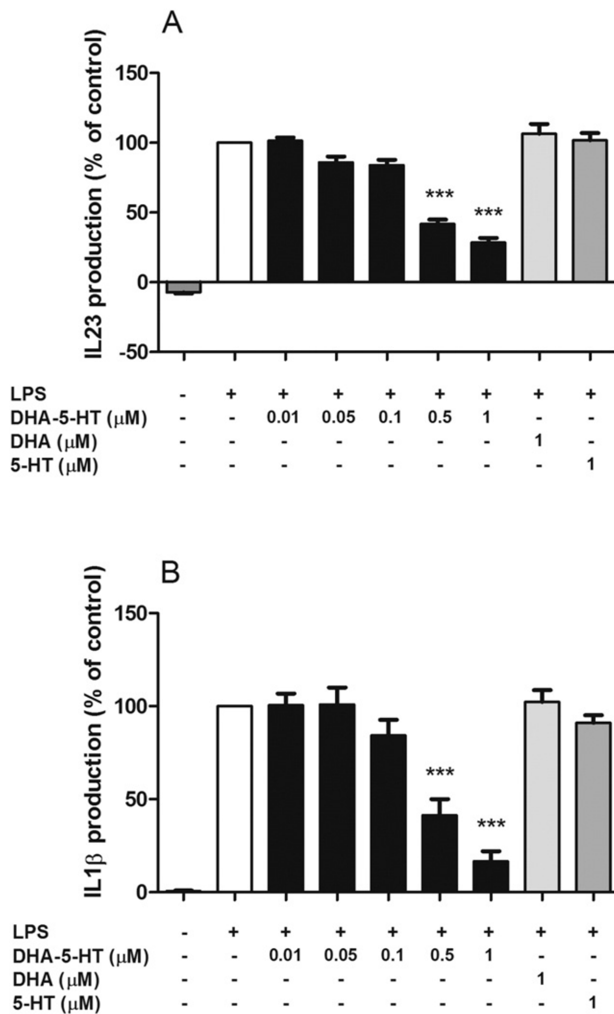

Fig. 4. Inhibitory effects of DHA-5-HT on levels of IL-23 and IL-1β produced by LPS-stimulated RAW264.7 macrophages. Concentration-response effects of 24 h treatment with DHA-5-HT (range from 0.01 to 1 μM); DHA (1 μM) or 5-HT (1 μM)) for IL-23 (A) and IL-1β (B) in RAW264.7 macrophages stimulated with 1 μg/mL LPS (+ LPS). Figures depict means ± SEM (n = 3 independent experiments) presented as percentages of the + LPS control (− LPS; is control without LPS). Levels were as follows: IL-23 (pg/mL) + SEM; − LPS:0; + LPS (1 μg/mL): 671 ± 139; + LPS (1 μg/mL) with 1 μM DHA-5-HT: 181 ± 20; IL-1β (pg/ml) + SEM; − LPS: 0.7 ± 0.4; + LPS (1 μg/mL): 88 ± 13; + LPS (1 μg/mL) with 1 μM DHA-5-HT: 14 ± 4.

macrophages' innate immune response that establish and influence the adaptive CD4 + Th17 response. A broad analysis of the expression of immune-related genes in the microarray data revealed IL-12b and IL-23a to be the strongest downregulated immune-related genes when LPS-stimulated macrophages were exposed to DHA-5-HT. IL-12b and IL-23a encode for the IL-12p40 and IL-23 alpha subunit p19, respectively, which together form the heterodimeric cytokine IL-23 [37,38]. IL-23 produced by macrophages is able to stimulate a subset of CD4 + T cells that produce IL-17, the so-called Th17 cells [39]. These cells are

Table 2A
Gene expression of matrix metallopeptidases changed by 24 h DHA-5-HT exposure in LPS-stimulated RAW264.7.

Gene name	Description	Gene array fold change	*P*-value
Mmp13	Matrix metallopeptidase 13	−2.86	3.5E-08
Mmp8	Matrix metallopeptidase 8	−1.78	0.000308
Mmp25	Matrix metallopeptidase 25	−1.54	5.17E-05

currently the focus of many studies as it has been established that Th17 cells play a dominant deteriorating role in many autoimmune inflammatory disorders, like psoriasis, inflammatory bowel disease, multiples sclerosis and others [40–42]. The importance of the IL-23-IL-17 signaling axis in autoimmune diseases is also evident from the many scientific studies and ongoing clinical trials that specifically target IL-23 or IL-17 [41]. The data presented here showed that DHA-5-HT suppressed the release of IL-23. Also, expression of TGF-β3 as well as production levels of IL-6, PGE2 and IL-1β were found to be reduced by DHA-5-HT. Interestingly, those latter inflammatory mediators have all been shown to drive the development of Th17 cells in a concerted way. While IL-6 and TGF-β3 seem to be involved in the development of pathogenic Th17 cells [43–45], IL-1β and IL-23 have been reported to synergistically enhance establishment and expansion of these cells [39,41,46,47]. PGE2 can induce the expression of IL-23 [48–50] and PGE analogs have been reported to exacerbate IBD symptoms, increase DC-derived IL-23 levels and support the Th17 phenotype in a mice model of colitis [51].

Additionally, gene-expression of several members of the interleukin (IL)-1 cytokine family [52], namely IL-1α, IL-1β and IL-1f6 (also known as IL-36α) were found to be inhibited (Table 1A). Interestingly, IL-36α (a recently discovered relatively new pro-inflammatory member of the (IL)-1 cytokine family) was reported to induce NOS2, COX-2 and MMP13 expression in human osteoarthritic chondrocytes [53]. Also, the co-stimulatory molecules CD80, CD69, CD40 and CD86, known to be present on antigen-expressing immune cells, like macrophages and DC, and engaged in the establishment of T cell sensitization [54,55] were downregulated.

Interestingly, also in the etiology of cancer the importance of the IL-23-IL-17 axis is becoming more and more evident. For example, it has been suggested to be a feature of tumor cells [38] to predominantly induce the IL-23-IL-17 signaling axis upon inflammation. IL-23 produced by tumor cells favor their growth by enhancing migration and angiogenesis. Interestingly, in our study, LPS-induced migration of the RAW264.7 cell was found to be inhibited by DHA-5-HT, as well as MCP-1 and CCL-20 chemokine production. Also, IL-23 has been mentioned to promote tumor growth [56], and IL-23 produced by innate lymphoid cells has been shown to induce *de novo* gut tumorigenesis in a transgenic mouse model [57]. Moreover, the IL-1 β/IL-6 axis and IL-17A have been reported to be involved in colitis associated-tumorigenesis [58,59].

Emerging evidence from both humans as well as animal studies indicates a beneficial role for n-3 PUFAs in relation to intestinal disorders like IBD [16,45,60–62] and cancer-associated colitis and have been linked to prevention of colorectal cancer [63,64]. Several underlying mechanisms and mediators, among which COX-2 and IL-17 [65–67], have been proposed to be involved in these effects. Several lines of evidence, in particularly those from experimental studies suggest these effects to be partly mediated by metabolites of n-3 PUFAs [16]. These include for instance the resolvins and maresins [68,69]. DHA has also been reported to exhibit anti-inflammatory effects *via* the Nrf2-antioxidant response element signaling pathway [70]. Remarkably, this cellular defense pathway against oxidative or electrophilic stress also came out of our GSEA as highest positive overlapping gene set. Interestingly, effective concentrations for DHA were approximately 50–100 times [70] higher compared to the effective concentrations we found for its metabolite DHA-5-HT. Next to stimulation of anti-oxidant and xenobiotic metabolizing enzymes the Nrf2 pathway is also thought to be involved in the suppression of pro-inflammatory cytokine production, and Nrf2-deficient mice with increased colonic tissue inflammation have been shown to be more susceptible to DSS-induced colitis [71]. Moreover, some other cannabinoid compounds have been shown to induce the Nrf2 pathway as well [72,73].

Currently, the number of fatty acid amides belonging to the long chain-fatty acid-derived subclass of fatty acid amides shown to possess immune-modulatory properties is growing [4]. Although the specific

Table 2B
Gene expression of CC chemokines changed by 24 h DHA-5-HT exposure in LPS-stimulated RAW264.7.

Gene name	Description	Gene array fold change	*P*-value
Ccl22	Chemokine (C-C motif) Ligand 22	−2.20	0.000143
Ccl7	Chemokine (C-C motif) Ligand 7	−1.63	0.00052
Ccl6	Chemokine (C-C motif) Ligand 6	−1.34	0.00096
Ccl17	Chemokine (C-C motif) Ligand 17	−1.22	0.047008

biological role of the best studied member of this subclass, DHEA, is still far from clear, recent *in vivo* studies suggest that it has, besides others [74–76], analgesic properties [77,78]. Decreases in physical pain were found to be associated with increased plasma levels of DHEA after modifying n-6/n-3 intake levels in humans [77] and in rats the DHEA precursor glycerophospho-containing DHEA was correlated with antihyperalgesic phenotypes [78]. Furthermore, DHEA, as well as a number of other DHA-derived conjugates, were identified as agonist or antagonist of the TRPV1-4 receptor family members in BV2 microglia cells [14]. In macrophages, DHEA has been shown to modulate inflammation by affecting the activity of COX-2 [6]. However, while DHEA modulates activity of COX-2 without affecting its gene-expression, DHA-5-HT altered COX-2 gene expression and displayed higher potencies in suppressing inflammatory mediators compared to DHEA. Likely, DHA-5-HT exerts its effects upstream of COX-2, possibly *via* the Nrf2 pathway. Furthermore, as both parent compounds DHA and serotonin did not display immune-modulatory effects at the protein level and at the gene-expression level at nanomolar and low micromolar concentrations, DHA-5-HT might have a specific biological role.

At this stage it is difficult to oversee the full *in vivo* relevance of particularly endogenously present DHA-5-HT. We determined DHA-5-HT in homogenates from mice intestinal tissue [17]. Levels were found to be around 0.02 ng/g tissue in the jejunum, which seems considerably lower than the concentrations used in the present study. However, as a general principle, caution is required when making such an *in vitro-in vivo* comparison as local and cellular concentrations can be very different. Additionally, release might increase during inflammatory conditions as has been shown for other acyl ethanolamines [13]. Given the potent anti-inflammatory properties of DHA-5-HT compared to its structural analogues it is tempting to speculate that this compound plays a prominent role as signaling lipid *in vivo*. However, future *in vivo* studies have to further reveal whether this compound is more of physiological or of pharmacological relevance.

Concluding, our group has previously identified DHA-5-HT as an endogenous compound appearing to be primarily formed in the intestine under the influence of dietary DHA, as for example present in fatty fish and fish oils. The data reported here revealed that DHA-5-HT suppressed the LPS-induced IL-23 signaling cascade in macrophages. By using microarray studies it was assessed that most immune related genes suppressed by DHA-5-HT are involved in the innate signaling

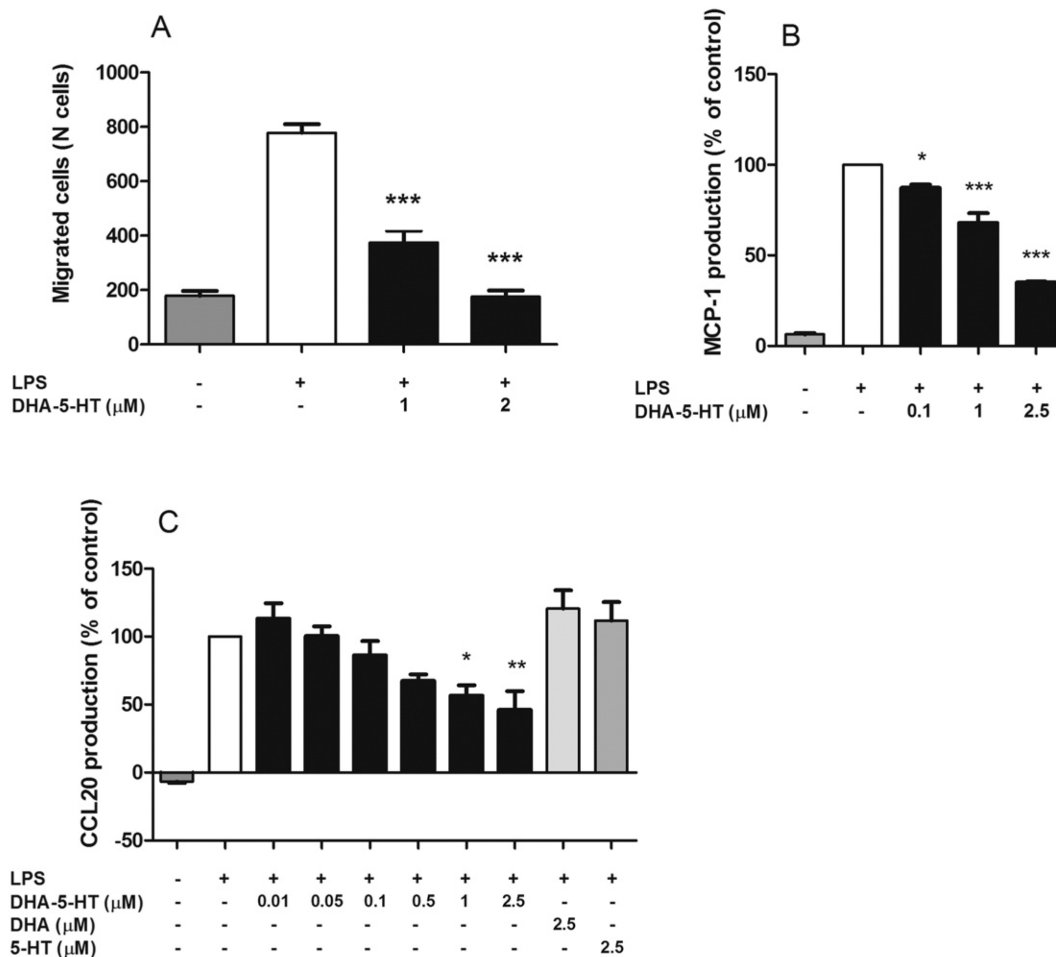

Fig. 5. Inhibitory effects of DHA-5-HT on migration of- and on chemokine levels (MCP-1 and CCL-20) produced by RAW264.7 macrophages. DHA-5-HT (1 μM, 2 μM) reduced migration of RAW264.7 after 4 h towards 0.1 μg/mL LPS in a transwell assay (A). Concentration-response effects of 24 h of incubation with DHA-5-HT (concentration range of 0.1, 1 and 2.5 μM) on MCP-1 (B) and DHA-5-HT (range from 0.01 to 2.5 μM); DHA (2.5 μM) and 5-HT (2.5 μM) on CCL-20 (C) in RAW264.7 stimulated with 1 μg/mL LPS (+ LPS). Figures depict means ± SEM (n = 3 independent experiments) presented as percentages of the + LPS control (− LPS; control without LPS). Levels were as follows: CCL-20 (pg/mL) + SEM; − LPS:0; + LPS (1 μg/mL): 265 ± 51; + LPS (1 μg/mL) with 2.5 μM DHA-5-HT: 135 ± 60; MCP-1 (ng/mL) + SEM; − LPS: 5 ± 0.5; + LPS (1 μg/mL): 75 ± 7; + LPS (1 μg/mL) with 2.5 μM DHA-5-HT: 2 ± 0.1.

Table 2C
Gene expression of CXC chemokines changed by 24 h DHA-5-HT exposure in LPS-stimulated RAW264.7.

Gene name	Description	Gene array fold change	*P*-value
Cxcl11	Chemokine (C-X-C motif) Ligand 11	−1.80	0.000626
Cxcl3	Chemokine (C-X-C motif) Ligand 3	−1.78	0.000335
Cxcl10	Chemokine (C-X-C motif) Ligand 10	−1.61	4.74E-04
Cxcl2	Chemokine (C-X-C motif) Ligand 2	−1.27	1.64E-02

driving the IL-23-IL-17 axis. Hence, also production of IL-6, PGE2, IL-1β and IL-23, all pivotal mediators known to trigger development and maintenance of pathogenic IL-17 cells in a concerted way, were decreased. These findings suggest that DHA-5-HT might play a role in intestinal immune regulation and that its administration (or potentially that of its precursors) might deliver options for treatment in intestinal disorders. Further studies are warranted to elucidate the specificity of DHA-5-HT mediated effects and to translate this to *in vivo* effects.

Transparency document

The Transparency document associated with this article can be found, in online version.

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The authors declare that there is no conflict of interest.

Table 3A
Gene-set-enrichment analysis showing negative gene sets with highest overlap for gene sets changed by DHA-5-HT.

Pathway	Source	NES	FDR q-val
TGF beta signaling pathway	Wikipathways 113	−2.24	0.000
Inflammatory bowel disease IBD	KEGG	−2.22	0.000
Type II interferon signaling IFNG	Wikipathways 1253	−2.22	0.000
Cytokine Cytokine receptor interaction	KEGG	−2.15	0.002
IL1R Pathway	Biocarta	−2.11	0.004
Signal transduction by L1	Gene ontology	−2.11	0.003
Leishmaniasis	KEGG	−2.08	0.005
Inflampathway	Biocarta	−2.07	0.005
Pertussis	KEGG	−2.05	0.005
Cytokines and inflammatory response	Wikipathways 222	−2.04	0.005
CTLA4 inhibitory signaling	Gene ontology	−2.04	0.005

Table 3B
Gene-set-enrichment analysis showing positive gene sets with highest overlap for gene sets changed by DHA-5-HT.

Pathway	Source	NES	FDR q-val
NRF2 targets	Gene ontology	2.92	0.000
Activation of the pre replicative complex	Gene ontology	2.66	0.000
G1 to S cell cycle control	Wikipathways 413	2.55	0.000
DNA replication pre initiation	Gene ontology	2.55	0.000
M G1 transition	Gene ontology	2.53	0.000
DNA replication	Wikipathways 150	2.53	0.000
Meitotic G1 G1 S phases	Gene ontology	2.50	0.000
G1 S transition	Gene ontology	2.49	0.000
Activation of ATR in response to replication stress	Gene ontology	2.48	0.000
S phase	Gene ontology	2.41	0.000

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbalip.2016.09.012.

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