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Docosahexaenoic acid downregulates interferon gamma-induced expression of CXCL16 in human aortic smooth muscle cells

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ABSTRACT

CXCL16 is a chemokine that is expressed in both transmembrane and secreted isoforms. Both variants have been implicated in atherosclerosis. Increased CXCL16 expression on the surface of human aortic smooth muscle cells induced by interferon gamma (IFNγ) signaling results in enhanced oxidized low density lipoprotein uptake and enhanced recruitment of pro-inflammatory cells. Docosahexaenoic acid (DHA), an omega-3 fatty acid, is known to inhibit IFNγ signaling in inflammatory cells. Therefore, we have investigated the effects of DHA treatment on the ability of IFNγ to induce CXCL16 expression in human aortic smooth muscle cells. We observed that DHA treatment significantly reduced IFNγ-induced CXCL16 expression. As a result, the pro-atherosclerotic functions of CXCL16 were also inhibited. Furthermore, IFNγ-induced STAT1 phosphorylation was inhibited by DHA, suggesting a potential mechanism. In conclusion, our data suggest inhibition of IFNγ signaling as one of the mechanisms behind the beneficial effects of DHA during atherosclerosis. These findings may prove to be important in other disease fields that identify IFNγ as a regulator.

Introduction

Chemokines are small proteins that mediate numerous functions including growth stimulation and cell migration [1]. Chemokines direct cell migration through gradient and signaling based mechanisms. While almost all chemokines are secreted from cells, CXCL16 and CX_3CL1 may be alternatively found as multidomain transmembrane proteins [2,3]. Membrane bound CXCL16 is cleaved by the disintegrin-like metalloproteinase ADAM-10 to become the secreted form [4,5]. CXCL16 in either form is a ligand for CXCR6, which was initially discovered as an human immunodeficiency virus co-receptor [2,6]. CXCL16 has also been heavily implicated in atherosclerosis [7]. CXCL16 was first identified as a scavenger receptor for oxidized low density lipoprotein (oxLDL) [8]. Over-expression of CXCL16 on the surfaces of macrophages and human aortic smooth muscle cells (HASMCs) results in increased uptake of oxLDL [9,10]. This can lead to HASMC apoptosis and damage to the aortic wall [11]. Damage to the aorta results in recruitment of pro-inflammatory cells, including macrophages and leukocytes. Transmembrane CXCL16 also functions as an adhesion molecule for $CXCR6^+$ leukocytes.

Over-expression of CXCL16 also results in an increase of the secreted variant. Secreted CXCL16 recruits $CXCR6^+$ inflammatory cells, including different subsets of T lymphocytes [12] and dendritic cells [13]. Infiltrating T lymphocytes secrete IFNγ into the region [14]. While increases in other oxLDL scavenger receptors, such as CD36, LOX-1, and SR-A, were reported, the increase in CXCL16 expression was the most significant [9]. As a result, IFNγ has been implicated as one of the key regulators of atherosclerosis [15]. For these reasons, our study focuses on CXCL16 expression induced by IFNγ.

The omega-3 polyunsaturated fatty acid docosahexaenoic acid (DHA), abundant in fish oils, has been linked to beneficial cardiovascular effects [16]. The observation that Greenland Eskimos have a relatively low incidence of chronic heart disease despite a high saturated fat intake has been a basis for the scientific speculation into the benefits of DHA [17]. Epidemiological [18] and randomized clinical trials [19] have reported significant decreases in morbidity and mortality from heart disease in subjects with diets supplemented with DHA. Incorporation of DHA into the cellular membrane disrupts signaling of IFNγ [20]. Therefore, we hypothesized that DHA inhibits the upregulation of CXCL16 brought about by IFNγ which would result in a decreased atherosclerotic phenotype.

Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; AA, arachidonic acid; IFNγ, interferon gamma; DiI, 3,3′-dioctadecylindocarbocyanine; oxLDL, oxidized low density lipoprotein; HASMCs, human aortic smooth muscle cells.

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Through *in vitro* experiments, we concluded that DHA downregulates IFNγ-induced expression of both the transmembrane and secreted forms of CXCL16 in HASMCs. Decreased expression of transmembrane CXCL16 resulted in decreased oxLDL uptake. Furthermore, inhibition of IFNγ-induced CXCL16 secretion leads to decreased migration of $CXCR6^+$ cells. In conclusion, our data suggests that incorporation of the omega-3 fatty acid DHA inhibits IFNγ signaling and is a potential mechanism behind the beneficial effects of DHA in atherosclerosis.

Materials and methods

Cells and reagents. HASMCs were purchased from American Tissue Culture Collection (ATCC, Rockville, MD) and maintained in Ham's F-12 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin, and 30 μg/ml endothelial cell growth supplement (Millipore). THP1 cells were maintained in RPMI-1640 (Invitrogen) supplemented with 10% FBS and penicillin/streptomycin. 3,3′-Dioctadecylindocarbocyanine–labeled oxLDL (DiI–oxLDL) was purchased from Biomedical Technologies. Fatty acids were purchased from Nuchek Prep and complexed with fatty acid free-bovine serum albumin (FAF-BSA) as previously described [21]. IFNγ was purchased from R&D systems.

Flow cytometry. For transmembrane CXCL16 expression, cell monolayers were treated in serum-free Ham's F-12 medium with the fatty acids for 24 h at 37 °C and 5% CO_2. After washing the cells, IFNγ (10 ng/ml) or the vehicle control were added in Ham's F-12 medium supplemented with 0.5% FAF-BSA at 37 °C for 48 h. Following the incubations, the cells were washed with cold phosphate buffered saline (PBS), cold PBS containing 0.1% BSA, and cold PBS containing 1.0% BSA consecutively. The cells were then stained with a goat polyclonal antibody to CXCL16 (R&D Systems) for 1 h. The cells were washed and stained with the secondary anti-goat antibody conjugated to FITC (BD Biosciences). The cells were washed, suspended in PBS, and analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with an air-cooled argon laser emitting at a 488 nm wavelength. Fluorescence was detected through a 575 ± 26 band pass filter and quantified using CellQuest Software (Becton Dickinson, San Jose, CA). Results are expressed as the percent increase in mean fluorescence intensity compared to the normal goat IgG isotype control.

For oxLDL uptake, the cells were treated with DHA and IFNγ in the same manner as for surface CXCL16 expression. Following the incubation period, the cells were treated with 10 μg/ml DiI–oxLDL in Ham's F-12 supplemented with 0.5% FAF-BSA for 4 h at 37 °C. The cells were then trypsinized and washed repeatedly to remove excess DiI–oxLDL. Analysis by flow cytometry was performed as described above for surface CXCL16 expression.

Secreted CXCL16 expression. Secreted CXCL16 expression was analyzed by sandwich ELSIA. Confluent HASMCs in 24 well plates were treated with fatty acids in the same manner as for surface CXCL16 expression and oxLDL uptake experiments. The cells were then washed and treated with 300 μl per well IFNγ (10 ng/ml) or the vehicle control in Ham's F-12 medium supplemented with 0.5% FAF-BSA for 72 h at 37 °C. The supernatants were harvested, clarified by centrifugation, and loaded at volumes of 100 μl in 96 well plates that were coated with goat polyclonal antibodies to CXCL16 (0.4 μg/ml). The supernatants were incubated for 4 h at room temperature. The wells were then washed three times and treated with biotinylated antibody to human CXCL16 (100 ng/ml; R&D Systems) for 1 h. The wells were washed three times and treated with streptavidin-HRP (R&D Systems) for 30 min. The cells were washed three times and treated with the color substrate mixture (R&D Systems) for 30 min. OD_{600} readings were taken, and CXCL16 concentration was determined through standard concentration curves generated with recombinant human CXCL16 (R&D Systems).

Cell migration assays. HASMCs were treated with fatty acids and IFNγ as described for the ELISAs; however, the IFNγ (10 ng/ml) and vehicle control was applied at volumes of 700 μl per well. Following the 72 h incubation, the supernatants were harvested and clarified by centrifugation. $CXCR6^+$ THP1 cells (2×10^6/ml) were placed in 5 μm Transwell chambers (Costar) at volumes of 100 μl. The inserts were placed into wells containing 600 μl of each supernatant or controls. The transwell plates were incubated for 2 h at 37 °C. Migration was halted by removing the transwell inserts. Migrated THP1 cells in the bottom chambers were counted by flow cytometry. Chemotactic index was determined by dividing the number of migrated cells in each condition by the number of cells that spontaneously migrated when fresh medium was added to the lower chamber.

Intracellular flow cytometry. HASMCs were treated for 24 h with DHA or the mock control in serum-free F-12 medium at the indicated concentrations. The cells were washed and treated with IFNγ (10 ng/ml) for escalating time periods. At each time point, the wells were aspirated, and the cells were fixed for 5 min at 4 °C with cold 3% paraformaldehyde. The cells were permeabilized with cold 90% methanol at 4 °C for 30 min. Intracellular stainings were performed with a mouse monoclonal antibody to phosphorylated signaling transduction and transactivation 1 (STAT1) protein (Santa Cruz) and followed by secondary treatment with a FITC-conjugated anti-mouse antibody. The cells were then washed and analyzed by flow cytometry. Results were quantified as the percent increase in mean fluorescence intensity, compared to a control staining with no primary antibody to phosphorylated STAT1.

Statistics. All experiments were performed at least three times each in triplicate and expressed as mean ± SE. Comparisons were done using Student's *t*-test. Significance was defined as $p < 0.05$.

Results

DHA downregulates IFNγ-induced CXCL16 surface expression

Increased surface CXCL16 expression is one of the major factors in atherosclerosis and is regulated by IFNγ [10]. We observed that treatment of HASMCs with 25 and 50 μM DHA significantly reduced the IFNγ-induced CXCL16 surface expression as measured by flow cytometry (Fig. 1A). Interestingly, the baseline levels of CXCL16 were unaffected by the DHA treatment (Fig. 1B), suggesting that the DHA specifically inhibited the IFNγ-induced CXCL16 expression rather than decreasing overall CXCL16 expression. DHA did not appear to be toxic to the cells, and this was confirmed with WST-1 proliferation assays (data not shown). As previously reported [20], other polyunsaturated fatty acids, such as eicosapentaenoic acid and arachidonic acid did not inhibit IFNγ-induced CXCL16 expression, suggesting that the effect is specific for DHA (Fig. 1C). In contrast, treatment of the HASMCs with arachidonic acid enhanced surface CXCL16 expression in both the vehicle and IFNγ treated cells. The biological significance of this observation will require further investigation.

DHA inhibits IFNγ-induced oxLDL uptake

A major functional consequence of transmembrane CXCL16 over-expression is increased oxLDL uptake [9]. This results in facilitation of apoptosis and the release of pro-inflammatory factors. We analyzed the effects of DHA on oxLDL uptake in HASMCs treated with IFNγ by incubating the treated cells with fluorescent DiI–oxLDL. We observed that the IFNγ-stimulated cells treated

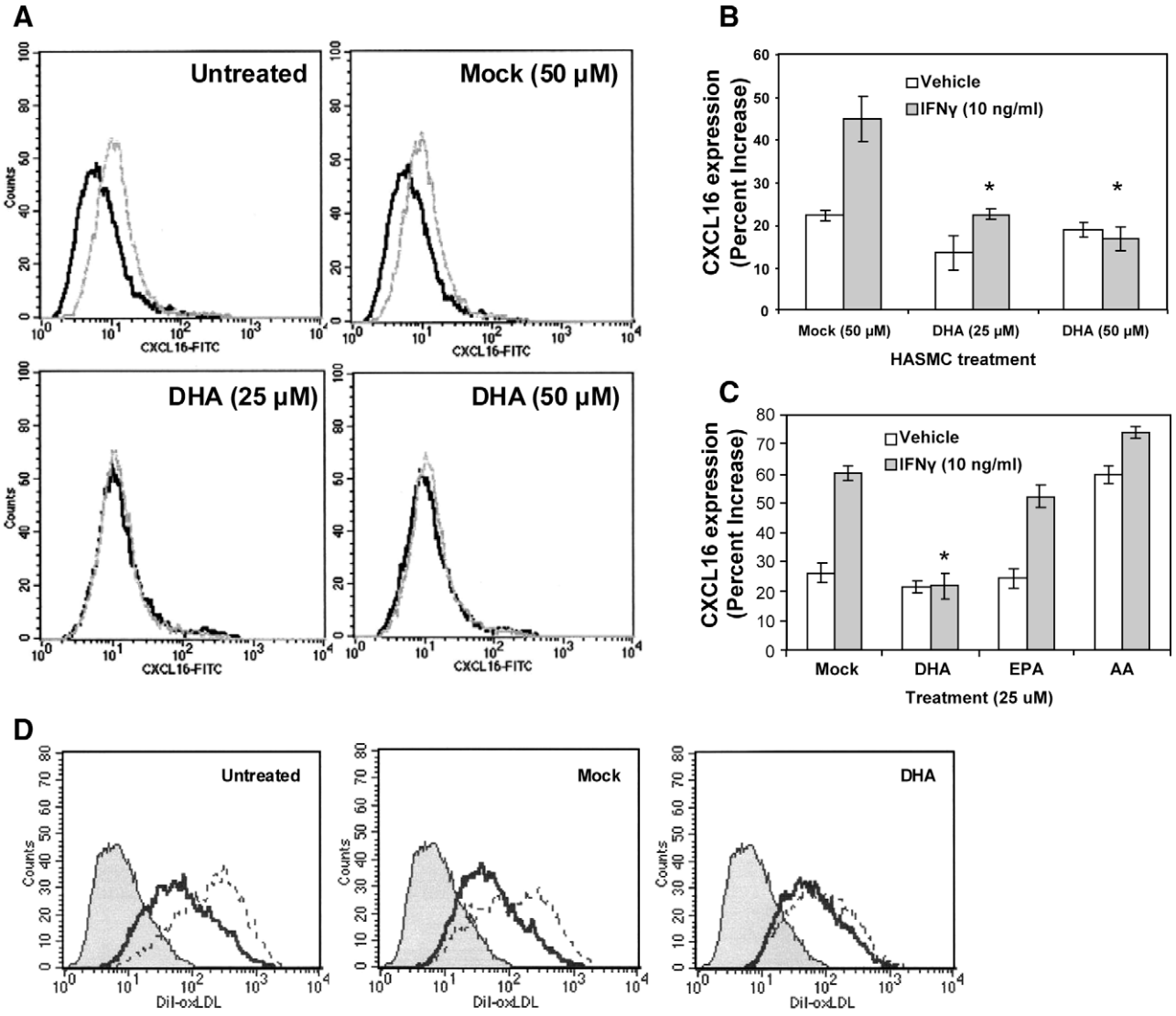

Fig. 1. Effects of DHA on interferon gamma-induced CXCL16 surface expression. (A) HASMCs were treated for 24 h with DHA and mock control as indicated. The cells were then washed and treated with either the vehicle control (black line) or 10 ng/ml IFNγ (grey line) for 48 h. The cells were harvested and stained with a polyclonal antibody to CXCL16. (B) CXCL16 expression was quantified as the percent mean fluorescence intensity increase. (C) Cells were treated with eicosapentaenoic acid (EPA) and arachidonic acid (AA) along side the mock control and DHA at concentrations of 25 μM prior to IFNγ stimulation. (D) Effects of DHA on oxidized low density lipoprotein uptake. HASMCs were treated for 24 h with 25 μM DHA or the mock control. Following treatment, the cells were washed and treated with either the vehicle control (solid line) or 10 ng/ml IFNγ for 48 h. The cells were then incubated for 4 h in the presence (solid and dashed lines) or absence (filled histogram) of 10 μg/ml DiI–oxLDL. Following incubation, the cells were washed, harvested, and analyzed by flow cytometry. Results are representative of three separate experiments. $^{*}p < 0.05$, compared to the mock control under the same conditions.

with 25 μM DHA displayed reduced oxLDL uptake, compared to those treated with the mock control (Fig. 1D). As with the CXCL16 expression, the oxLDL uptake of vehicle-stimulated cells was unaffected by DHA treatment (Fig. 1D). This observation suggests that the effect of DHA was specific for IFNγ-stimulated cells. Furthermore, we also observed that increased DiI–oxLDL uptake upon IFNγ stimulation was completely blocked by an antibody to CXCL16 (data not shown). This confirmed previous reports [10] and suggests that while other oxLDL scavenger receptors may contribute to oxLDL uptake, IFNγ-regulated CXCL16 is the primary scavenger receptor that is involved in oxLDL uptake.

DHA inhibits IFNγ-induced CXCL16 secretion

CXCL16 promotes atherosclerosis through an alternate mechanism. While most chemokines are strictly secreted from the expressing cells, CXCL16 may be found in both a membrane bound and a secreted form [2]. Secreted CXCL16 recruits CXCR6^{+}, pro-inflammatory cells that promote formation of atherosclerotic plaques. By using ELISA, we observed that treatment of HASMCs with DHA resulted in a dose dependent decrease of IFNγ-stimulated CXCL16 secretion by approximately 50–60% (Fig. 2). However, the baseline levels of secreted CXCL16 were unaffected.

DHA decreases the IFNγ-induced chemotaxis of CXCR6^{+} cells

Recruitment of pro-inflammatory cells is a mechanism behind the pro-atherosclerotic effects of secreted CXCL16 [2,9,13]. We examined the effect of DHA treatment on the chemotaxis function of CXCL16 secreted from HASMCs using the THP1 monocytic cell lines that express significant levels of CXCR6 (Fig. 3A), a ligand for CXCL16. Through the use of a neutralizing antibody to CXCL16, we confirmed previous reports that migration of CXCR6^{+} cells was regulated by a significant increase in CXCL16 secretion (Fig. 3B)

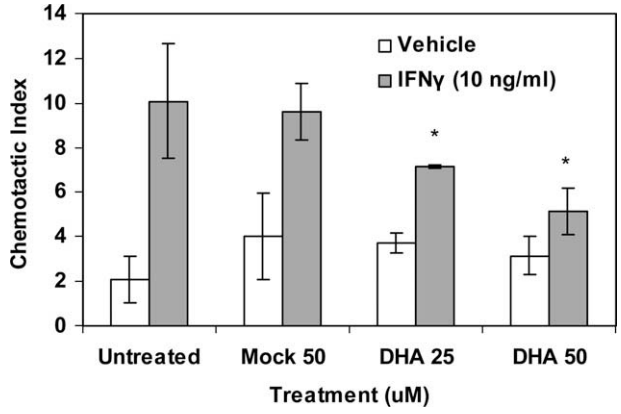

Fig. 2. Effects of DHA on interferon gamma-induced secretion of CXCL16. HASMCs were treated for 24 h with DHA (25 or 50 μM) or the mock control. Following treatment, the cells were washed and incubated for 72 h with IFNγ (10 ng/ml) or the vehicle control. The resulting supernatants were used to determine the CXCL16 concentration as described in "Materials and methods". Results are reported as the mean CXCL16 concentration (pg/ml) ± SD of three assays performed in triplicate. $^{*}p < 0.005$, compared to the mock control.

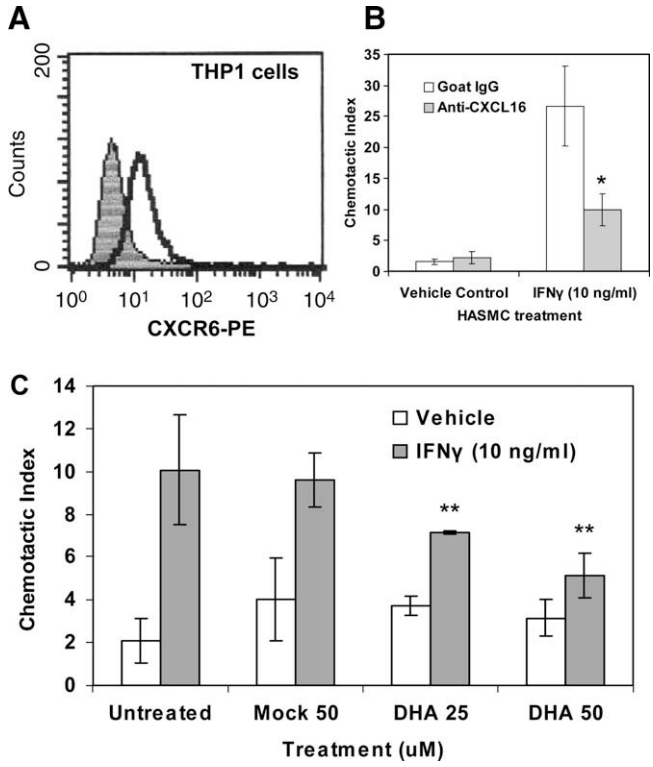

Fig. 3. Effects of DHA on migration of THP1 cells to the supernatant of HASMCs. (A) CXCR6 expression on THP1 cells (grey, isotype; black line, CXCR6) as analyzed by flow cytometry. (B) THP1 migration in response to supernatants from IFNγ-stimulated HASMCs. Supernatants were treated with CXCL16 or isotype antibodies to determine migration resulting from CXCL16 secretion. (C) THP1 migration in response to supernatants from DHA-treated HASMCs subsequently stimulated with IFNγ. Chemotactic index was determined by dividing the number of migrated cells by the average number of cells that spontaneously migrated when fresh chemotaxis medium was added to the lower wells. Error bars represent the standard deviation of three independent assays performed in triplicate. $^{*}p < 0.05$, compared to the normal goat IgG treated sample. $^{**}p < 0.05$, compared to the mock control under the same conditions.

[22]. HASMCs were then treated with DHA or the mock control prior to IFNγ stimulation (Fig. 3C). We observed that treatment of the HASMCs with DHA significantly decreased by approximately 50% the levels of THP1 migration toward the supernatants of IFNγ treated smooth muscle cells. Migration of THP1 cells to supernatants of unstimulated HASMCs was unaffected by DHA.

Docosahexaenoic acid decreases phosphorylation of STAT1

Phosphorylation of the signaling transduction and transactivation 1 (STAT1) protein is a critical step in the IFNγ cascade. We performed intracellular flow cytometry experiments to investigate the effects of DHA on STAT1 phosphorylation. We observed that stimulation of HASMCs with IFNγ for 1 h significantly increased STAT1 phosphorylation (Fig. 4A). Treating the HASMCs for 24 h with DHA resulted in a dose dependent decrease in STAT1 phosphorylation (Fig. 4B). Overall STAT1 expression was unaffected by the DHA treatment (data not shown).

Discussion

IFNγ signaling stimulates expression of CXCL16, a scavenger receptor for oxLDL as well as a molecule that stimulates cell migration [9,10]. Over-expression of IFNγ leads to heightened expression of both CXCL16 variants in HASMCs and therefore IFNγ is regarded as one of the key regulators of atherosclerosis [15]. Increased expression of CXCL16 leads to increased uptake of oxLDL and increased recruitment of pro-inflammatory cells, such as macrophages and neutrophils. It is interesting to note that complete elimination of CXCL16 through murine gene targeting resulted in enhanced pro-atherosclerotic conditions, suggesting that CXCL16

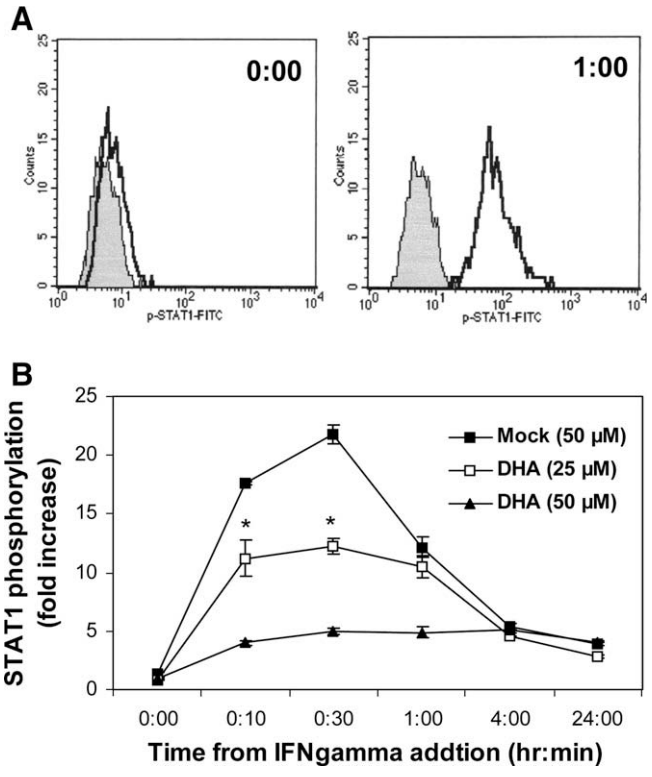

Fig. 4. Effects of DHA on STAT1 phosphorylation. (A) Phosphorylation of STAT1 upon IFNγ treatment. Cells were treated for zero (left panel) or one (right panel) hour with IFNγ. Cells were then fixed, permeabilized, and stained either with a primary antibody to phosphorylated STAT1 followed by a FITC-conjugated secondary antibody (solid line) or the FITC-conjugated secondary antibody alone (grey histogram). (B) Time course of STAT1 phosphorylation in DHA-treated HASMCs. Data is quantified as the fold increase of the mean fluorescent intensity compared to stainings with only the secondary anti-mouse-FITC antibody ± SD of three duplicate assays. $^{*}p < 0.05$, compared to the mock control at the same time point.

also exerts an atheroprotective effect [23]. These observations suggest that it is important to have a baseline level of CXCL16 expression; however, its over-expression will also lead to pro-atherosclerotic conditions. Recent reports have been published to address this conflict. The uptake of oxLDL in CXCL16$^+$ macrophages drives the expression of the anti-atherosclerosis proteins ATP binding cassette transporter A1, ATP binding cassette transporter G1, and apolipoprotein E [24]. Because of these conflicting data on CXCL16 expression and atherosclerosis, we suggest that therapies that target CXCL16 must not completely antagonize or eliminate the protein, but rather down-modulate the increased expression to the baseline levels. Our data suggests that concentrations as low as 25 μM significantly reduce IFNγ-induced transmembrane and secreted CXCL16 without affecting its baseline expression.

Binding of IFNγ to its receptor results in dimerization between IFNγR1 and IFNγR2. Following dimerization, the Janus kinases (JAK) are phosphorylated and activated in an autocrine mechanism. STAT1 is phosphorylated by the JAK2 protein. Cholesterol rich lipid rafts in the cellular membrane have been implicated in IFNγ signaling [25]. The lipid raft domain acts as a bridge between the STAT1 and JAK2 protein and is required for effective signaling. DHA incorporation into the cell membrane is known to disrupt the lipid raft domains [26,27], suggesting that DHA inhibition of IFNγ-stimulated STAT1 phosphorylation as one of the mechanisms for DHA inhibition IFNγ-induced CXCL16 expression. We have suggested in a previous report that DHA inhibits the CXCL12/CXCR4 signaling axis also through disruption of the lipid raft domains [21]. The IFNγ receptor and CXCR4 are receptors that require dimerization for effective signaling [28,29]. While CXCR4 requires the lipid rafts for proper signaling, it is unknown whether or not this is the case for IFNγ receptors.

The enzyme disintegrin-like metalloproteinase ADAM-10 is responsible for cleavage of the transmembrane isoform of CXCL16 to generate the secreted variant [4,5]. Interestingly, we observed that while the transmembrane CXCL16 was reduced almost completely to the baseline level by DHA treatment (Fig. 1), there was still a significant amount of secreted CXCL16 in the supernatants of DHA-treated cells (Fig. 2). This suggests that DHA treatment could be having a stimulatory effect on the activity of ADAM-10. Further investigation will be required to determine the biological significance and mechanism for observations.

Numerous studies are emerging that implicate CXCL16 in other inflammatory diseases. For example, IFNγ treatment increased CXCL16 expression and function in kidney podocytes enhanced progression of glomerular kidney disease [22]. The results of our study suggest that in addition to atherosclerosis, DHA may be helpful for treating these diseases as well. Plasma levels of omega-3 fatty acids have been measured as high as 200–400 μM in humans consuming moderate to high fish intake over several months [30], suggesting that the concentrations used in this study fall well within the physiological range. Whether or not DHA inhibits IFNγ-induced expression of CXCL16 *in vivo* will require further analysis.

Acknowledgments

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