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Omega-3 fatty acids attenuate dendritic cell function via NF-κB independent of PPARγ☆

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Abstract

Long-chain n-3 polyunsaturated fatty acids (n-3 PUFA) have been shown to modulate the immune response and have therapeutic effects in inflammatory disorders. PUFA are also peroxisome proliferators-activator receptor-gamma (PPARγ) ligands; a family of ligand-activated transcription factors, which when activated antagonise the pro-inflammatory capability of nuclear factor κB (NF-κB). PPARγ plays a role in dendritic cell (DC) maturation and n-3 PUFA have been shown to affect DC maturation by decreasing activation of NF-κB. While n-3 PUFA can function as PPAR ligands, it is not known whether the NF-κB-mediated immunomodulatory properties of n-3 PUFA are PPARγ-dependent. In this study we examined whether the immunomodulatory effects of n-3 PUFA on DC activation were mediated through activation of PPARγ. Treatment of murine bone marrow derived DCs with docosahexaenoic acid (DHA; 25 μM) and eicosapentaenoic acid (EPA; 25 μM) attenuated LPS-induced DC maturation. This was characterised by suppression of IL-12 production and expression of CD40, CD80, CD86 and MHC II and enhanced production of IL-10 and expression of IL-10R. This was coincident with enhanced PPARγ expression, suppressed NF-κB activity and increased the physical interaction and cellular colocalization between NF-κB with PPARγ. To understand the functional implication of the physical association of PPARγ with NF-κB, we determined whether the specific PPARγ inhibitor, GW9662 could abolish the anti-inflammatory effect of n-3 PUFA Inhibiting PPARγ did not impede the NF-κB-mediated anti-inflammatory cytokine profile induced by EPA and DHA alone. Thus n-3 PUFA activate PPARγ and interact with NF-κB in DC. However, the anti-inflammatory effects of EPA and DHA on DCs are independent of PPARγ.

Keywords: Dendritic cells; LC n-3 PUFA; NF-κB; PPARγ

1. Introduction

Dendritic cells (DCs) play a critical role in directing adaptive immune responses, including T helper (Th) cell responses. Activation of DCs by inflammatory stimuli, such as lipopolysaccharide (LPS), induces maturation and homing to lymph nodes where DCs present antigen to naïve T cells [1]. This maturation process is characterized by the production of cytokines and by increased expression of major histocompatibility complex (MHC) class II molecules and co-stimulatory molecules (CD40, CD80, CD86). The differentiation of naïve $CD4^+$ T cells into Th cell subsets is determined in part by the cytokines produced by DCs. Interleukin (IL)-12 promotes Th1 differentiation, IL-4 induces Th2 cells, IL-10 promotes the induction of type 1 T regulatory T (Tr1) cells, while IL-23 production by DCs is involved in generating or expansion of IL-17-producing CD4+ T (Th17) cells [2–5]. Since expression of these cytokines, together with the maturation status of the DC have a pivotal role in directing Th cell responses, modulating DC activation can have a profound effect on the quality and quantity of the adaptive immune response.

Recently it has been demonstrated that peroxisome proliferators-activator receptor-gamma (PPARγ) affects DC maturation [6]. PPARγ is a ligand-activated transcription factor that antagonises nuclear factor κB (NF-κB), activator protein-1 (AP-1) and signal transducers and activators of transcription factor (STAT) transcriptional activity [7]. The phenotypic effects of agonist-induced PPARγ activation on DC maturation are well characterised [6,8,9]. The PPARγ ligand, rosiglitazone, suppresses production of IL-12 and expression of CD80 in DCs [8]. Furthermore, other PPARγ ligands, troglitazone or 15d-PGJ2, decrease DC IL-12 production and increase expression of CD80 and CD40 expression, concomitant with up-regulation of CD86 [6]. Thus, activation of PPARγ in DCs may influence Th cell differentiation and may represent a therapeutic target against T cell-mediated diseases.

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PPARγ can bind a broad range of natural and synthetic ligands, including polyunsaturated fatty acids (PUFA) and prostaglandin derivatives [10]. However, the role of PPAR in the immunomodulatory properties of n-3 PUFA has not been determined. We have demonstrated that the PUFA, conjugated linoleic acid (CLA), exerts potent anti-inflammatory effects on DC activation characterised by inhibition of IL-12 and enhancement of IL-10 production [11]. EPA and DHA also inhibit DC maturation, as evidenced by altered expression levels of CD40, CD80 and CD86 [12]. Furthermore, DHA and EPA have been shown to activate PPARγ in several cell types, for example, in adipocytes [13]. Whether these effects are PPARγ−dependent is unknown.

This study examined the hypothesis that EPA and DHA exert their immunomodulatory effects in DCs via NF-κB through activation of PPARγ. We show that DHA, and to a lesser extent EPA, increase expression of PPARγ in DCs. Interestingly, we demonstrate that EPA and DHA inhibit NF-κB, modulate cytokine production and expression of co-stimulatory molecules. Whilst PPARγ colocalizes with NF-κB, the immunomodulatory effects of EPA and DHA are independent of PPARγ activation.

2. Material and methods

2.1. Animals and materials

BALB/c mice were purchased form Harlan (Bicester, UK) and were used at 10–14 weeks of age. Animals were maintained according to the regulations of the European Union and the Irish Department of Health. EPA, DHA, lauric acid (LA) and the PPARγ inhibitor (GW9662) were purchased from Sigma (Sigma-Aldrich, UK). *Escherichia coli* LPS (serotype 127:B8) was purchased from Alexis Biochemicals (UK). The PPARγ agonist rosaglitazone was purchased from Alpha Technologies. The fatty acids and the PPARγ inhibitor and agonist were dissolved in sterile dimethyl sulfoxide (DMSO) (Sigma-Aldrich, UK) and stored at –20°C in the dark.

2.2. Isolation and culture of bone marrow-derived DC

Bone marrow-derived immature DCs were prepared by culturing bone marrow cells obtained from the femurs and tibia of mice in Roswell Park Memorial Institute medium (RPMI) 1640 medium with 5% fetal calf serum supplemented with 10% supernatant from a granulocyte-macrophage colony-stimulating factor (GM-CSF)F-expressing cell line (J558-GM-CSF). The cells were cultured at 37°C for 3 days and the supernatant was carefully removed without disturbing the cell monolayer and replaced with fresh medium with 10% GM-CSF cell supernatant. On Day 7 of culture, cells were collected, counted and used for assays. DMSO (vehicle control), EPA (25 μM) DHA (25 μM) or LA (50 μM) (saturated fatty acid control) was added to the cells on day 1 of culture for all experiments. These concentrations were found to be non-toxic to the cells and have been reported in previous studies [14].

2.3. Effect of EPA and DHA on DC cytokine production

DC (1×10^6/ml) were cultured in 24-well plates with LPS (100 ng/ml) or medium alone for 0–24 h. In certain experiments DCs were incubated in the presence or absence of the specific PPARγ inhibitor GW9662 (10 μM) or rosaglitazone (10 μM). At the end of the relevant incubation periods, supernatants were removed and IL-10 and IL-12p70 concentrations were measured using DuoSet ELISA kits from R&D Systems (UK) according to manufacturers instructions.

2.4. Effect of EPA and DHA on NF-κB levels

Following treatment with EPA, DHA, LA or DMSO, DCs were cultured in 6 well plates with LPS (100 ng/ml) or medium alone for 0–5 h. Supernatants were removed and whole cell protein extracts were prepared. Alternatively, cells were washed in ice cold phosphate-buffered saline (PBS) and lysed in 10 mmol/L HEPES pH 7.9, 1.5 mmol/L $MgCl_2$, 10 mmol/L KCL, 0.5 mmol/L phenylmethylsufonyl fluorid (PMSF), 0.5 mmol/L dithiothreitol (DTT) and 0.1% v/v Nonidet P-40. Following centrifugation, the supernatant was removed and stored at −80°C as the cytosolic extract. To prepare the nuclear extract, the pellet was resuspended in 20 mmol/L HEPES pH 7.9, 1.5 mmol/L $MgCl_2$, 420 mmol/L NaCl, 0.2 mmol/L EDTA, 25% glycerol and 0.5 mmol/L PMSF, incubated on ice for at least 15 min with repeated vortexing and then centrifuged. To the supernatant, 10 mmol/L HEPES pH 7.9, 50 mmol/L KCL, 0.2 mmol/L EDTA, 20% glycerol, 0.5 mmol/L DTT, 0.5 mmol/L PMSF were added and samples were mixed well and stored at −80°C as the nuclear extract. The concentration of protein in the nuclear and cytoplasmic samples was quantified by Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA).

2.5. Western blot analysis and Immunoprecipitation

Proteins and pre-stained protein markers (precision plus protein standards; Bio-Rad) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto 0.45uM Biotrace polyvinylidene fluoride transfer membrane (Pall Life Sciences, New York, NY, USA). Membranes were blocked using 10% non-fat dried milk in PBS with 0.05%Tween (PBS-T) and incubated overnight at 4°C with either anti-NF-κBp65 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:500) or anti-PPARγ (Santa Cruz Biotechnology; 1/1000) antibodies. Membranes were washed with PBS-T and incubated for 1 h at room temperature with either peroxidase conjugated anti-mouse IgG or anti-rabbit IgG (Sigma, Dorset, UK; 1:1000). After further washing protein complexes were visualized with supersignal (Pierce, Northumberland, UK). Membranes were exposed to film for 1-10 min and processed using an Agfa X-ray processor. Protein bands were quantified using the GeneSnap acquisition and GeneTools analysis software (GeneGenius Gel Documentation and Analysis System, Cambridge, UK). Where required, membranes were stripped by incubating them in stripping buffer (Tris pH 6.8, β-mercaptoethanol, SDS) for 30 min at 50°C, before probing with a subsequent antibody. NF-κBp65 and PPARγ were expressed as arbitrary units. For immunoprecipitation, nuclear and cytosolic protein fractions were incubated with primary antibody (NF-κBp65, Santa Cruz Biotechnology) for 4 h at 4°C. This was then incubated with protein A agarose beads (Sigma) for 2 h. Samples were washed four times in protein lysis buffer and resuspended in 25 μl 3×SDS sample buffer and boiled for 5 min. These proteins were then resolved on an SDS-PAGE gel, transferred to a nitrocellulose membrane and detected as described for western blot analysis.

2.6. Effect of EPA and DHA on DC surface marker expression by flow cytometry

DCs were treated with EPA, DHA LA or DMSO from day 1 of culture and then cultured in 24 well plates (1×10^6 cells) with LPS (100 ng/ml) or medium alone for 24 h in the presence or absence of the specific PPARγ inhibitor, GW9662. Cells were then washed and used for immunofluorescence analysis. The expression of CD40, CD80, CD86, MHCII, IL-10R, and CD48 on DCs was assessed using an anti-mouse CD11c (hamster IgG1, Caltag), CD40 (hamster IgM, BD Biosciences), CD80 (hamster IgG, BD Biosciences), CD86 (rat IgG2a, BD Biosciences), MHCII (rat IgG2a, BD), IL-10R (rat IgG1, BD Biosciences), CD48 (rat IgG2a, Acris) and appropriately labelled isotype-matched antibodies, which acted as controls. After incubation for 30 min at 4°C, cells were

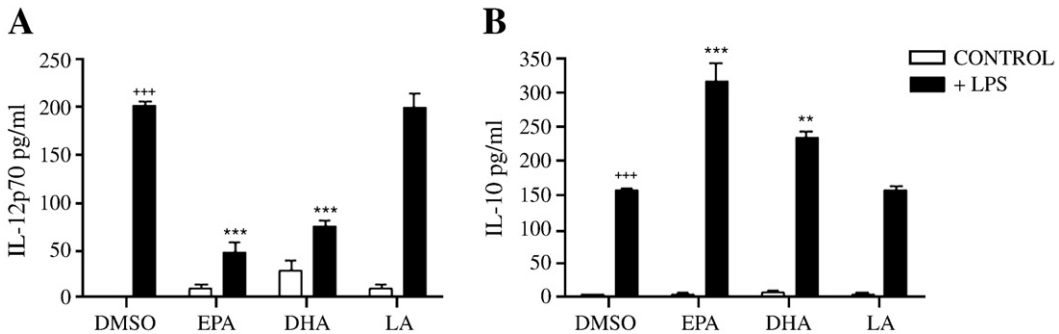

Fig. 1. EPA and DHA modulate LPS-induced cytokine release in murine DCs. Bone marrow-derived DCs were incubated for 7 days with DMSO (vehicle control), 25 μM EPA, 25 μM DHA, or 50 μM LA (saturated fatty acid). Supernatants were recovered 24 h after LPS-stimulation (100 ng/ml) and the levels of IL-12p70 (A) and IL-10 (B) were measured using specific immunoassays. The results are mean (±S.E.M.) for three independent experiments. ***$P<.001$; **$P<.01$ vs. DMSO vehicle control determined by one-way ANOVA test.

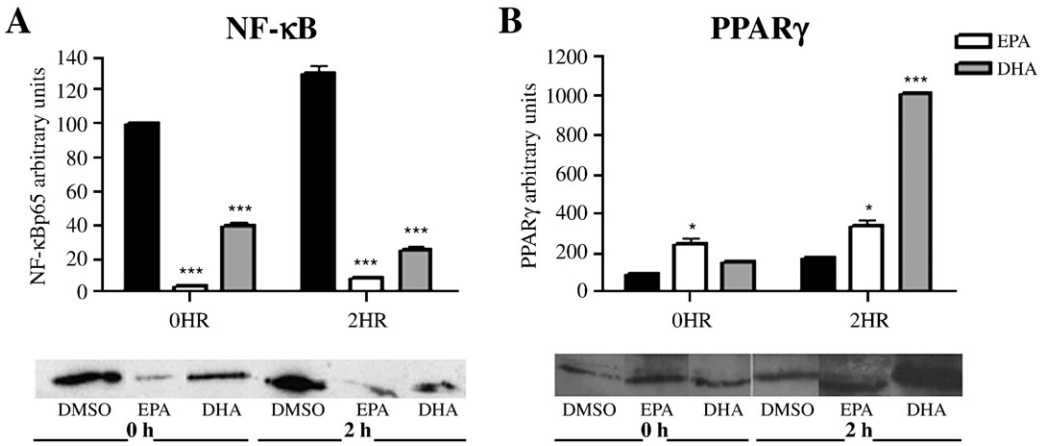

Fig. 2. EPA and DHA modulate total cellular expression and of NF-κB (A) and PPARγ (B). DCs treated for 7 days with either DMSO (vehicle control), EPA (25 μM), DHA (25 μM), or LA (50 μM) were stimulated with LPS (100 ng/ml) for 0 and 2 h. Total cellular levels of NF-κBp65 (A) and PPARγ (B) were determined by Western blot. Densitometric analysis was conducted on immunoblots and results are expressed as a fold change relative to control (DMSO). Representative immunoblots are shown below the graphed data. *Lane 1*: 0 h DMSO, *Lane 2*: 0 h EPA, *Lane 3*: 0 h DHA, *Lane 4*: 2 h DMSO, *Lane 5*: 2 h EPA, *Lane 6*: 2 h DHA. ***P<.001, *P<.05 vs. DMSO vehicle control determined by one-way ANOVA test.

washed and immunofluorescence analysis was performed on a FACsCalibur (BD Biosciences) using Cell Quest software.

2.7. Immunofluorescence staining, confocal imaging and quantification

DCs were fixed in 4% paraformaldehyde (Sigma) for 30 min. Cell membranes were then permeabilised using 0.1% Triton X-100 (Sigma) for 10 min and blocked for 10 min with 5% bovine serum albumin (Sigma). Cells were then incubated with primary antibodies (1/100 dilution, NF-κBp65 and 1/100 dilution PPARγ; Santa Cruz Biotechnology) for 1 h. Cells were washed with PBS and then incubated with secondary antibodies (1/400 dilution fluorescein isothiocyanate-conjugated goat anti-rabbit IgG and 1/400 dilution rhodamine-conjugated goat anti-rabbit IgG; Abcam, Cambridge, UK) for 1 h. Multiple-labelling immunofluorescence images of DCs treated with DMSO, EPA or DHA and stimulated with LPS for either 0, 2 or 5 h were captured using a BioRad MRC 1024 confocal microscope. Confocal analysis parameters were identical for each image with 8–15 non-overlapping sections per slide. Images were then acquired using Volocity 4 3D imaging software (Improvision Software, Coventry UK). The amount of NF-κBp65 and PPARγ in DC was quantified using Volocity 4 software (Improvision Software).

2.8. Statistics

One-way analysis of variance (ANOVA) was used to determine significant differences between conditions. When this indicated significance (P<.05), post hoc Student-Newmann-Keul test analysis was used to determine which conditions were significantly different from each other. There was no significant difference between cells alone and DMSO (vehicle control) treated cells; therefore, DMSO was used as the reference treatment.

3. Results

3.1. EPA and DHA attenuate the LPS-induced pro-inflammatory profile in DCs

Given the critical role of IL-12 and IL-10 in the generation of Th cell responses, we examined whether EPA and DHA could modulate the production of these cytokines by murine DCs. Bone marrow-derived DCs were cultured with DMSO (vehicle control), EPA (25 μM), DHA (25 μM), or LA (50 μM) for 7 days prior to stimulation with LPS. Activation of DCs with LPS resulted in significant production of IL-12p70 and IL-10 24 h after treatment (P<.001; Fig. 1A & B). The production of LPS-induced IL-12p70 was significantly suppressed in cells pre-treated with EPA and DHA (P<.001; Fig. 1A), but not with the saturated fatty acid, LA. In contrast, pre-treatment of DCs with EPA and DHA, but not LA, significantly enhanced LPS-induced production of IL-10 (P<.001; P<.01; Fig. 1B). EPA and DHA had no effect on LPS-induced tumor necrosis factor α (TNF-α) production by DCs (data not shown). We then examined the effects of EPA and DHA on NF-κB, given that it plays a key role regulating IL-12 production by DCs. EPA and DHA significantly decreased total NF-κBp65 expression in un-stimulated DCs (P<.001; Fig. 2A) and attenuated LPS stimulated NF-κBp65 levels at 2 h (P<.001).

3.2. EPA and DHA increase PPARγ expression and modulate the interaction of PPARγ with NF-κB

PPARγ is a nuclear hormone receptor that displays anti-inflammatory effects when activated. As EPA and DHA are partial agonists of PPARγ, we examined the role of PPARγ in the anti-inflammatory effects of EPA and DHA in DCs. EPA and DHA significantly increased PPARγ expression in LPS-stimulated DC (P<.05, P<.001; Fig. 2B). Furthermore, EPA increased basal DC PPARγ expression prior to LPS stimulation (P<.05). Previous reports have suggested that PPARγ physically interacts and regulates NF-κB [15], and since EPA and DHA reciprocally regulated NF-κBp65 and PPARγ expression we examined potential interactions. Cell lysates were immunoprecipitated with an anti-NF-κBp65 antibody and subsequently immunoblotted with an anti-PPARγ antibody. In resting cells there was no observable

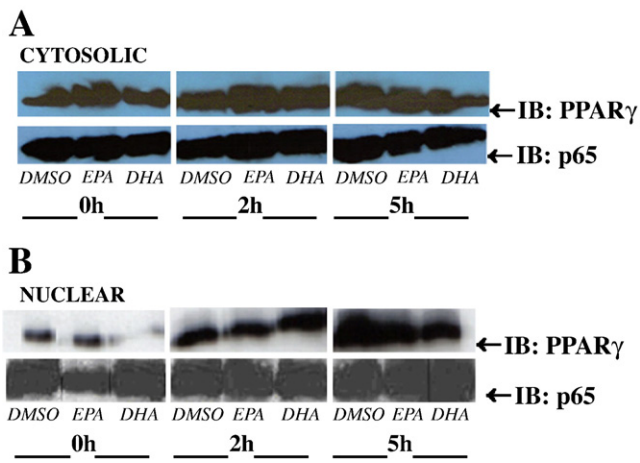

Fig. 3. EPA and DHA modulate the interaction of PPARγ with NF-κBp65. DCs were treated for 7 days with DMSO, EPA or DHA. Protein prepared from cytosolic (A) and nuclear (B) extracts were immunoprecipitated for NF-κBp65 and subsequently probed for PPARγ. The same membrane was stripped and re-probed with an anti-NF-κBp65 antibody. Immunoblots are representative of three separate experiments.

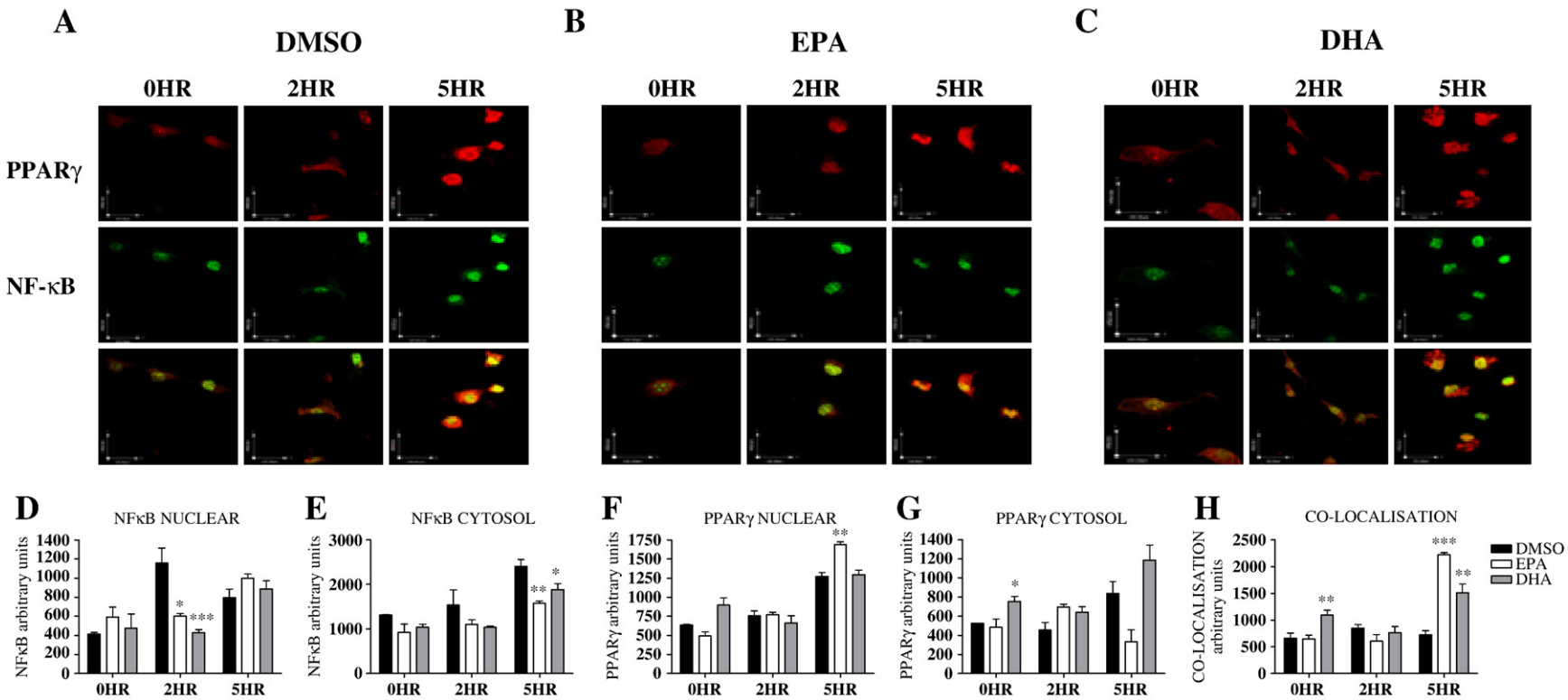

Fig. 4. EPA and DHA modulate the interaction of PPARγ with NF-κBp65. Fatty acid-treated DCs were stimulated with LPS (100 ng/ml) for 0, 2 and 5 h before being fixed and stained. Multiple-labelling immunoflourescence images are shown for NF-κBp65 (green) and PPARγ (red) and colocalization (yellow). Graphs represent the mean value of immunofluorescence intensity for NF-κBp65 (D and E) and PPARγ (F and G) in both nuclear and cytosolic cellular regions. Pearson's correlation, calculated using Volocity 4 software was used to measure colocalization (H). ***P<.001; **P<.01; *P<.05 vs. DMSO vehicle control determined by one-way ANOVA test.

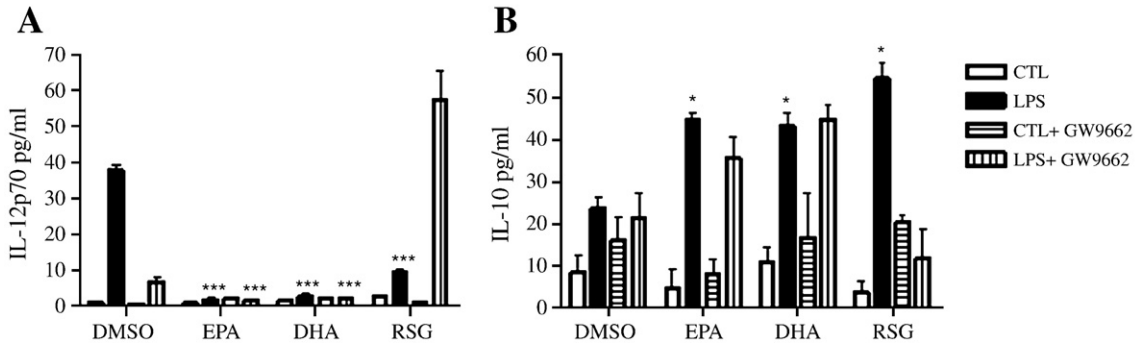

Fig. 5. The PPARγ inhibitor, GW9662, does not reverse PUFA-mediated changes in IL-12p70 and IL-10 production in DCs. DCs were cultured in either, DMSO, DHA, EPA or rosaglitazone (10 μM) in the presence or absence of the PPARγ inhibitor, GW9662 (10 μM) for 7 days and then stimulated with LPS (100 ng/ml). Supernatant was removed 24 h later and the levels of IL-12p70 (A) and IL-10 (B) assessed. Results are means±SE of quadruplicate assays and represent three independent experiments. ***P<.001, *P<.05 vs. DMSO vehicle control determined by one-way ANOVA test.

difference between treatments in cytosolic fractions (Fig. 3A). At 2 h post LPS stimulation, there was more PPARγ associated with NF-κB in the cytosolic fraction of EPA and DHA treated DCs compared to the vehicle control (Fig. 3A). In nuclear lysates, there was increased association between PPARγ and NF-κBp65 2 h post-LPS stimulation, particularly in DHA-treated cells (Fig. 3B).

Confocal microscopy was employed to further examine PPARγ and NF-κB nuclear and cytosolic colocalization (Fig. 4). EPA and DHA-treated DCs showed significantly less nuclear NF-κBp65 localization 2 h post-LPS stimulation (P<.05 and P<.001, respectively; Fig. 4D) and cytosolic NF-κBp65 expression 5 h post-LPS stimulation (P<.01 and P<.05, respectively; Fig. 4E). In contrast, DHA-treated resting DCs had significantly greater cytosolic PPARγ levels at 0 h (P<.001; Fig. 4G). Also EPA- and DHA-treated DCs showed greater cytosolic PPARγ expression 2 h post LPS stimulation, albeit not significant (Fig. 4G). Nuclear PPARγ expression was significantly increased 5 h post-LPS stimulation in EPA-treated DCs compared to the vehicle control DCs (P<.01; Fig. 4F). An important aspect of the confocal analysis was to study the colocalization indicative of potential physical interactions between NF-κBp65 (green) and PPARγ (red), wherein colocalization

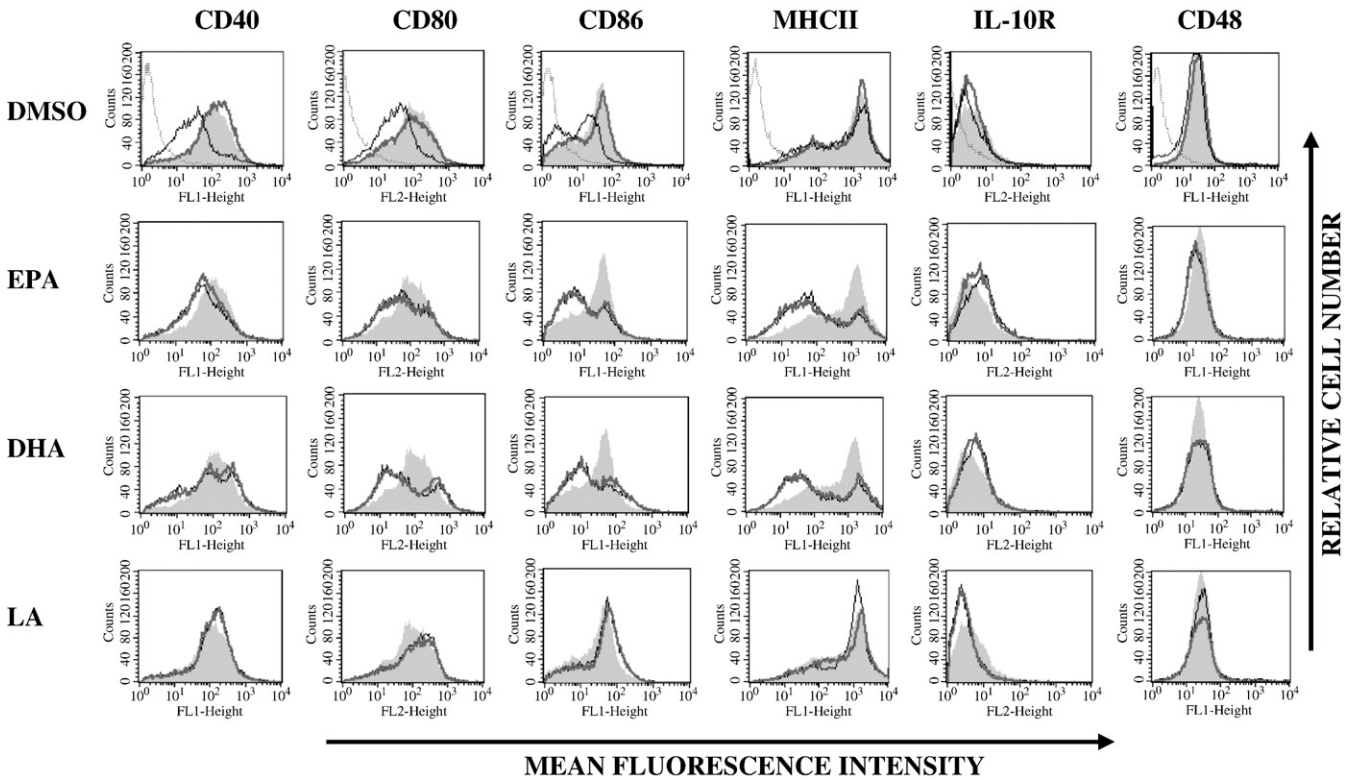

Fig. 6. EPA and DHA modulate the expression of CD40, CD80, CD86, MHCII, and IL-10R on the surface of LPS-stimulated DCs independent of PPARγ. DCs were cultured for 7days in the presence of fatty acids (EPA, DHA or LA) or vehicle control. Immature DC were exposed to the specific PPARγ inhibitor, GW9662 (10 μM) for 1 h prior to LPS stimulation (100 ng/ml). Following incubation for 24 h, cells were washed and stained with antibodies specific for CD40, CD80, CD86, MHCII, IL-10R and CD48, or with isotype matched controls. Results of immunofluorescence analysis are shown for DMSO/LPS-treated DC (filled histogram), PUFA/LPS-treated DC (thin black line), and fatty acid/LPS- or DMSO/LPS-treated DC exposed to the PPARγ inhibitor (solid grey line). Histograms for isotype controls are shown as dotted lines in the first row only. Profiles are shown for a single experiment and are representative of three experiments.

appears yellow. (Fig. 4H). The degree of colocalization was calculated using Volocity 4 software which used the Pearson Correlation. Clearly most colocalization occurs in the nucleus, indicating that PPARγ may interact directly with NF-κBp65 following nuclear translocation. Interestingly EPA and DHA-treated DCs showed significantly greater association between PPARγ and NF-κB ($P<.001$; $P<.01$; Fig. 4H). This increased interaction was also observed in resting cells treated with DHA ($P<.01$). These data suggest that the interaction between PPARγ and NF-κBp65 could be responsible for the functional effects of EPA and DHA.

3.3. Modulation of cytokine production by EPA and DHA are PPAR-γ independent

It has been demonstrated that PPARγ activation inhibits LPS-induced inflammatory response in DCs [6]. Therefore, having demonstrated that both EPA and DHA modulated DC cytokine response and activated PPARγ, we investigated whether the effects of EPA and DHA on cytokine production were impeded by the specific PPARγ antagonist, GW9662. In these experiments DCs were treated for with DMSO, EPA, DHA or the PPARγ agonist, rosaglitazone in the presence or absence of GW9662 and then activated with LPS. Consistent with the data shown in Fig. 1, EPA and DHA significantly suppressed IL-12p70 ($P<.001$; Fig. 5A) and enhanced IL-10 production by DCs ($P<.05$; Fig. 5B). In the presence of GW9662, EPA and DHA retained the ability to significantly inhibit LPS-induced IL-12p70 production by DC ($P<.001$; Fig. 5A and B) while the effects of rosaglitazone were completely reversed. Furthermore, EPA and DHA continued to enhance LPS-induced IL-10 in the presence of the PPARγ inhibitor, while the increase in IL-10 in rosaglitazone-treated group was completely abrogated. These data suggest that modulation of LPS-induced DC cytokine response by fatty acids is independent of PPARγ activation.

3.4. EPA and DHA modulate expression of cell surface markers on DC via a PPARγ independent mechanism

Several studies have indicated that PPARγ may attenuate LPS-induced up-regulation of DC cell surface maturation markers. Furthermore n-3 PUFA have previously been shown to inhibit DC maturation [12]. We therefore explored the possibility that while PPARγ is not involved in the modulation of cytokines by EPA and DHA, it may play a role in mediating their effects on DC maturation. The data in Fig. 6 demonstrate that activation of DCs with LPS for 24 h enhances expression of CD40, CD86, CD80, MHC II and CD48 (top panel). Pretreatment of DCs with EPA and DHA resulted in significant suppression of LPS-induced expression of CD80, CD86, MHC II and in the case of EPA a small reduction in CD40 expression. This suppression was maintained in the presence of the PPARγ inhibitor, demonstrating that these effects were independent of PPARγ activation. The saturated fatty acid control LA, had no suppressive effect on expression of these markers and indeed slightly enhanced expression of CD40, CD80 and CD86 in LPS-stimulated DCs. The expression of CD48 was relatively unchanged by any of the treatments. Both EPA and DHA enhanced the expression of the IL-10R on resting and LPS-stimulated DCs.

4. Discussion

PPARγ is widely expressed by a number of immune cells, wherein it controls various aspects of both innate and adaptive immunity through specific effects on individual cell populations. PPARγ is a negative regulator of macrophages and monocytes, suppressing oxidative burst [16] and inflammatory cytokine production [17]. Furthermore, PPARγ regulates Th cell responses [18]. Importantly, DC PPARγ activation by PGJ_2, rosaglitazone and pioglitazone, attenuates DC activation and maturation, suppressing IL-12 production and co-stimulatory molecule expression [19,20]. Modulation of DC maturation status affects subsequent Th cell activation resulting in the development of $CD4^+$ Th cell anergy [21]. LC n-3 PUFA are anti-inflammatory and this study sought to determine if these effects are mediated by PPARγ.

Both LC n-3 PUFA suppressed LPS-induced DC IL-12p70 expression, but enhanced IL-10 production. This observation agrees with our previous demonstration that another PUFA, CLA, suppressed LPS-induced IL-12 and enhanced IL-10 expression in DCs [11]. Our data concurs with other reports which show that DHA and EPA suppress IL-12p70 and IL-12p40 in murine and human DCs [12,22]. IL-12, produced by innate immune cells, is a key cytokine in the development of Th1 responses and has been implicated in the pathogenesis of infectious, inflammatory and autoimmune diseases, such as inflammatory bowel disease [23,24]. Specifically, EPA and DHA reduce circulating and splenic IL-12p70 and IL-12p40 production in mice following infection with *Listeria monocytogenes* [25] and decrease adaptive Th1-driven responses in a murine sensitization model [26].

The suppression of IL-12 by PPARγ ligands including troglitazone, rosaglitazone and PGJ_2, is well established [6,21,27]. Given that EPA and DHA suppressed DC IL-12 production, we examined whether this was due to PPARγ activation. Our recent work demonstrated that CLA suppressed DC IL-12p70 production in a PPARγ dependent fashion [28]. Other studies have shown that EPA and DHA activate PPARγ in adipocytes and vascular cells [13,29,30]. The inhibitory effect of PPARγ on IL-12 involves its interaction with NF-κB [15], a key transcription factor in DC activation and function [19]. Therefore the effects of EPA and DHA on expression of NF-κBp65 and the physical interaction between PPARγ and NF-κB were determined. EPA and DHA increased DC PPARγ expression; decreased total cellular, cytosolic and nuclear NFκBp65 protein in resting and LPS-stimulated DCs. This suppressive effect of EPA and DHA on NF-κB activity is consistent with studies in macrophages [14,31], pancreatic tumor cells [32], neutrophils [33] and Jurkat T cells [34].

Few studies have examined the interaction between PPARγ and NF-κB. Recent reports demonstrated that c-Rel, a member of the NF-κB complex, regulates DC IL-12 expression [35] and interestingly activation of PPARγ in DCs down-regulates c-Rel expression [19]. Therefore PPARγ may inhibit IL-12 production via the NF-κB pathway. Given our evidence that EPA and DHA activated PPARγ and suppressed NF-κB, we explored the interaction between PPARγ and NF-κB. Immunoprecipitation and confocal microscopy studies clearly demonstrated that EPA and DHA treatment increased the physical association between PPARγ and NF-κB in DCs. However PPARγ inhibitors failed to attenuate the anti-inflammatory effects EPA and DHA, including the reduction in IL-12 and enhancement of IL-10 production in DC. This concurs with recent data wherein EPA and DHA suppressed murine macrophage IL-12 production independently of PPARγ [36]. The lack of effect of PPARγ may be due to a possible role for IL-10 in mediating the anti-inflammatory effects of EPA and DHA. IL-10 is a potent anti-inflammatory cytokine, which inhibits innate immune cell IL-12, TNF-α and IL-1 expression [37]; however, all of the studies that examine the effects of PPARγ ligands on DC function have all reported that they have no effect on IL-10 production. The significant induction of IL-10 by EPA and to a lesser extent DHA in the present study concurs with other studies with CLA in murine DCs [11], and n-3 PUFA in whole blood cells and T cells [38,39].

The maturation of a DC following activation is a crucial step in their development into fully potent antigen presenting cells capable of interacting with and activating naïve T cells. DC maturation is associated with enhanced expression of MHC class II and co-stimulatory molecules, including CD40, CD80 and CD86, which are necessary for interaction with T cells. Given that we observed potent

effects of EPA and DHA on cytokine production by DC, we examined whether they could modulate expression of these molecules, and indeed whether these effects where PPARγ-dependent. LPS-mediated activation of DCs resulted in maturation, characterized by increased expression of MHC II, CD40, CD80 and CD86. DC maturation was significantly suppressed in EPA and DHA-treated DCs, which concurs with recent studies [22,40]. These effects may modulate the ability of DCs to present antigen, an affect which has previously been reported for n-3 PUFA [41]. Activation of PPARγ by ligands such as troglitazone, 15d-PGJ_2 and rosaglitazone, suppresses CD80 but enhances CD86 following DC activation, suggesting that the modulated expression of these molecules on DCs, has consequences for the subsequent generation of Th cell responses [6,8]. Indeed, it has recently been reported by Klotz et al. [21] that these effects plus those on cytokine production resulted in DCs capable of inducing $CD4^+$ T cell anergy. Our finding that EPA and DHA down-regulate CD86 expression on DCs, which is contrary to effects seen with PPARγ ligands, suggests that the effects of PUFA are independent of PPARγ. Clear evidence of this is provided by our inhibitor studies which demonstrate DHA and EPA continue to exert their suppressive effect on expression of MHC II, CD40, CD80 and CD86 in the presence of GW9962, a specific PPARγ inhibitor. Given that NF-κB plays a role in regulating the expression of these molecules, and our evidence of suppression of NF-κB, suggests that EPA and DHA may exert these effects through NF-κB.

In conclusion, our findings demonstrate that EPA and DHA suppress NF-κB and enhance PPARγ expression in LPS-stimulated DCs, both transcription factors colocalize but this is without a functional effect. EPA and DHA inhibit LPS-induced IL-12 production and expression of MHC II, CD40, CD80 and CD86 while enhancing production of IL-10 and expression of the IL-10R, effects which are not reversed in the presence of a specific PPARγ inhibitor. These data indicate that while n-3 PUFA may be ligands for PPARγ, PPARγ activation does not mediate their anti-inflammatory effects in DCs.

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