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Docosahexaenoic acid attenuates macrophage-induced inflammation and improves insulin sensitivity in adipocytes-specific differential effects between LC *n-3* PUFA☆

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Abstract

Objective: Adipose tissue inflammation with immune cell recruitment plays a key role in obesity-induced insulin resistance (IR). Long-chain (LC) *n-3* polyunsaturated fatty acids (PUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have anti-inflammatory potential; however, their individual effects on adipose IR are ill defined. We hypothesized that EPA and DHA may differentially affect macrophage-induced IR in adipocytes.
Methods: J774.2 macrophages pretreated with EPA or DHA (50 μM for 5 days) were stimulated with lipopolysaccharide (LPS, 100 ng/ml for 30 min–48 h). Cytokine secretion profiles and activation status of macrophages were assessed by enzyme-linked immunosorbent assay and flow cytometry. Pretreated macrophages were seeded onto transwell inserts and placed over 3T3-L1 adipocytes for 24–72 h; effects on adipocyte–macrophage cytokine cross-talk and insulin-stimulated ^{3}H-glucose transport into adipocytes were monitored.
Results: DHA had more potent anti-inflammatory effects relative to EPA, with marked attenuation of LPS-induced nuclear factor (NF)κB activation and tumor necrosis factor (TNF)α secretion in macrophages. DHA specifically enhanced anti-inflammatory interleukin (IL)-10 secretion and reduced the expression of proinflammatory M1 (F4/80^{+}/CD11^{+}) macrophages. Co-culture of DHA-enriched macrophages with adipocytes attenuated IL-6 and TNFα secretion while enhancing IL-10 secretion. Conditioned media (CM) from DHA-enriched macrophages attenuated adipocyte NFκB activation. Adipocytes co-cultured with DHA-enriched macrophages maintained insulin sensitivity with enhanced insulin-stimulated ^{3}H-glucose transport, GLUT4 translocation and preservation of insulin-receptor substrate-1 expression compared to co-culture with untreated macrophages. We confirmed that IL-10 expressed by DHA-enriched macrophages attenuates the CM-induced proinflammatory IR phenotype in adipocytes.
Conclusions: We demonstrate an attenuated proinflammatory phenotype of DHA-pretreated macrophages, which when co-cultured with adipocytes partially preserved insulin sensitivity.

Keywords: Insulin resistance; inflammation; DHA; LC *n-3* PUFA; IL-10; Adipose tissue macrophage

1. Introduction

Chronic low-grade inflammation and recruitment of immune cells including macrophages and T lymphocytes into adipose tissue are classical hallmarks of obesity [1–3]. Studies indicate that inflammation impairs insulin sensitivity via inhibition and down-regulation of key components of the insulin-signalling cascade [4–9], ultimately leading to insulin resistance (IR) and type 2 diabetes mellitus. Infiltration of proinflammatory F4/80^{+}/CD11c^{+} macrophages into adipose tissue contributes to adipose tissue inflammation and IR [3,10], with recent studies demonstrating protection of mice with genetic ablation of CD11c^{+} cells from high-fat diet (HFD)-induced IR [11]. Macrophage-derived proinflammatory mediators, including tumor necrosis factor (TNF)α, interleukin (IL)-6 and IL-1β, cross-talk with resident adipocytes inducing IR [4,5,12,13].

Adipose tissue macrophages (ATM) are broken down into two major subsets: classically activated proinflammatory "M1" (F4/80^{+}/CD11c^{+}) or alternatively activated anti-inflammatory (F4/80^{+}/CD11c^{-}) "M2" phenotypes. Resident M2 macrophages likely switch to a proinflammatory M1 phenotype augmenting adipose inflammation and IR [14]. Lumeng et al. established an *in vitro* co-culture model of macrophages with adipocytes to mimic their interactions *in vivo* and confirmed that presence of macrophages induces marked IR in adipocytes [8]. Given the prominent pathogenic role of inflammation during obesity-induced IR, anti-inflammatory interventions offer promising therapeutic potential.

Long chain (LC) *n-3* polyunsaturated fatty acids (PUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have anti-

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inflammatory effects in a range of immune cells including macrophages [15,16]. However, the potential impact of LC *n-3* PUFA on IR is controversial. In obese IR mice, LC *n-3* PUFA show positive effects on glucose and insulin metabolism [17,18], while in man, the data conflict. Epidemiological data suggest that habitual dietary fish intake is inversely associated with IR [19]. Short-term, high-dose LC *n-3* PUFA supplementation in subjects with impaired glucose tolerance and diabetes reported positive effects on insulin sensitivity [20,21]. Long-term, lower-dose LC *n-3* PUFA have not shown positive effects [22,23]. EPA and DHA are distinct fatty acids (FA). Previous work has shown that DHA more potently inhibited macrophage cytokine expression and nuclear factor (NF)κB activity relative to EPA [15]. Therefore, within the context of adipose biology, inflammation and IR, we hypothesized that EPA and DHA may differentially affect inflammation-related IR within a macrophage–adipocyte co-culture model. We demonstrate that DHA specifically promotes a less proinflammatory macrophage phenotype with increased IL-10 secretion, which protects co-cultured adipocytes from IR. Complimentary analysis from a large human study found that high plasma DHA, but not EPA, levels were associated with reduced HOMA-IR.

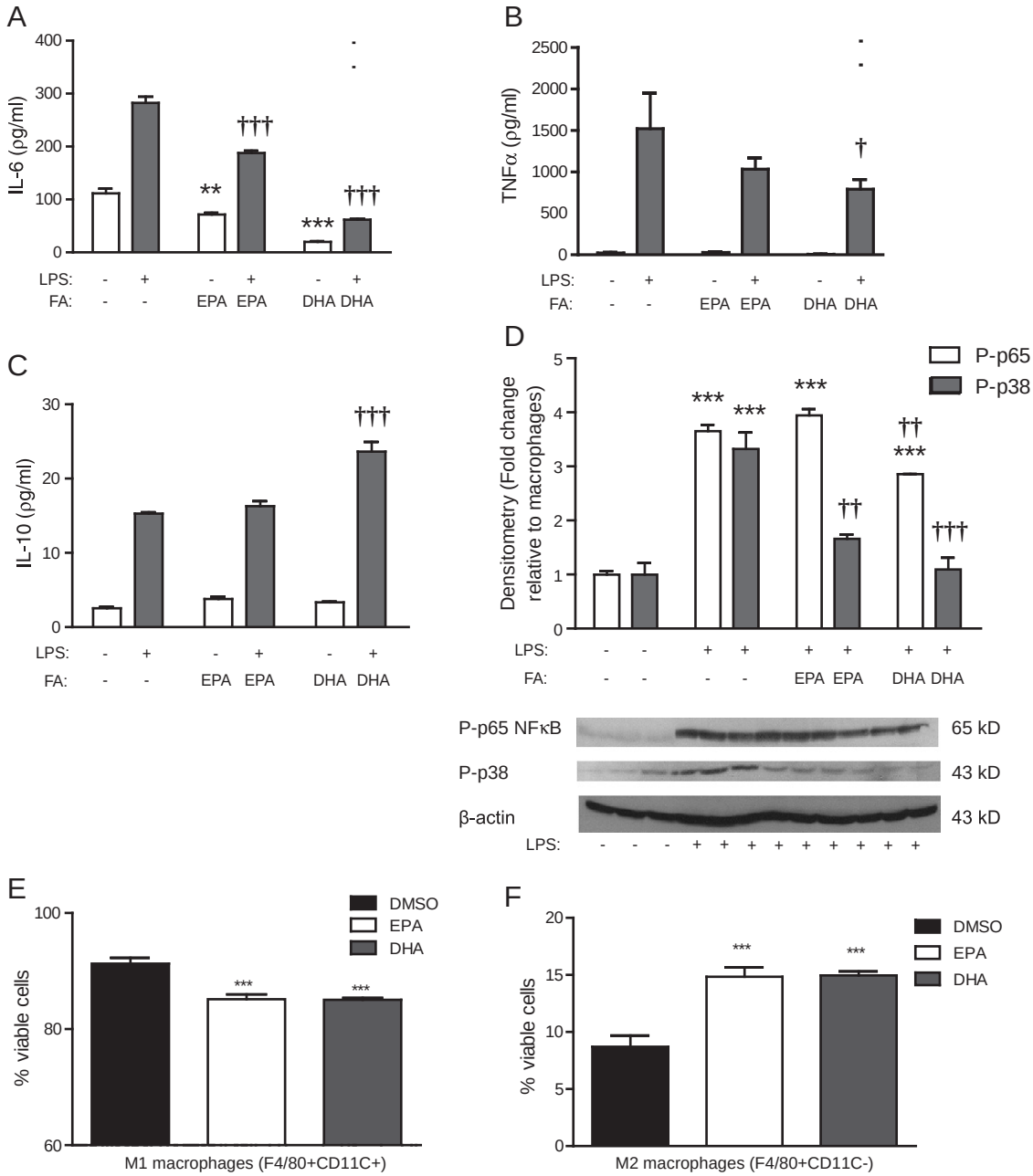


Fig. 1. DHA attenuates macrophage inflammation more potently than EPA. J774.2 macrophages chronically pretreated with EPA, DHA (50 μM) or DMSO vehicle control (5 days) were stimulated ±LPS (100 ng/ml) for 30 min. Cells were washed in PBS, and fresh media were added and left to incubate with cells for a further 48 h. (A) IL-6, (B) TNFα and (C) IL-10 secretion was determined by ELISA (picogram [pg]/ml, n=4, $^{**}P\leq.01$, $^{***}P\leq.001$ wrt untreated, $^{\dagger}P\leq.05$, $^{\dagger\dagger\dagger}P\leq.001$ wrt to LPS-stimulated). Macrophages were lysed in RIPA buffer after 30-min LPS stimulation. Levels of phosphorylated (D) p65 NFκB (ser536) and p38 MAP kinase (Thr180/Tyr182) were determined by immunoblotting. Correct loading of protein was confirmed by blotting for β-actin. Blots were quantified by densitometry, and fold change relative to untreated adipocytes is presented (n=3, $^{***}P\leq.001$ wrt untreated, $^{\dagger\dagger}P\leq.01$, $^{\dagger\dagger\dagger}P\leq.001$ wrt to LPS treated, E=EPA and D=DHA). EPA- and DHA-enriched macrophages were labeled with antibodies for F4/80 and CD11c. The macrophage activation status was analyzed using flow cytometry. (E) F4/80^{+}CD11c^{+} was associated with an M1 proinflammatory polarization state, while (F) F4/80^{+}CD11c^{-} indicated an M2 anti-inflammatory phenotype (n=5, $^{***}P\leq.001$ wrt untreated). Data presented as mean±S.E.M. ANOVA was performed, and intergroup differences were tested by post hoc Bonferroni statistical analysis.

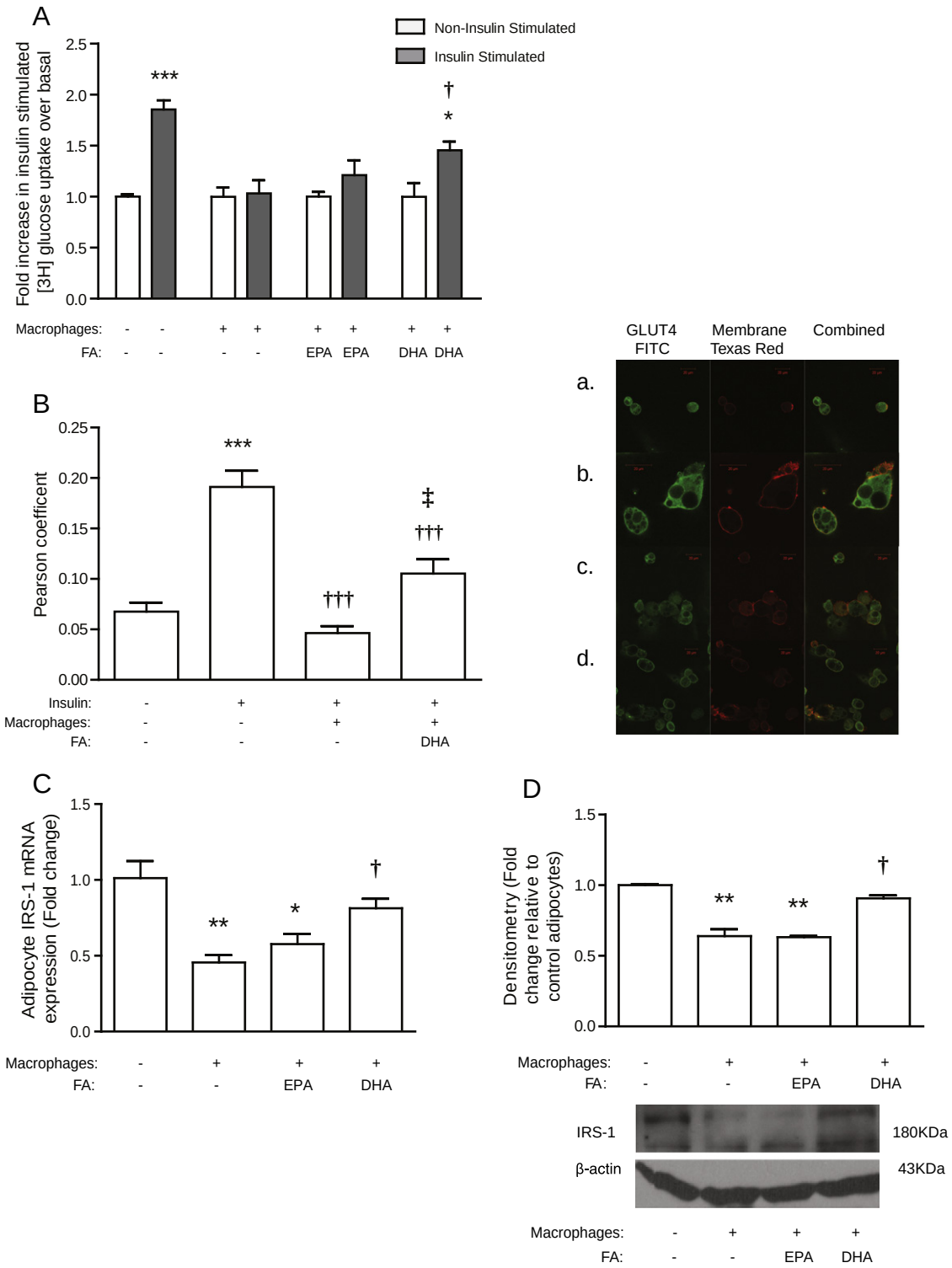


Fig. 2. DHA attenuated macrophage-induced IR in co-cultured adipocytes. J774.2 macrophages chronically pretreated with EPA, DHA (50 μM) or DMSO control (5 days) and stimulated with LPS (100 ng/ml, 30 min) were washed and placed in transwell inserts above fully differentiated 3T3-L1 adipocytes for 72 h. (A) Insulin-stimulated ^{3}H-glucose transport into adipocytes was subsequently monitored. Fold change relative to basal glucose uptake is presented (n=3, $^{*}P\leq.05$, $^{***}P\leq.001$ wrt untreated basal glucose uptake, $^{\dagger}P\leq.05$ wrt to insulin-stimulated adipocytes co-cultured with untreated LPS-stimulated macrophages). (B) GLUT4 translocation was monitored in co-culture adipocytes±insulin (10 nM) for 40 min. The membrane was labeled with EZ-link Sulfo-NHS-LC-biotin followed by Texas red avidin D (red), and GLUT4 was labeled with a primary antibody followed by a corresponding FITC-conjugated secondary antibody (green). Translocation of GLUT4 to the membrane is shown as yellow (63× magnification) (a) adipocytes (n=182), (b) adipocytes+insulin (n=168), (c) adipocytes co-cultured with LPS-stimulated macrophages+insulin (n=209), (d) adipocytes co-cultured with LPS-stimulated macrophages pretreated with DHA+insulin (n=251; $^{***}P\leq.001$ wrt untreated adipocytes, $^{\dagger\dagger\dagger}P\leq.001$ wrt adipocytes+insulin, ‡wrt adipocytes co-cultured with untreated LPS-stimulated macrophages+insulin). EPA-/DHA-enriched macrophages were LPS stimulated for 48 h and placed over mature adipocytes in transwell inserts for 24 h. RNA and protein were harvested from the co-cultured adipocytes. Expression of IRS-1 was quantified using (C) RT-PCR real-time analysis and (D) immunoblotting (n=3, $^{*}P\leq.05$, $^{**}P\leq.01$ wrt adipocytes alone, $^{\dagger}P\leq.05$ wrt adipocytes co-cultured with untreated LPS-stimulated macrophages). Correct loading of protein was confirmed by blotting for β-actin; representative blots are shown. The relative protein levels were quantified by densitometry, and fold change relative to untreated adipocytes is presented. Data presented as mean±S.E.M. ANOVA was performed, and intergroup differences were tested by post hoc Bonferroni statistical analysis.

2. Materials and methods

2.1. Cell lines and reagents

3T3-L1 adipocytes and J774.2 macrophages were purchased from the European Collection of Animal Cell Cultures (ECACC, Salisbury, UK). NIH-3T3 fibroblasts stably transfected with a luciferase-reporter construct in the NFκB promoter region were purchased from Panomics (Cambridge, UK). Cell culture materials were purchased from Lonza (Slough, UK). Recombinant-mouse cytokines and IL-10 neutralizing antibody were purchased from R&D systems (Abington, UK). Reagents were purchased from Sigma-Aldrich (Poole, UK) unless otherwise stated.

2.2. Cell culture

Cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), glutamine (2 mM) and antibiotics. A total of 100 μg/ml

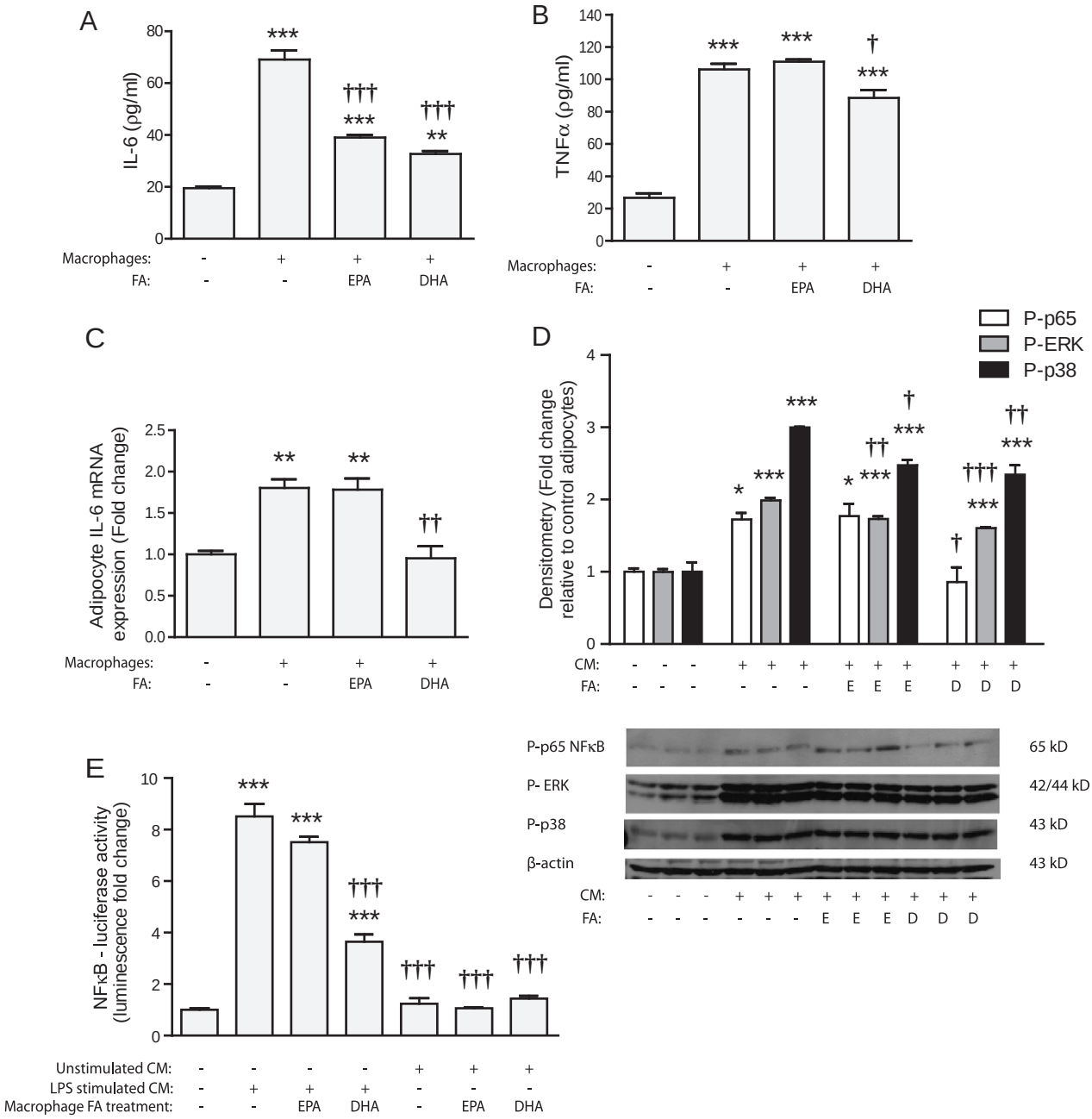
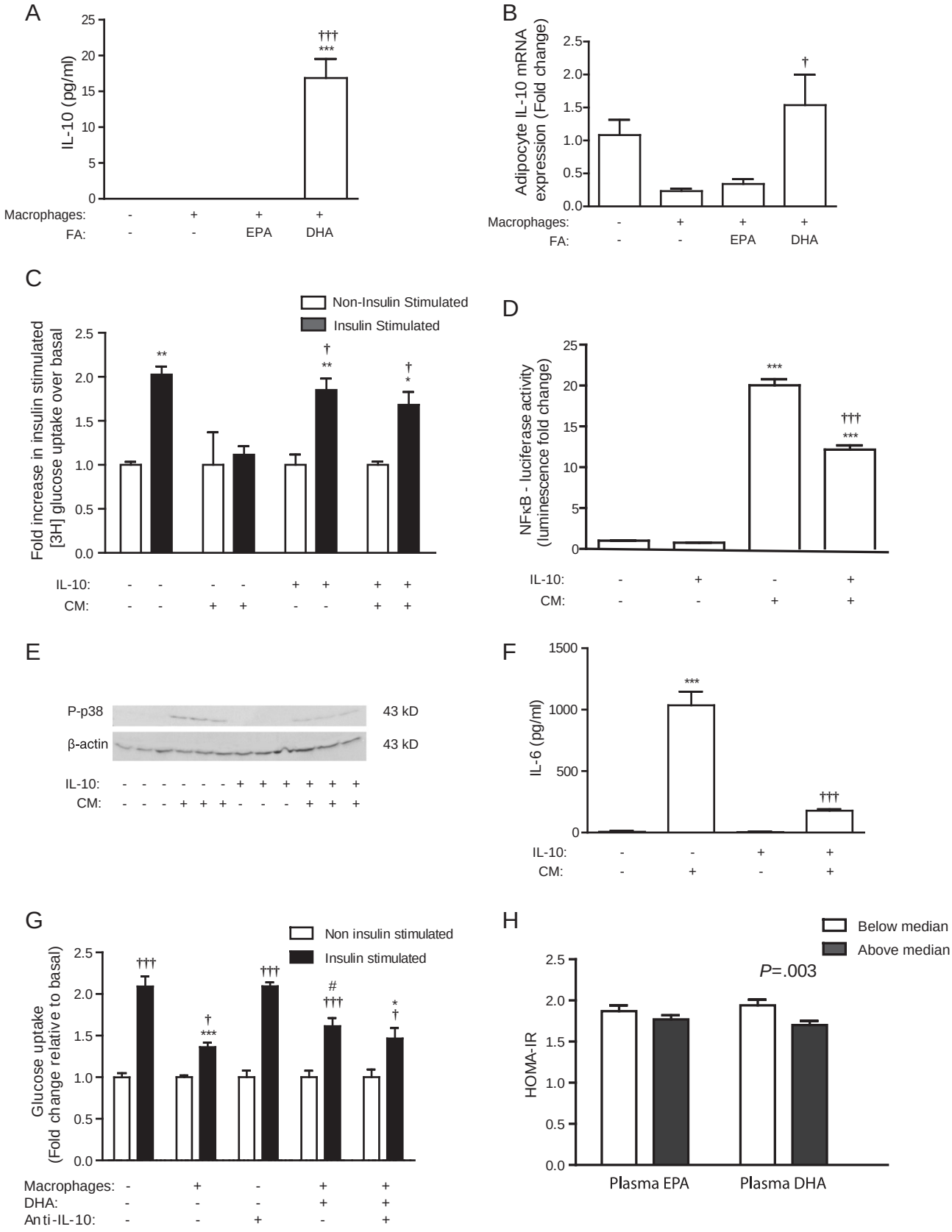


Fig. 3. DHA attenuates adipocyte–macrophage co-culture inflammation more potently than EPA. J774.2 macrophages chronically pretreated with EPA, DHA (50 μM) or DMSO control (5 days) were stimulated with LPS (100 ng/ml) for 48 h and placed in transwell inserts above mature 3T3-L1 adipocytes for 24 h. Supernatants were harvested, and (A) IL-6 and (B) TNFα secretion was determined by ELISA (pg/ml, $n=3$, $^{**}P\leq.01$, $^{***}P\leq.001$ wrt adipocytes alone, $^{\dagger}P\leq.05$, $^{\dagger\dagger\dagger}P\leq.001$ wrt adipocytes co-cultured with untreated LPS-stimulated macrophages). RNA was harvested from the co-cultured adipocytes, and mRNA expression of (C) IL-6 was quantified using RT-PCR real-time analysis ($n=3$, $^{**}P\leq.01$, wrt adipocytes alone, $^{\dagger\dagger}P\leq.01$ wrt adipocytes co-cultured with untreated LPS-stimulated macrophages). EPA- and DHA-enriched macrophages were stimulated with LPS (100 ng/ml) for 30 min. LPS stimulus was removed, cells were washed, and CM from the macrophages were collected over the subsequent 24 h. CM were incubated with mature 3T3-L1 adipocytes for 30 min. Cells were lysed in RIPA buffer, and expression of phosphorylated (D) p65 NFκB (ser536), ERK (Thr202/Tyr204) and p38 (Thr180/Tyr182) was determined by immunoblotting. Correct loading of protein was confirmed by blotting for β-actin. Bands were quantified by densitometry; fold change relative to untreated adipocytes is presented ($n=3$, $^{*}P\leq.05$, $^{***}P\leq.001$ wrt adipocytes alone, $^{\dagger}P\leq.05$, $^{\dagger\dagger}P\leq.01$, $^{\dagger\dagger\dagger}P\leq.001$ wrt adipocytes incubated with CM of untreated LPS-stimulated macrophages, E=EPA and D=DHA). (E) NFκB gene reporter fibroblasts were incubated for 6 h with CM from macrophages enriched with EPA and DHA±LPS. NFκB-dependent luciferase activity was measured using a Luminometer ($n=4$, $^{***}P\leq.001$ wrt adipocytes alone, $^{\dagger\dagger\dagger}P\leq.001$ wrt fibroblasts incubated with CM of untreated LPS-stimulated macrophages). Data presented as mean±S.E.M. ANOVA was performed, and intergroup differences were tested by post hoc Bonferroni statistical analysis.

Hygromycin B was added to NIH-3T3 fibroblasts media (Roche Co., Clare, Ireland). 3T3-L1 differentiation was induced in fully confluent 3T3-L1 preadipocytes by adding 3-isobutyl-1-methylxanthine (0.5 mM), dexamethasone (0.25 μM) and insulin (1 μg/ml) for 6 days.

2.3. LC n-3 PUFA treatment of macrophages

Macrophages were chronically pretreated with EPA, DHA (50 μM) or dimethyl sulfoxide (DMSO) vehicle control for 5 days. EPA (≥99% purity) and DHA (≥98%



purity) (Sigma-Aldrich) were dissolved in DMSO and added directly to the growth media containing 10% FBS. FA content and the M1/M2 phenotype were analyzed by gas–liquid chromatography (GC) (Supplemental Table I) and flow cytometry, respectively. EPA/DHA-enriched macrophages were stimulated with lipopolysaccharide (LPS) (100 ng/ml) for 30 min–48 h. After 30-min stimulation, cells were harvested for immunoblotting. Cytokine profiling was assessed by enzyme-linked immunosorbent assay (ELISA) (R&D systems).

2.4. Protein extraction and immunoblotting

Cells were washed in phosphate-buffered saline (PBS), and total cell lysates were extracted with radioimmunoprecipitation assay buffer (RIPA) buffer containing protease and phosphatase inhibitor cocktail. A total of 10 μg of protein was separated by sodium dodecyl sulfate electrophoresis, and proteins were transferred to a nitrocellulose membrane. Membranes were blocked using 5% bovine serum albumin (BSA) in tris buffered saline (TBS) and incubated overnight at 4°C with antibodies specific to IRS-1, p-p65 (Ser536), p-p38 (Thr180/Tyr182) and p-ERK1/2 (Thr202/Tyr204) (Cell Signalling Technology, MA, USA). Correct loading of protein was confirmed by blotting for β-actin. Membranes were washed and incubated in appropriate secondary antibody, and blots were visualized by chemiluminescence (Pierce Biotechnology, Dublin, Ireland).

2.5. RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR)

Total cell RNA was extracted using an RNeasy Mini kit from Qiagen (West Sussex, UK). cDNA was synthesized from 1.5 μg RNA using SuperScript VILO (Invitrogen). Gene expression was quantified by real-time RT-PCR on an ABI PRISM 7900HT sequence detection system from Applied Biosystems (Warrington, UK). Predeveloped primer probe kits from Applied Biosystems were used. The comparative Ct method was used to calculate gene expression levels. The Ct values of both the calibrator and samples of interest were normalized to 18S.

2.6. Flow cytometry

Pretreated macrophages were blocked in PBS containing 0.2% BSA for 15 min and labeled with antibodies for macrophage markers F4/80 and CD11c (AbD Serotec, Oxford, UK). Cells were washed in PBS/0.2% BSA and analyzed by flow cytometry using a Cyan ADP with Summit v4.3 software. Unstained, single stains and fluorescence minus one were used for setting compensations and gates. $F4/80^+/CD11c^+$ cells were classified as M1 macrophages, while $F4/80^+/CD11c^-$ cells were classified as M2 macrophages.

2.7. Fatty acid analysis

Lipid was isolated from cells in accordance to the Bligh and Dyer method [24,25]. Total lipids were transmethylated, and fatty acids were then separated and detected by a Shimadzu gas chromatography (GC) 2010 (Mason Technologies, Dublin, Ireland) equipped with a flame ionization detector, a fused silica capillary column (Omegawax, 30-m×0.25-mm inner diameter, 0.2-μm film) and GC solutions software. Fatty acids were identified according to their retention times in comparison to two fatty acid methyl ester standards (Supplemental Table I).

2.8. Macrophage–adipocyte co-culture model

EPA/DHA-enriched macrophages were stimulated ±LPS (100 ng/ml) for 30 min, washed and seeded onto transwell inserts (3×10^5/ml) above mature adipocytes for 24–72 h of co-culture. At 24 h, GLUT4 translocation was determined by confocal microscopy, and media were collected for cytokine profiling, while adipocytes were harvested for real-time RT-PCR and immunoblotting. At 72 h, insulin-stimulated ^{3}H-glucose uptake into adipocytes was monitored.

2.9. Indirect co-culture model

EPA/DHA-enriched macrophages were stimulated with LPS for 30 min and washed, and conditioned media (CM) from macrophages (2×10^5cells/ml) were collected after 24 h. Mature adipocytes were incubated with CM for 30 min. Additionally, adipocytes were pretreated ±IL10 (20 ng/ml) for 24 h prior to treatment ±CM for 30 min–24 h. After 30-min incubation, whole cell protein was collected for immunoblotting. At 3 h, insulin-stimulated ^{3}H-glucose uptake into adipocytes was monitored, and at 24 h, media were collected for cytokine analysis.

2.10. NFκB-luciferase promoter–reporter assay

NIH-3T3 fibroblasts (1×10^5cells/ml) were incubated for 6 h with CM from EPA/DHA-enriched macrophages. In addition, NIH-3T3 fibroblasts were pretreated ±IL-10 (10 ng/ml) for 1 h and stimulated with macrophage CM for 6 h. Cells were lysed, and NFκB-dependent luciferase activity was measured using a Luminometer (Promega, Southampton, UK).

2.11. Confocal GLUT4 translocation analysis

Adipocytes were cultured on 13-mm cover glass within the lower compartment of the transwell co-culture system. After co-culture with macrophages, inserts were removed, and 10 nM of insulin was added to each well for 40 min. Adipocytes were washed and fixed with 4% paraformaldehyde. Cells were incubated with 5% BSA, and the cell surface was labeled with 0.5 mg/ml EZ-link Sulfo-NHS-LC-biotin followed by Texas red avidin D (Pierce Biotechnology) as described previously [26]. Cells were permeabilized using Triton X-100 (0.1%) and then incubated with BSA (5%). Cells were incubated with GLUT4 primary antibody (Millipore) followed by a corresponding fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Abcam, Cambridge, UK). The cover glass was mounted onto slides using a Dako mounting medium (Dako, CA, USA). Images were taken, and co-localization was analyzed using an LSM510 META confocal microscope (Carl Zeiss MicroImaging Inc., Hertfordshire, UK). Pearson correlation coefficient describes the degree of overlap between the two patterns, i.e., the co-localization.

2.12. Deoxy-D-glucose, 2-[^{3}H(N)] glucose uptake assay

Adipocytes were incubated in serum free media with 0.2% BSA (fraction V, Calbiochem, Merck Chemicals Ltd., Nottingham, UK) for 4 h followed by a glucose-free step-down in PBS/0.2% BSA for 30 min. Cells were then stimulated ±insulin (100 nM) for 15 min prior to addition of ^{3}H-glucose tracer (5 μCi/ml of deoxy-D-glucose, 2-[^{3}H(N)] in 1 mM glucose) for 10 min. Cells were washed twice and lysed in RIPA buffer. A portion of the lysate was used to determine ^{3}H-glucose levels by liquid scintillation counting. Fold increase in glucose transport in response to insulin over basal is presented.

2.13. Homeostasis model assessment (HOMA)-IR in human plasma samples

The LIPGENE case–control cohort of subjects with or without the metabolic syndrome was used to determine potential differences between plasma EPA and DHA and IR [28]. HOMA, a measure of IR, was calculated as [(fasting plasma glucose×fasting serum insulin)/22.5] [27]. HOMA was examined according to plasma median levels for EPA (median=0.81% of total measured fatty acids, n=857<median, n=856>median) and for DHA (median=2.36% of total measured fatty acids, n=857<median,

Fig. 4. IL-10 pretreatment attenuates macrophage CM-induced adipocyte inflammation and IR. J774.2 macrophages chronically pretreated with EPA, DHA (50 μM) or DMSO control (5 days) were stimulated with LPS (100 ng/ml) for 48 h and placed in transwell inserts above mature 3T3-L1 adipocytes for 24 h. Supernatants were harvested, and (A) IL-10 secretion was determined by ELISA (pg/ml, n=3, $^{***}P\leq.001$ wrt adipocytes alone, $^{\dagger\dagger\dagger}P\leq.001$ wrt adipocytes co-cultured with untreated LPS-stimulated macrophages). RNA was harvested from the co-cultured adipocytes, and mRNA expression of (B) IL-10 was quantified using RT-PCR real-time analysis (n=3, $^{\dagger}P\leq.01$ wrt adipocytes co-cultured with untreated LPS-stimulated macrophages). J774.2 macrophages were stimulated with LPS (100 ng/ml) for 30 min, cells were washed, and CM from the macrophages were collected over the subsequent 24 h. (C) Insulin-stimulated ^{3}H-glucose transport into adipocytes was monitored in adipocytes pretreated with IL-10 (20 ng/ml) for 24 h and then stimulated with CM for 3 h (n=3, $^{*}P\leq.05$, $^{**}P\leq.01$ relative to basal glucose uptake, $^{\dagger}P\leq.05$ wrt insulin-stimulated adipocytes co-cultured with untreated LPS-stimulated). (D) NFκB-dependent luciferase activity was measured using NFκB-gene reporter fibroblasts pretreated with IL-10 (10 ng/ml) for 1 h and then stimulated with CM for 6 h. NFκB-dependent luciferase activity was measured using a Luminometer (n=4, $^{***}P\leq.001$ wrt fibroblasts alone, $^{\dagger\dagger\dagger}P\leq.001$ relative to CM stimulated fibroblasts). Adipocytes were pretreated with IL-10 (20 ng/ml) for 24 h and stimulated with CM for 30 min–24 h. After 30 min, cells were lysed in RIPA buffer, and expression of phosphorylated (E) p38 (Thr180/Tyr182) was determined by immunoblotting and equal loading confirmed by blotting for β-actin. After 24 h, (F) IL-6 secretion was determined by ELISA (n=3, $^{***}P\leq.001$ wrt adipocytes alone, $^{\dagger\dagger\dagger}P\leq.001$ wrt adipocytes incubated with CM of untreated LPS stimulated macrophages). (G) J774.2 macrophages chronically pretreated with DHA (50 μM) or DMSO control (5 days) were stimulated with LPS (100 ng/ml) for 30 min and placed in transwell inserts above mature 3T3-L1 adipocytes for 72 h in the presence or absence of an IL-10 neutralizing antibody (100 ng/ml). Insulin (100 nM)-stimulated glucose transport into adipocytes was subsequently monitored (n=3–9, $^{*}P<.05$, $^{***}P<.001$ wrt control+insulin; $^{\#}P<.05$ wrt macrophages+insulin; $^{\dagger}P<.05$, $^{\dagger\dagger\dagger}P<.001$ wrt insulin stimulated, n=3-9). (H) HOMA, a measure of IR, was examined below and above the plasma EPA (n=392 and n=461) and DHA (n=857 and n=856) median within the LIPGENE case–control cohort. P values for linear regression were adjusted for potential confounding factors. Data presented as mean±S.E.M. ANOVA was performed, and intergroup differences were tested by post hoc Bonferroni statistical analysis.

n=856>median) within the LIPGENE case–control cohort of 877 matched pairs of metabolic syndrome cases and controls as previously described [28].

2.14. Statistical analysis

Data reported as mean±S.E.M. Statistical analysis was performed with Prism (GraphPad, CA, USA). The distributions were assessed, and variables were normalized. Multiple comparisons were performed by one-way or two-way analysis of variance (ANOVA). Significant intergroup differences were subsequently tested by post hoc Bonferroni statistical analysis. A statistical probability of $P \leq .05$ was considered significant.

3. Results

3.1. DHA attenuates macrophage inflammation

Firstly, we examined the effects of chronic EPA and DHA treatment on LPS-stimulated J774.2 macrophages. EPA/DHA enrichment reduced LPS-stimulated IL-6 secretion from macrophages (Fig. 1A), while DHA further inhibited TNFα secretion (Fig. 1B) and NFκB p65 phosphorylation (Fig. 1D). DHA pretreatment enhanced LPS-induced IL-10 secretion from macrophages (Fig. 1C). Both EPA and DHA attenuated LPS-induced p38 MAP kinase phosphorylation (Fig. 1D). GC profiling of macrophages confirmed EPA and DHA incorporation into the whole cell lipid fraction (Supplemental Table 1). The polarization status of macrophages was further probed after macrophage enrichment with EPA or DHA and demonstrated a marked reduction in the number of proinflammatory M1 ($F4/80^+/CD11c^+$) macrophages with a concomitant increase in the number of anti-inflammatory M2 ($F4/80^+/CD11c^-$) macrophages in response to both fatty acids (Fig. 1E and F). Nonetheless, increased IL-10 secretion was only evident with DHA despite equivalent M1/M2 macrophage polarization status with EPA.

3.2. DHA attenuated macrophage-induced IR in adipocytes

Given the potent anti-inflammatory effects of LC *n-3* PUFA on macrophages, we used a co-culture model to examine their effects on macrophage-induced IR in 3T3-L1 adipocytes. Insulin-stimulated ^3H glucose transport and GLUT4 translocation to the adipocyte membrane (Fig. 2A and B) were attenuated after co-culture with LPS-stimulated macrophages. DHA macrophage pretreatment improved insulin sensitivity of co-cultured adipocytes as indicated by increased ^3H glucose uptake and GLUT4 translocation from the cytoplasm to the membrane. EPA pretreatment did not attenuate the negative effect of macrophages on adipocyte glucose uptake or GLUT4 translocation (data not shown). Adipocyte IRS-1 mRNA and protein expression was reduced when co-cultured with macrophages (Fig. 2C and D); however, DHA enrichment of macrophages prevented this. A similar trend was observed for GLUT4 expression, although nonsignificant (data not shown).

3.3. DHA attenuates adipocyte–macrophage co-culture inflammation

Within the co-culture model, LPS-stimulated macrophages significantly increased IL-6 secretion, which was attenuated by EPA/DHA macrophages (Fig. 3A). TNFα secretion was significantly increased by the presence of LPS-stimulated macrophages and partially attenuated by DHA (Fig. 3B). Macrophage-mediated induction of adipocyte IL-6 mRNA was markedly suppressed by DHA macrophages (Fig. 3C). DHA-enriched, but not EPA-enriched, macrophage CM markedly reduced p65 NFκB phosphorylation in 3T3-L1 adipocytes compared to macrophage CM alone (Fig. 3D). Phosphorylation of ERK and p38 MAP kinase was reduced by CM from both EPA- and DHA-enriched macrophages compared to CM from untreated macrophages (Fig. 3D). DHA-enriched macrophage CM markedly reduced NFκB-dependent luciferase activity in 3T3-fibroblasts; EPA-enriched macrophage CM did not (Fig. 3E).

3.4. IL-10 pretreatment attenuates CM-induced adipocyte IR, while IL-10 neutralization blocks the beneficial effects of DHA on macrophage-induced adipocyte IR

We hypothesized that IL-10 may contribute to the attenuated inflammatory environment and reduced IR in co-cultured adipocytes. IL-10 secretion was enhanced by DHA-pretreated macrophages in the co-culture system (Fig. 4A). Adipocyte IL-10 mRNA expression was significantly increased when co-cultured with DHA-enriched macrophages (Fig. 4B). IL-10 did not affect insulin-stimulated ^3H glucose transport in the absence of CM. However, IL-10 pretreatment maintained insulin-stimulated ^3H glucose uptake into adipocytes exposed to CM, compared to adipocytes exposed to CM alone (Fig. 4C). IL-10 pretreatment attenuated CM-induced NFκB activation in NIH-3T3 gene-reporter fibroblasts (Fig. 4D). CM-induced adipocyte p38 MAP kinase phosphorylation (Fig. 4E) and IL-6 secretion (Fig. 4F) were also attenuated by IL-10. Neutralization of IL-10 in a transwell co-culture system partially blocked the beneficial effects of DHA on macrophage-induced adipocyte IR. Fig. 4G demonstrates that co-culture with macrophages results in a 73.4%±5.08% reduction in adipocyte insulin sensitivity. Pretreatment with DHA partially restored insulin sensitivity (29.4%±4.6% reduction of insulin sensitivity compared to control). Co-incubation with an IL-10 neutralizing antibody partially prevented these beneficial effects of DHA (57.3%±12.9% reduction in insulin sensitivity).

Our results suggest that differential effects between EPA and DHA may be partially mediated via DHA-derived IL-10. We therefore reexamined detailed FA data from a large human cohort of 877 matched pairs of metabolic syndrome cases and controls [24] with respect to possible differential effects of EPA and DHA. This showed that plasma DHA (above median of 2.36% of total measured fatty acids), but not EPA (above median of 0.81% of total measured fatty acids), levels were associated with significantly lower IR as defined by HOMA-IR (1.94±0.07 vs. 1.70±0.05, P=.009) (Fig. 4H).

4. Discussion

We demonstrated that chronic pretreatment of macrophages with DHA, but not EPA, attenuated the proinflammatory, insulin desensitizing effect of macrophages when co-cultured with 3T3-L1 adipocytes. This effect may be ascribed to enhanced IL-10 and reduced IL-6 secretion from DHA-enriched macrophages, which alters macrophage-derived cytokine cross-talk with adipocytes. Consistent with this, we demonstrated that the proinflammatory, insulin desensitizing effects of macrophage CM were attenuated by IL-10, while the beneficial effects of DHA were partially circumvented in the presence of an IL-10 neutralizing antibody.

Previously, we demonstrated that DHA more effectively reduced cytokine secretion and nuclear NFκB p65 levels and impeded p65 nuclear binding to a greater extent than EPA in THP-1 macrophages [15,16]. In this study, we extended our findings and demonstrated that chronic DHA pretreatment drives macrophages towards a less inflammatory phenotype with reduced IL-6 secretion and NFκB p65 phosphorylation and enhanced IL-10 secretion. Our data compliment a recent study demonstrating that LC *n-3* PUFA supplementation in mice fed an HFD reduced infiltration of $CD11c^+$ and $CD11b^+$ ATM, with lower M1 proinflammatory genes but increased M2 anti-inflammatory gene expression in adipose tissue [18]. The authors ascribed improved insulin sensitivity to inhibition of macrophage-induced inflammation in adipose. Indeed, we show that EPA and DHA

have equal effects in reducing F4/80$^+$ CD11c$^+$ M1 macrophages and enhancing F4/80$^+$ CD11c$^-$ M2 macrophages *in vitro*. We further define the DHA specific effects of LC *n-3* PUFA on cellular cross-talk between adipocytes and macrophages pretreated with DHA versus EPA in a co-culture model. There are striking similarities with respect to the differential effects of DHA versus EPA on adipocyte–macrophage biology within the context of IR and that of vascular endothelial cell functionality in atherosclerosis. In a recent study, Wang et al. (2011) demonstrated that DHA was a more potent attenuator of adhesion molecular expression, mediated through the I-κB/NF-κB dependent pathway [29]. The similarity between disease contexts is not surprising given the common NF-κB involvement.

Lumeng et al. established that macrophage-secreted factors induce IR in adipocytes via down-regulation of insulin signaling targets [8]. Consistent with this, we observed that the proinflammatory environment induced by macrophages resulted in an IR state in co-cultured adipocytes coincident with a marked reduction in IRS-1 expression and GLUT4 translocation to the cell membrane. Furthermore, we demonstrated that insulin-stimulated ^{3}H glucose uptake, IRS-1 expression and GLUT4 translocation to the membrane were restored by DHA pretreatment of macrophages prior to co-culture. In contrast, macrophage EPA pretreatment was insufficient to protect adipocytes from macrophage-induced IR.

We hypothesized that protection from the insulin desensitizing effects of DHA-enriched macrophages was due to altered balance of pro- and anti-inflammatory cytokine secretion into co-culture media coincident with attenuated inflammatory pathway activation in adipocytes. We confirmed that chronic DHA treatment more effectively reduced IL-6 and TNFα secretion in the co-culture and adipocyte IL-6 mRNA expression, while EPA and DHA both down-regulated macrophage-induced MAP kinase activity (p38 and ERK) in adipocytes. DHA-enriched macrophage CM exclusively inhibited adipocyte p65 NFκB phosphorylation and NFκB-dependent luciferase activity in gene reporter fibroblasts. These findings demonstrate less inflammatory cytokine cross-talk between DHA-enriched macrophages and adipocytes.

Only DHA-enriched macrophages enhanced IL-10 secretion when co-cultured with adipocytes coincident with enhanced adipocyte IL-10 mRNA expression. We speculated that IL-10 from the DHA-enriched macrophages may partially mediate the anti-inflammatory and insulin-sensitizing effects and demonstrated partial loss of protection with DHA treatment in the presence of an IL-10 neutralizing antibody. Furthermore, pretreatment of adipocytes with IL-10 prevented CM-induced IR and was associated with marked reduction in IL-6 secretion, lower NFκB activity and reduced adipocyte p38 phosphorylation. Thus, IL-10 in concert with reduction in proinflammatory cytokines such as IL-6 mediated the protective effect of DHA. Lumeng et al. observed that pretreatment of 3T3-L1 adipocytes with IL-10 blocked acute TNFα-induced IR [14]. However, our model using macrophage CM reflects a more physiologically relevant milieu of pro- and anti-inflammatory mediators secreted from macrophages in adipose tissue.

Taken together, our results provide novel insights into different EPA and DHA specific effects on macrophage–adipocyte cross-talk, which suggest that DHA is more effective at attenuating macrophage-induced adipocyte IR. In humans, the effects of LC *n-3* PUFA on obesity-induced IR are inconsistent. Different doses with variable EPA/DHA mixes may contribute to divergent responses. Reanalysis of a large human cohort [28] with respect to possible differential effects of EPA and DHA showed that high plasma DHA, but not EPA, was associated with lower HOMA-IR. Studies indicate a positive dose-dependent relationship between dietary LC *n-3* PUFA intake with EPA/DHA plasma levels resulting in lower peripheral blood mononuclear cell proinflammatory cytokine secretion [30]. There is no doubt that the DHA concentrations used in the *in vitro* co-culture model are higher in comparison to potential plasma or adipose tissue concentrations in man even following LC *n-3* PUFA supplementation. While this is a potential limitation of our work, *in vitro* studies rely on a greater short-term lipid intervention which cannot account for the longer, time-dependent accumulation of LC *n-3* PUFA which occurs in man. Further work is required to examine if chronic DHA supplementation in humans can bias macrophages to a less inflammatory phenotype and provide protection from macrophage-induced IR within the adipose tissue of obese individuals.

5. Conclusion

We demonstrate that DHA promotes an anti-inflammatory macrophage phenotype with increased IL-10 secretion, which protects co-cultured adipocyte from IR.

Supplementary materials related to this article can be found online at doi:10.1016/j.jnutbio.2011.06.014.

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