Journal of Alzheimer's Disease 35 (2013) 697–713
DOI 10.3233/JAD-130131
IOS Press

Omega-3 Fatty Acids Enhance Phagocytosis of Alzheimer's Disease-Related Amyloid-β_{42} by Human Microglia and Decrease Inflammatory Markers

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Accepted 12 February 2013

Abstract. The use of supplements with omega-3 (ω3) fatty acids (FAs) such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) is widespread due to proposed beneficial effects on the nervous and cardiovascular systems. Many effects of ω3 FAs are believed to be caused by down-regulation and resolution of inflammation. Alzheimer's disease (AD) is associated with inflammation mediated by microglia and astrocytes, and ω3 FAs have been proposed as potential treatments for AD. The focus of the present study is on the effects of DHA and EPA on microglial phagocytosis of the AD pathogen amyloid-β (Aβ), on secreted and cellular markers of immune activity, and on production of brain-derived neurotrophic factor (BDNF). Human CHME3 microglial cells were exposed to DHA or EPA, with or without the presence of $A\beta_{42}$. Phagocytosis of $A\beta_{42}$ was analyzed by flow cytometry in conjunction with immunocytochemistry using antibodies to cellular proteins. Secreted proteins were analyzed by ELISA. Both DHA and EPA were found to stimulate microglial phagocytosis of $A\beta_{42}$. Phagocytosis of $A\beta_{42}$ was performed by microglia with a predominance of M2 markers. EPA increased the levels of BDNF in the culture medium. The levels of TNF-α were decreased by DHA. Both DHA and EPA decreased the pro-inflammatory M1 markers CD40 and CD86, and DHA had a stimulatory effect on the anti-inflammatory M2 marker CD206. DHA and EPA can be beneficial in AD by enhancing removal of $A\beta_{42}$, increasing neurotrophin production, decreasing pro-inflammatory cytokine production, and by inducing a shift in phenotype away from pro-inflammatory M1 activation.

Keywords: Amyloid, brain-derived neurotrophic factor, cytokine, DHA, EPA, interleukin, M1, M2, resolution

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ISSN 1387-2877/13/$27.50

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative and progressive disease characterized by the impairment and death of neurons, increased levels of the amyloid-β (Aβ) peptide [1], increased presence of intracellular tangles composed of hyperphosphorylated forms of the microtubule protein tau [2], and chronic inflammation [3–5]. Inflammation is prominent in AD as evidenced by activated microglia and astrocytes [6], and increased levels of pro-inflammatory cytokines in the brain [3, 5].

On a clinical level, AD is characterized by cognitive deficits that initially affect learning and memory, but later in the disease are manifested by a global cognitive decline. The clinical symptoms mirror the pathological changes in the brain, where the neuronal loss and plaque/tangle pathology begin in memory-related areas (entorhinal cortex, hippocampus), and then spread to other parts of the cortex [7, 8].

It is believed that increased levels of Aβ represent one of the culprits for the disease [9]. Aβ is produced by intramembranous cleavage of the Aβ protein precursor (AβPP), yielding a peptide that is prone to aggregation, thus forming oligomers, fibrils, and plaques [10]. Studies on AD brains and data from animal studies show the presence of activated glial cells around the plaques [5, 11, 12]. It is unclear whether inflammation is an initial driving force in the pathogenesis of AD or a consequence of the disease. Aβ has been shown to induce inflammation [13–19], with release of cytokines and other inflammatory factors. In turn, inflammatory cytokines have been shown to increase the production of Aβ [15, 20, 21], suggesting the presence of a vicious, self-reinforcing circle between inflammation and Aβ production and, thus, a role for inflammation in the primary pathology of AD.

Clearance of debris by phagocytosis and secretion of neurotrophic growth factors from activated astrocytes and microglia are activities related to inflammation that can protect and improve the function of neurons [22–24]. However, in the case of chronic inflammatory states, such as in AD, the detrimental and tissue-damaging activities of inflammation are dominating [25]. Early evidence of a role for inflammation in AD pathogenesis originates from epidemiological studies, showing reduced prevalence of AD in patients under treatment with non-steroidal anti-inflammatory drugs (NSAIDs) ([26], see [27]).

Dietary supplementation with the ω3 fatty acids (FAs) docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) is very common, and has been ascribed several health benefits related to the nervous system [28–31]. Furthermore, the levels of polyunsaturated fatty acids (PUFAs, i.e., ω3) have been shown to be reduced in postmortem brain tissue [32], and in plasma samples [33], from AD patients. Several studies have shown that DHA and EPA can exert anti-inflammatory activities, and their beneficial effects are commonly ascribed to this property (see [34, 35]). *In vitro* studies have shown a suppressing effect by EPA on inflammatory cytokine production in human macrophages [36], and DHA has been shown to down-regulate inflammatory proteins *in vitro* [37] and *in vivo*. Dietary supplementation with DHA-rich ω3 FAs resulted in increased plasma concentrations of DHA (and EPA) in AD patients, and this was associated with reduced release of interleukin (IL)-1β, IL-6, and granulocyte colony-stimulating factor from peripheral blood mononuclear cells *ex vivo* [39]. DHA is the ω3 FA that has received most attention for its beneficial effects on factors related to AD [40–44]. Authors of this study performed the first completed intervention study in which AD patients were treated with ω3-supplements (the OmegAD study) [45]. A beneficial effect was not found, except in a subgroup with mild symptoms, suggesting the importance of early intervention. Similar studies performed after this study have provided little support for a curative effect of ω3 FAs on established AD, but rather emphasizing the findings of a potential benefit on early AD [46].

DHA and EPA are intimately associated with the resolution phase of inflammation. In this final stage of the immune response, cleaning, healing, and regeneration of the tissue take place for the restoration of homeostasis, concurrent with a down-regulation of the inflammatory response. The increased phagocytic activity in the resolution phase is not associated with inflammatory activation (non-phlogistic phagocytosis) (e.g., [47]). Resolution is induced by specialized pro-resolving mediators (SPMs), that are derivatives of FAs [34, 35, 48]. The SPMs include the resolvin E series, derived from EPA [48], and the resolvin D series, derived from DHA [44, 49]. Studies on mice have shown that healing and regeneration of the tissue is promoted by increased levels of growth factors, emphasizing the role of growth factors in resolution [50, 51].

An effective treatment for AD is lacking. Reducing the levels of Aβ and the inflammation in the AD brain, while at the same time increasing the neurotrophic signaling, is a reasonable approach for the treatment of AD. The capacity to phagocytose Aβ by microglia has been demonstrated in *in vitro* studies

[52]. Interestingly, this phagocytic capacity was shown to be reduced in microglia incubated with human cryostat sections containing amyloid plaques [53], suggesting that the environment in AD brain tissue has "anti-resolving" properties. Increased clearance of Aβ can be accomplished by stimulating microglia into increased phagocytosis *in vitro* [54]. Phagocytosis in the acute phase of inflammation may have neurotoxic consequences, not desirable from a treatment point of view. However, the prominent phagocytic activity during the resolution phase of inflammation is a promising therapeutic target. Therefore, we have performed a series of experiments on human microglial cells, in which the capacity of DHA and EPA to stimulate phagocytosis of $A\beta_{42}$ was the primary outcome. The effects on cell-associated and secreted markers and effectors of immune activation were also investigated. The M1/M2 polarization axis is commonly applied to the interpretation of the responses of macrophages and similar cells, such as microglia. To establish the effect on phenotype polarization, we investigated the M1 activation marker CD40 [55, 56] and the co-stimulatory factor CD86. We also analyzed the M2 markers CD163 and CD206, as well as the deactivating receptor CD200R. CD163 and CD206 also have a direct link to phagocytosis by their ability to mediate this through recognition of pathogens or debris [57, 58].

We analyzed the secretion of the important memory-related neurotrophin brain-derived neurotrophic factor (BDNF), a protein shown to be decreased in the brain of AD-patients [59, 60], and of the inflammatory (M1) cytokines IL-6 and tumor necrosis factor (TNF)-α, shown to be increased in AD [3, 61]. The anti-inflammatory M2 cytokine IL-10 was also measured.

MATERIALS AND METHODS

Chemicals

DHA and EPA were purchased from Nucheckprep (Elysian, USA). $A\beta_{42}$ conjugated with HiLyteFluor-488 or biotin was obtained from Anaspec (Fremont, USA). Dimethylsulfoxide (DMSO), Triton-X100, and bovine serum albumin (BSA) were purchased from Sigma (Stockholm, Sweden). Normal donkey serum and fluorescence mounting medium were purchased from DakoPatts (Stockholm, Sweden). Donkey anti-rabbit Northern Light-647 conjugated antibodies, and ELISA-kits for IL-6 IL-10, TNF-α, and BDNF were purchased from R&D systems (Abingdon, United Kingdom). Cell culture medium, phosphate-buffered saline (PBS), GlutaMaxII, fetal calf serum (FCS), and PBS-based enzyme-free cell dissociation buffer, were purchased from Invitrogen (Stockholm, Sweden). Cell culture bottles and multi-well plates were purchased from BD Biosciences (Stockholm, Sweden).

Cell cultures

Human microglial cells (CHME3) were obtained as a kind gift from Prof. M. Tardieu, Neurologie pédiatrique, Hôpital Bicêtre, Hôpitaux de Paris, Paris, France. The CHME3 cells were cultured in T75 or T175 bottles in culture medium (DMEM/high glucose w/o sodium pyruvate, supplemented with GlutaMaxII and 10% heat-inactivated FCS). The cells were subcultured at confluence using enzyme-free cell dissociation buffer, after washing once with PBS without Mg^{2+} and Ca^{2+}.

For flow cytometry and analysis of secretory products, the CHME3 microglial cells were seeded in 6-well plates. The CHME3 cells are quick proliferators, and care must be taken so the cultures do not become over-confluent. In our experience, starting the experiments at 60–70% confluency of the cells produce cultures of 80–85% confluency after 24 h, thus avoiding over-confluency.

$A\beta_{42}$ was dissolved in DMSO to a concentration of 5 mg/ml, and stored in darkness at +4°C until use in a final concentration of 1 μg/ml for all experiments on cells. DHA and EPA were diluted in 95% EtOH to a concentration of 200 mM and stored in a nitrogen atmosphere. Before the experiments, the cells are washed with serum-free medium, and then treated with $A\beta_{42}$ or vehicle (DMSO) together with DHA, EPA, or vehicle (95% EtOH), also in serum-free medium. The final concentration of DMSO in the cultures was 0.02%, and the final concentration of EtOH was 0.00125%.

Assessment of aggregational forms of $A\beta_{42}$ by immunoblotting

$A\beta_{42}$ peptide was diluted with DMEM/high glucose culture medium to the final concentration of 1 μg/ml (used in the cell experiments) and 5 μg/ml, and seeded into a 24-well plate. Medium without $A\beta_{42}$ was used as control. All the samples were incubated at +37°C and 5% CO_2 in a cell culture incubator for 6 and 24 h. Freshly dissolved $A\beta_{42}$ peptide was prepared before start of the electrophoresis. In order to keep the state of aggregation of $A\beta_{42}$ at the time points studied, all the samples were treated with the cross-linker glutaraldehyde at a final concentration of 2.5%. Equal volume

of sample buffer (75 μl) was added to each sample (75 μl) and incubated 10 min before loading the gel. We found that $A\beta_{42}$ is prone to stick to the plastic of the cell culture well. In order to detach this, each well was flushed with 75 μl sample buffer by trituration, and then mixed with an equal volume of the medium previously removed. Proteins were separated by electrophoresis, followed by overnight transfer to 0.22 mm nitrocellulose membrane. After transfer, the membranes were incubated with 6E10 primary antibody diluted 1:700 (Nordic Biosite, Sweden). The 6E10 antibody is raised against the COOH-terminus of Aβ peptide. The membranes were then incubated with horse-radish peroxidase (HRP)-conjugated sheep anti-mouse secondary antibody diluted 1:1000 (GE Healthcare, Sweden), after which chemiluminescence (ECL™ Prime, GE Healthcare) reagent was applied and the membranes analyzed with a CCD camera (Fujifilm LAS-3000 luminescent image analyzer).

The amount of fibrillar $A\beta_{42}$ in the culture medium was analyzed using the Thioflavin-T (ThT) assay. Culture medium incubated with 0, 1, and 5 μg/ml $A\beta_{42}$ for 6 and 24 h, as well as medium in which the $A\beta_{42}$ was freshly dissolved, were analyzed. In short, ThT was prepared in a stock solution of 25 mM, diluted in PBS to 50 μM, in which the medium to be analyzed was prepared to a final concentration of 25 μM ThT, and incubated at +37°C for 20 min, followed by analysis in a Tecan plate reader (excitation wavelength: 440 nm; emission wavelength: 480 nm). To control the validity of the ThT assay, $A\beta_{42}$ (0, 2.5, 25, and 250 μg/ml) was incubated in PBS for 96 h and analyzed with the same method.

Quantification of Aβ42 phagocytosis and cellular markers by flow cytometry

To investigate the influence of DHA and EPA on phagocytosis of $A\beta_{42}$ the CHME3 microglial cells were exposed to 1 μg/ml of this peptide together with DHA or EPA, or vehicle (EtOH). The cells were harvested at 2, 6, and 24 h, and analyzed by flow-cytometry for phagocytosis of $A\beta_{42}$ and the presence of inflammatory and phenotype markers. The levels of secreted products were analyzed by ELISA (see below).

After the treatments, the CHME3 microglial cells were dissociated with PBS-based enzyme-free dissociation buffer, and centrifuged at 1500× *g* for 10 min. The cells were then resuspended and fixed in 300 μl of 1% para-formaldehyde in PBS for 40 min at room temperature. Subsequently, the cells were washed by addition of 10 ml PBS followed by centrifugation at 1500× *g* for 10 min, removal of supernatant, resuspension in 300 μl of PBS, and then stored at +4°C in darkness.

Analysis of phenotype markers by directly conjugated antibodies

After harvesting and fixation, the cells were incubated with phytoerythrin (Biolegend)-conjugated antibodies to CD40 (diluted 1:200), CD86 (diluted 1:100) and CD200R (diluted 1:100), respectively, and with AlexaFluor647 (Biolegend)-conjugated antibodies to CD163 (diluted 1:50) and anti-CD206 (diluted 1:100). After incubation with the antibodies at +4°C for 40 min, the cells were washed by adding PBS and centrifuged at 2000× *g* for 10 min, after which the pelleted cells were resuspended and analyzed with flow-cytometry using the corresponding isotype control (Biolegend) to set the limit of background for each antibody.

Enzyme-linked immunosorbent assay (ELISA)

The levels of IL-6, IL-10, TNF-α, and BDNF were analyzed in the cell culture medium obtained from experiments terminated at 24 h. After dissociation and centrifugation as described above, the culture medium was stored at −20°C until analysis with commercially available ELISA-kits. To detect the low levels of IL-10 and TNF-α in the medium, the assay protocol was adjusted so that the samples were incubated overnight in +4°C in addition to the 2 h at room temperature. In the case of IL-10 and TNF-α ELISAs, QuantaRed fluorescent substrate (Thermo Scientific) was used instead of the colorimetric substrate in the standard protocol. Analysis of optical density (OD), or fluorescence, was performed in a TECAN Safire2 plate reader.

Statistical analyses

The presented data are the result from 10–14 individual experiments. In each experiment, the data for each treatment were normalized to the averaged data of that particular parameter in that experiment. To allow for parametric statistical analysis, the normalized data were logarithmized (natural logarithm, e), after which the data were analyzed for systematic variance using ANOVA. When significant variance was found, pair-wise comparison between groups using Fisher's *post hoc* test was performed. The comparison between different variables was performed with the

non-parametric Wilcoxon matched-pairs test on non-normalized and non-logarithmized data. The graphs show the normalized and logarithmized data. All statistical analyses were performed in Statistica v10 (Statsoft).

RESULTS

Assessment of aggregational forms of $A\beta_{42}$

Immunoblotting was performed to investigate which aggregation forms of $A\beta_{42}$ that the microglial cells were exposed to in the experiments (Fig. 1). The concentration (1 μg/ml) used in the treatment experiments, as well as a higher concentration (5 μg/ml), were analyzed. No bands were visible in the control (medium without addition of $A\beta_{42}$) (data not shown). The results show a mixture of lengths of $A\beta_{42}$ oligomers. Bands corresponding to the size of monomers and dimers were seen at all incubation times, as well as in the freshly dissolved $A\beta_{42}$ preparation, of both concentrations (Fig. 1A). Due to the weak luminescence signal of the bands from incubation of 1 μg/ml $A\beta_{42}$, image analysis was performed on bands obtained from 5 μg/ml $A\beta_{42}$ (Fig. 1B). Bands corresponding to the size of tetramers (19.2 kD), hexamers (26.8 kD), and other large oligomers (>70 kD) were observed. Factorial ANOVA (Fig. 1B) showed that the intensity of bands corresponding to monomers was stronger than that of tetramers, hexamers, and large oligomers after 0, 6, and 24 h of incubation ($p < 0.005-0.05$), indicating a predominance of $A\beta_{42}$ monomers. No fibrillar $A\beta_{42}$ was detected by the ThT-assay at any time point or concentration tested (data not shown).

Effects of ω3 FAs on phagocytosis of $A\beta_{42}$

Exposure of the human CHME3 microglial cells to 1 μg/ml of $A\beta_{42}$ for 2, 6, and 24 h did not result in any visible changes in morphology or other apparent signs of activation. Neither did the treatment with DHA or EPA change the morphology.

Incubation of the microglia for 2 h with DHA in the concentrations of 0.1, 0.5, and 1 μM, significantly increased phagocytosis ($p < 0.005$, $p < 0.05$, and $p < 0.05$, respectively) as compared to control (treatment with $A\beta_{42}$ alone) (Fig. 2A). At 24 h, all concentrations increased the number of microglia showing phagocytosis of $A\beta_{42}$ ($p < 0.005$–0.05).

Treatment with EPA in the concentrations of 0.01, 0.5, and 1 μM for 2 h increased the number of $A\beta_{42}$-positive cells above control ($A\beta_{42}$ alone) ($p < 0.005$–0.05). Results from incubation for 6 h did not reach significant variance. Similar to DHA, the EPA-stimulated phagocytosis of $A\beta_{42}$ regained vigor after 24 h of incubation, showing a significant variance. The *post hoc* test showed that 0.01, 0.5, and 1 μM EPA increased the number of $A\beta_{42}$-positive cells with 24% above control level ($p < 0.005$–0.05) (Fig. 2B). At 24 h, significant differences between these concentrations indicate a bell-shaped dose-response curve.

Effects of ω3 FAs on cellular markers related to M1-activation

To investigate the immunological phenotype of the human microglia, markers for M1 (CD40 and CD86) activation were used.

CD40

The basal level (vehicle control) of CD40-positive microglia was 57.8% at 2 h, 53% at 6 h, and 58.4% at 24 h. DHA did not produce any changes in the number of positive cells until at 24 h (Fig. 3A), when a decrease was observed with 0.5 μM ($p < 0.05$) and 1 μM ($p < 0.01$), as compared to control.

The level of CD40-positive microglia when incubated with $A\beta_{42}$ alone was 40% at 2 h, 26.2% at 6 h, and 21.2% at 24 h. No significant effects could be seen upon co-incubation of the cells with $A\beta_{42}$ and DHA.

Incubation with EPA did not have statistically significant effects on the number of CD40-positive microglia, but in the experiments on EPA together with $A\beta_{42}$, a significant decrease in the number of CD40-positive cells could be seen at 6 h with 0.5 μM EPA ($p < 0.0005$, Fig. 3B), as compared with control ($A\beta_{42}$ alone).

CD86

The basal level (vehicle control) of CD86-positive microglia was 8.5% at 2 h, 7.5% at 6 h, and 10.4% at 24 h. At 24 h, treatment with DHA resulted in a statistically significant decrease in the number of CD86-positive cells as compared to control ($p < 0.05$) (Fig. 4A, left panel).

The level of CD86-positive microglia when incubated with $A\beta_{42}$ alone was 7.8% at 2 h, 4.2% at 6 h, and 6.6% at 24 h. For both DHA and EPA, co-incubation of the microglia with $A\beta_{42}$ induced more prominent effects than when each FA was added alone. Co-incubation of the cells with $A\beta_{42}$ and DHA gave a hint of a bell-shaped dose-response curve, which was supported by a significant variance in the data ($p < 0.0005$, Fig. 4A, right panel). At 6 h, the lowest

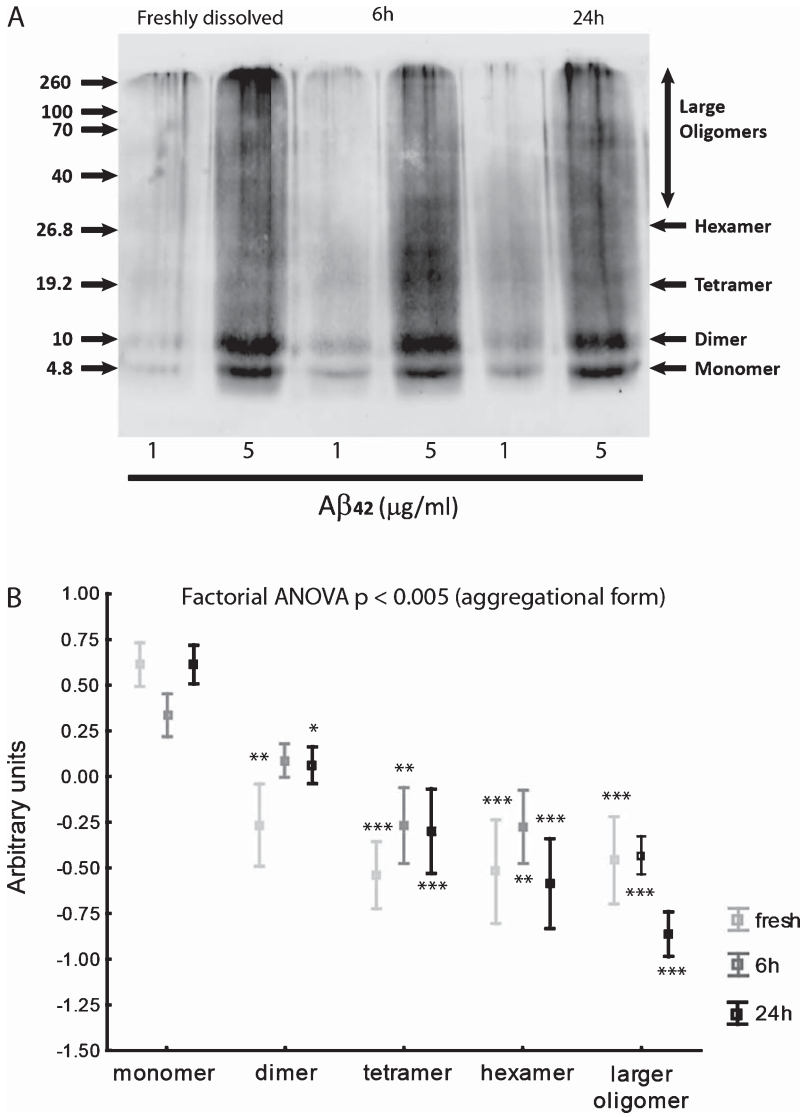

Fig. 1. Forms of aggregation of $A\beta_{42}$ in DMEM/high glucose culture medium. Immunoblotting of 5 μg/ml $A\beta_{42}$ after incubation for 0, 6, and 24 h in cell culture conditions (37°C, 5% CO_2) using the 6E10 antibody raised against the COOH-terminus of Aβ peptide (A). Quantification of chemiluminescence followed by statistical analysis by factorial ANOVA showed a significant variance by treatment at all times (B). The *post hoc* test shows high abundance of monomers. There was no significant variance by time of incubation. The graph shows data from 10 individual experiments. The data were logarithmized (ln). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$ as compared to control (monomeric $A\beta_{42}$). Squares indicate mean and error bars show ± standard error of the mean (SEM).

(0.05 μM), and highest (1 μM) concentrations of DHA resulted in a decrease in the number of CD86-positive microglia as compared to $A\beta_{42}$ alone ($p < 0.01$ and and $p < 0.005$, respectively). At 24 h, the reduction in CD86-positive cells induced by 0.05 and 1 μM DHA was still present ($p < 0.05$). The image of a bell-shaped dose-response relationship was even stronger than at 6 h and supported by significant variance in the data ($p < 0.05$).

The only effect of EPA alone on the number of CD86-positive microglia was a reduction by 0.5 μM at 24 h ($p < 0.05$, Fig. 4B, left panel). Co-incubation of the cells with EPA and $A\beta_{42}$ also (similar to DHA) resulted in data for which a bell-shaped dose-response relationship could be discerned. In contrast to DHA, the bell-shaped curve seen for EPA appeared already at 2 h (Fig. 4B, right panel). Thus, at 2 h the lowest (0.005 μM) and highest (1 μM) concentration of EPA

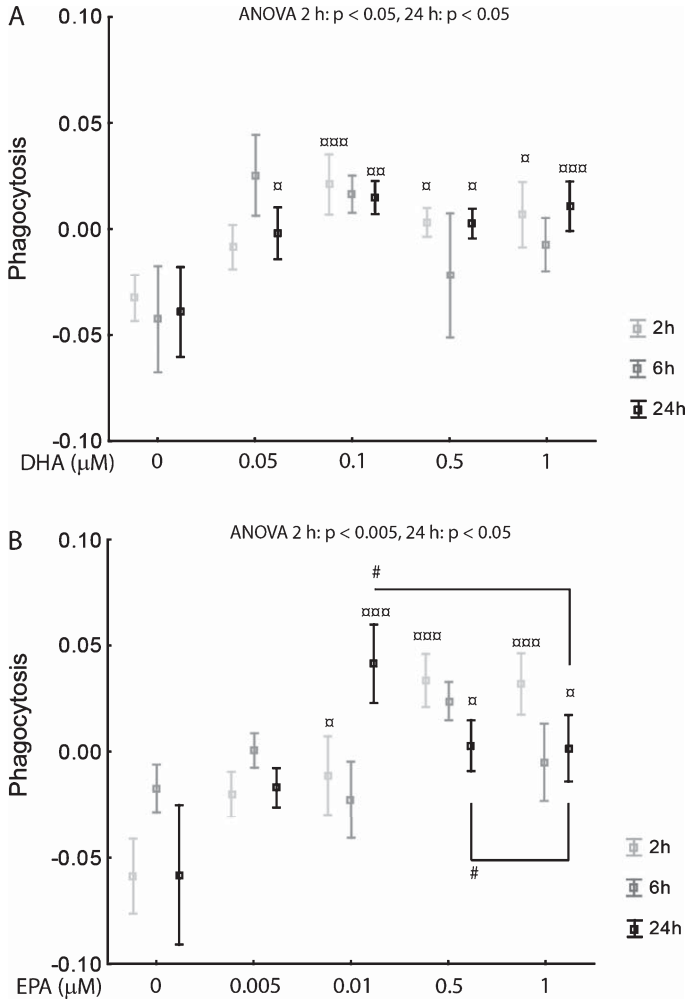

Fig. 2. Effects of DHA and EPA on microglial phagocytosis of $A\beta_{42}$ analyzed by flow-cytometry. Human CHME3 microglia were incubated with DHA (A) or EPA (B) in the presence of 1 μg/ml $A\beta_{42}$ for 2, 6, and 24 h. ANOVA showed significant variance at 2 and 24 h for both DHA and EPA. The *post hoc* test shows significant increase in phagocytosis by all concentrations of DHA, by 0.01, 0.5, and 1 μM EPA as compared to control ($A\beta_{42}$ alone), and a significant difference between different concentrations of EPA at 24 h. The graphs show data from 11 individual experiments. The data were logarithmized (ln). ¤$p < 0.05$, ¤¤$p < 0.01$, ¤¤¤$p < 0.005$ compared with $A\beta_{42}$ alone. #$p < 0.05$ for comparison between different concentrations of EPA (lines indicate the comparisons of concentrations). Squares indicate mean and error bars show ± standard error of the mean (SEM).

reduced the number of CD86-positive cells ($p < 0.05$ and $p < 0.005$, respectively). At 6 and 24 h, the effect of EPA on CD86 had dissipated.

Effects of ω3 FAs on cellular markers related to M2-activation

The number of cells bearing the M2 markers CD163 and CD206 was also investigated after exposure of the microglia to FAs with and without $A\beta_{42}$. There was a smaller number of microglia with M2 markers as compared to the M1 marker CD40 at basal conditions.

CD163

The basal level (vehicle) of CD163-positive microglia was 9% at 2 h, 12% at 6 h, and 10.8% at 24 h. The level of CD163-positive microglia when incubated with $A\beta_{42}$ alone was 8.6% at 2 h, 15% at 6 h, and 13.4% at 24 h. Treatment with DHA or EPA at any concentration tested did not induce any significant change in the number of CD163-positive cells. This was true both

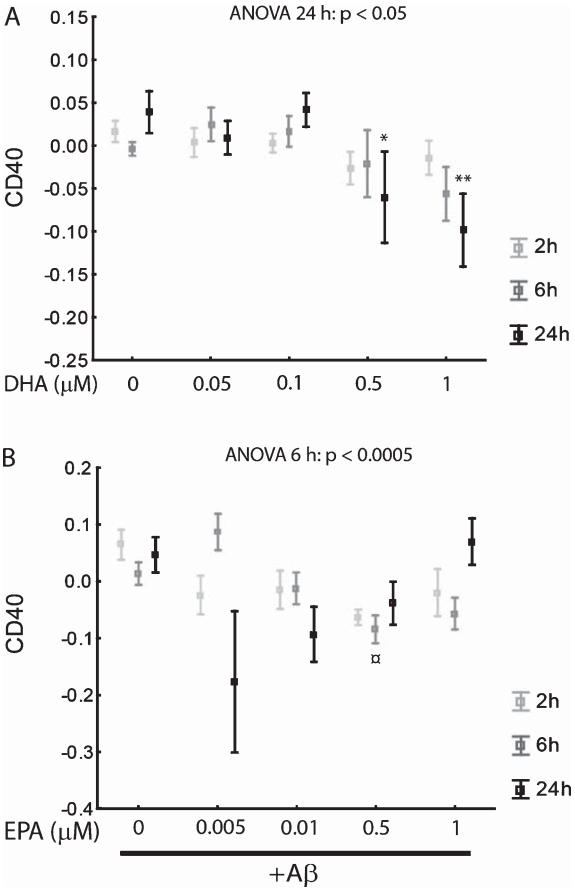

Fig. 3. Effects of DHA and EPA on the number of microglial cells labeled for CD40. Human CHME3 microglia were incubated with DHA (A) or EPA (B) for 2, 6, and 24 h, with (B) or without (A) 1 μg/ml $A\beta_{42}$. ANOVA showed no significant variance, except at 24 h, when treatment with DHA alone, and with EPA together with $A\beta_{42}$, resulted in significant effects. The *post hoc* test shows significant decrease in the number of CD40-positive microglia upon incubation with the FAs. The graphs show data from 11 individual experiments. The data were logarithmized (ln). $^{*}p < 0.05$, $^{**}p < 0.01$, compared with vehicle. ¤$p < 0.05$ compared with $A\beta_{42}$ alone. Squares indicate mean and error bars show ± standard error of the mean (SEM).

for cultures treated with FAs alone, and in combination with $A\beta_{42}$ (data not shown).

CD206

The basal level (vehicle control) of CD206-positive microglia was 14.3% at 2 h, 9.5% at 6 h, and 10.9% at 24 h. At 6 h, incubation with 0.5 and 1 μM DHA reduced the number of CD206-positive cells as compared to control ($p < 0.05$ and $p < 0.005$, respectively) (Fig. 5). This result was reversed at 24 h, when 0.1 and 0.5 μM DHA increased the number of CD206-positive microglia ($p < 0.005$ and $p < 0.05$, respectively). The highest dose of DHA resulted in a significant decrease in the number of CD206-positive cells, as well as with 0.1 and 0.5 μM DHA, indicating a bell-shaped dose-response curve at 24 h.

The level of CD206-positive microglia when incubated with $A\beta_{42}$ alone was 12.9% at 2 h, 7.7% at 6 h, and 9.8% at 24 h. Treatment with DHA together with $A\beta_{42}$ did not produce any significant changes in the number of CD206-positive microglia at any concentration or time point tested (data not shown).

Treatment with EPA alone or in combination with $A\beta_{42}$ did not produce any significant change in the number of CD206-positive microglia at any concentration or time point tested (data not shown).

Effects of ω3 FAs on the immunosuppressive receptor CD200R

The basal level (vehicle control) of CD200R-positive microglia was 8.9% at 2 h, 10.2% at 6 h, and 10% at 24 h. The level of CD200R-positive microglia when incubated with $A\beta_{42}$ alone was 5.9% at 2 h, 4.5% at 6 h, and 8% at 24 h.

Treatment with DHA alone or together with $A\beta_{42}$ did not produce any significant change in the number of CD200R-positive microglia at any concentration or time point tested (data not shown).

Upon incubation of the microglia with EPA alone, there was no significant change in the number of CD200R-positive microglia at any concentration or time point tested (data not shown). Incubation of the cells with both 1 μM EPA and $A\beta_{42}$ for 6 h resulted in a significant reduction in the number of CD200R-positive microglia as compared to $A\beta_{42}$ alone ($p < 0.005$) (Fig. 6).

Microglial phenotype associated with phagocytosis

In order to establish the phenotype of microglia performing phagocytosis of $A\beta_{42}$, the proportion of microglia showing immunoreactivity to each cellular marker was analyses in the phagocytic and non-phagocytic cell population, respectively (see Supplementary data; available here: http://dx.doi.org/10.3233/JAD-130131). Microglia belonging to the phagocytic group were found to express the M2 markers CD163 and CD206 to a larger extent, and to be less prone to express the M1 marker CD40,

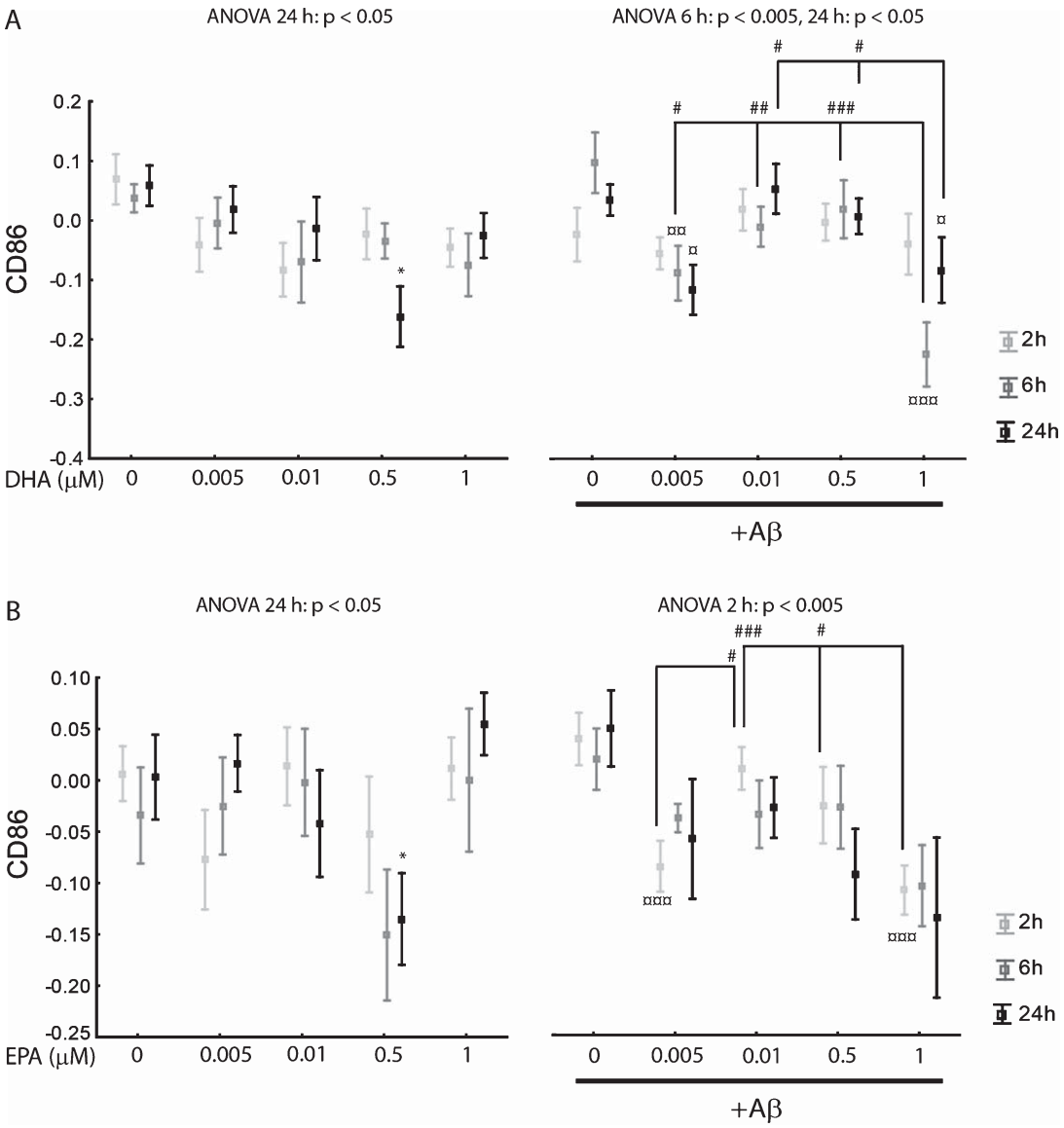

Fig. 4. Effects of DHA and EPA on the number of microglial cells labeled for CD86. Human CHME3 microglia were incubated with DHA (A) or EPA (B) for 2, 6, and 24 h, with or without 1 μg/ml $A\beta_{42}$. ANOVA showed significant variance for treatment of the microglia for 24 h with DHA both with and without $A\beta_{42}$, and for 6 h treatment with DHA together with $A\beta_{42}$. Regarding EPA, ANOVA showed significant variance for treatment at 24 h in cultures treated without $A\beta_{42}$, and for treatment at 2 h in cultures treated in the presence of $A\beta_{42}$. The *post hoc* test shows significant difference between several of the concentrations of DHA and EPA as compared to control (vehicle or $A\beta_{42}$), and in comparison between different concentrations of DHA or EPA, respectively. Thus, a decrease in CD86-positive cells was observed with both DHA and EPA alone, and when the FAs were added together with $A\beta_{42}$, bell-shaped dose-response curves can be seen. The graphs show data from 11 individual experiments. The data were logarithmized (ln). $^{*}p < 0.05$, compared with vehicle, ¤$p < 0.05$, ¤¤$p < 0.01$, ¤¤¤$p < 0.005$ compared with $A\beta_{42}$ alone, #$p < 0.05$, ###$p < 0.005$ for comparison between different concentrations of FA (lines indicate the comparisons of concentrations). Squares indicate mean and error bars show ± standard error of the mean (SEM).

by comparison of the number of cells labeled with a specific marker using the Wilcoxon matched-pairs test (see Supplementary Table 1). No significant treatment effect could be observed on the distribution of phagocytic microglia into M1 or M2 phenotypes.

Effects of ω3 FAs on the secretion of BDNF, IL-6, IL-10, and TNF-α

The secretory products BDNF, IL-6, IL-10, and TNF-α were measured in media collected after 24 h

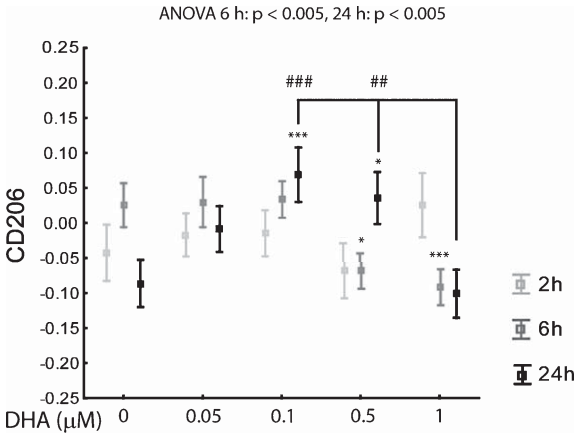

Fig. 5. Effects of DHA on the number of microglial cells labeled for CD206. Human CHME3 microglia were incubated with DHA for 2, 6, and 24 h. ANOVA showed significant variance for treatment of the microglia with DHA at 6 and 24 h. The *post hoc* test shows a significant decrease in the number of CD206-positive cells by 6 h treatment with 0.5 and 1 μM DHA as compared to control (vehicle), whereas an increase can be seen at 24 h with 0.1 and 0.5 μM DHA. At 24 h, the highest dose of DHA resulted in a significant decrease in the number of CD206-positive cells in comparison with control, as well as with 0.1 and 0.5 μM DHA, indicating a bell-shaped dose-response curve. The graphs show data from 11 individual experiments. The data were logarithmized (ln). $*p < 0.05$, $***p < 0.005$ compared with control, $\#\#p < 0.01$, $\#\#\#p < 0.005$ for comparison between different concentrations (lines indicate the comparisons of concentrations). Squares indicate mean and error bars show ± standard error of the mean (SEM).

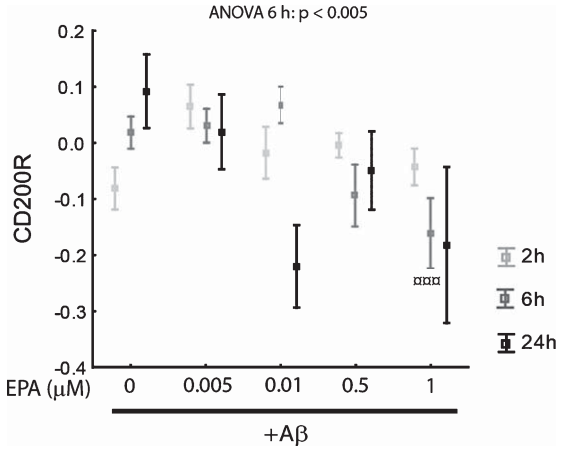

Fig. 6. Effects of EPA on the number of microglial cells labeled for CD200R. Human CHME3 microglia were incubated with EPA for 2, 6, and 24 h in the presence of 1 μg/ml $A\beta_{42}$. ANOVA showed significant variance at 6 h, and the *post hoc* test shows a significant decrease as compared to control ($A\beta_{42}$ alone). The graphs show data from 8 individual experiments. The data were logarithmized (ln). $¤¤¤p < 0.005$ compared with $A\beta_{42}$ alone. Squares indicate mean and error bars show ± standard error of the mean (SEM).

incubation with DHA and EPA, alone or together with $A\beta_{42}$, respectively.

BDNF

The mean basal secretion of BDNF from the CHME3 microglia was 463 pg/ml. $A\beta_{42}$ at 1 μg/ml did not affect this secretion. Treatment with DHA, alone or together with $A\beta_{42}$, did not alter the secretion of BDNF (data not shown). After exposure of the microglia with 0.005 and 0.01 μM EPA alone (Fig. 7A, left panel), there was an increase in BDNF levels in the medium ($p < 0.05$ and $p < 0.01$, respectively). This effect was not seen in the presence of $A\beta_{42}$ (Fig. 7A, right panel).

IL-6

The mean basal level of IL-6 in the microglial culture medium was 985 pg/ml. $A\beta_{42}$ at 1 μg/ml did not affect this secretion, and neither did DHA (data not shown). A small increase in the levels of IL-6 was seen after incubation with 0.005 and 0.01 μM EPA alone ($p < 0.05$, Fig. 7B, left panel). This effect was not seen in the presence of $A\beta_{42}$ ($p < 0.005$–0.05) (Fig. 7B, right panel).

TNF-α

The mean basal level of TNF-α in the microglial culture medium was 27.8 pg/ml. $A\beta_{42}$ at 1 μg/ml did not affect this secretion. There was no effect of DHA alone (Fig. 7C, left panel), but when co-incubated with $A\beta_{42}$, all concentrations of DHA produced a reduction in the TNF-α levels in the medium, as compared to $A\beta_{42}$ alone (Fig. 7C, right panel). The strongest effect was seen with 0.1 μM DHA ($p < 0.005$). Also, the levels of TNF-α after incubation with DHA and $A\beta_{42}$ were significantly lower as compared to the levels after treatment with DHA alone ($p < 0.005$–0.05). There was no effect of EPA alone, or together with $A\beta_{42}$, on the secretion of TNF-α (data not shown).

IL-10

The mean basal level of IL-10 in the microglial culture medium was 5.4 pg/ml. $A\beta_{42}$ at 1 μg/ml did not affect this secretion, and neither did DHA or EPA, alone or together with $A\beta_{42}$ (data not shown).

DISCUSSION

In the present study, microglia of the human cell line CHME3 were exposed to the ω3 FAs DHA and EPA in a context of AD. The data showed an increase in phagocytic removal of $A\beta_{42}$, and increased production

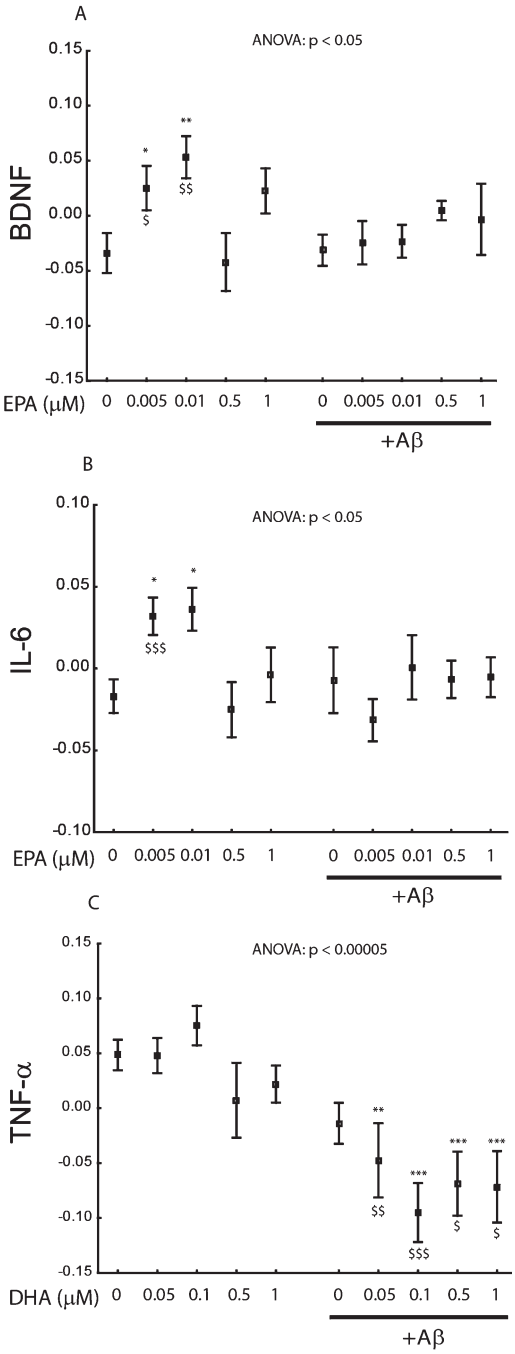

Fig. 7. Effects of EPA and DHA on the levels of brain-derived neurotrophic factor (BDNF) (A), interleukin (IL)-6 (B), and tumor necrosis factor (TNF)-α (C), respectively, in medium from cultures of microglia. Human CHME3 microglia were incubated with EPA (A, B) or DHA (C) for 24 h, with or without 1 μg/ml $A\beta_{42}$. ANOVA showed significant variance for the effects of EPA on the levels of BDNF (A) and IL-6 (B), and for the effects of DHA treatment on TNF-α levels (C). The *post hoc* tests revealed several significant differences between treatment with DHA or EPA in different concentrations compared to control (vehicle). The graphs show data from 10 individual experiments. The data were logarithmized (ln). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$ compared with control. $\$p < 0.05$, $\$\$p < 0.01$, $\$\$\$p < 0.005$ for comparison between the same concentration of EPA with and without $A\beta_{42}$. Squares indicate mean and error bars show ± standard error of the mean (SEM).

of growth factors, together with a decrease in inflammatory factors. These findings support the notion of beneficial effects of the FAs.

The microglia were incubated with 1 μg/ml $A\beta_{42}$ for time periods up to 24 h, during which no morphological changes indicating activation could be seen. In the experimental conditions used in this study, low-number oligomeric forms of $A\beta_{42}$ were present, but no fibrillar forms could be detected. The activation elicited by microglia exposed to Aβ is a function of the level and form of the Aβ, together with the expression pattern of microglial proteins that recognize Aβ. In a previous study on rat microglia, we found that the form of Aβ was determinant of both magnitude and type of cytokines secreted [17], emphasizing the importance of the form of Aβ. Studies on human THP-1 monocytes and murine microglia showed that Aβ-fibrils induce inflammatory activation in a CD36-dependent manner [62]. The fact that fibrils were absent under the conditions used in the present studies supports the view that the microglia were not activated.

Both DHA and EPA had significantly stimulatory effects on microglial phagocytosis of $A\beta_{42}$ and it appeared that this stimulation was biphasic, with an immediate stimulation, followed by a period of relative inertness, and then (at 24 h), stimulation again. Considering the plethora of cellular mechanisms that are influenced by DHA and EPA, it may be assumed that one set of mechanisms, already in place at the start of the experiments, was responsible for the stimulatory effect at 2 h. The effects on phagocytosis observed at 24 h could hypothetically have been induced by events, downstream of DHA and EPA, that require time to put the molecular machinery mediating enhanced phagocytosis in place. DHA and EPA are precursors for the SPMs resolvin, maresin, and neuroprotectin [49, 63], which have been shown to stimulate phagocytosis and down-regulate inflammation. SPMs may require some time to build up to relevant concentrations, which could be one explanation for the later effects on phagocytosis. Microglial functions influenced by DHA and EPA include ionic conductance [64], clustering of pathogen-associated molecular pattern receptors in lipid rafts [65], and activation of signaling molecules such as peroxisome proliferator-activated receptor (PPAR)-γ [66], all representing possible alternative explanations for the effects observed in this study. Activation of PPAR-γ has been shown to mediate both an anti-inflammatory effect and the stimulation of phagocytosis [67].

A study on mice fed with DHA showed that activated microglia are converted to a quiescent phenotype, and confirms an anti-inflammatory effect of DHA on microglia [68]. However, to our knowledge, the present study is the first to address the effects of ω3 FAs on microglial phagocytosis of $A\beta_{42}$ and on their M1/M2 phenotype. Several studies have shown stimulatory effects of ω3 FAs on other types of phagocytic cells of human or animal origin, such as caprine monocytes [69] and murine macrophages [70]. Also, Halvorsen and colleagues demonstrated that EPA increased adhesion of bacteria to human monocytes, indicating a positive effect on phagocytosis [71]. Studies on neutrophils and monocytes obtained from human subjects given fish oil containing DHA and EPA daily for 2 months [72] showed an increase in phagocytic activity. In contrast, 4 weeks treatment of mice with EPA or DHA did not affect the phagocytosis of *ex vivo* cultured cells as compared to placebo (olive oil) [73].

Studies on the effects of ω3 FAs on phagocytosis have, so far, been performed mainly on bacteria or apoptotic leukocytes. However, a recently published study on human macrophages *ex vivo* [74] showed a positive influence on the phagocytosis of Aβ in the presence of the DHA-derived resolvin D1. Interestingly, this SPM was shown to prevent the increase in transcription of inflammatory genes observed upon incubation with Aβ [74].

In parallel with the studies on phagocytosis of $A\beta_{42}$, the effects of DHA and EPA on microglial phenotype polarization were analyzed. Microglia expressing the M1 phenotype markers CD40 and CD86 were significantly decreased by treatment with DHA and EPA. Interestingly, this effect was enhanced upon co-incubation with $A\beta_{42}$. This leads us to hypothesize that $A\beta_{42}$ at the concentration used was not sufficient to evoke a detectable activation, but induced changes which made the microglia more responsive to DHA and EPA.

The microglial cells labeled with the M1 marker CD40 outnumbered the proportion of cells expressing the M2 markers CD163 and CD206 by approximately 10:1. The effects of DHA and EPA on CD163 were negligible. There was no effect of EPA on microglial expression of CD206, whereas DHA showed a biphasic effect, with a significant decrease in the number of CD206-positive cells at 6 h, followed by an increase at 24 h. Again, this result is suggestive of a polymodal action of the ω3 FAs. Since CD206 (macrophage mannose receptor 1) is associated with cellular ingestion, it may be speculated that the biphasic effects are associated with the effects on phagocytosis observed at the same time points, i.e., no effect at 6 h, but a stimulatory effect at 24 h. No study has yet investigated phagocyto-

sis or ingestion of Aβ mediated by CD206. However, mannose-binding lectin was shown to bind Aβ [75], suggesting that Aβ can be recognized by CD206. Titos et al. demonstrated an increase in M2 markers, including CD206, upon incubation of mouse macrophages with DHA [76]. Interestingly, similar results were observed with the DHA-derived resolvin D1 [76].

Altogether, analysis of the microglial phenotype showed that microglia performing phagocytosis of $A\beta_{42}$ had a lower expression of M1 markers, while the expression of M2 markers was higher. This indicates that under the conditions used microglia performing phagocytosis of $A\beta_{42}$ carry the M2 phenotype and that M2-polarized microglia are responsible for removing extracellular Aβ. It may be speculated that the inflammation present in the AD-brain, characterized by increased levels of proinflammatory factors, contributes to the overabundance of Aβ in the AD-brain by hindering attainment of the M2 phenotype which, as indicated by our data, may be more efficient in removing Aβ. Support for this theory is provided by findings of increased expression of M1 markers such as CD40 [56], and major histocompatibility complex Class II (MHC-II) [77, 78], in the AD brain. Additional support comes from *in vitro* studies showing M2 polarization and stimulated phagocytosis of Aβ in rat microglia that were stimulated with IL-4 [79], and from a study in which Th2 cytokines were found to reduce Aβ accumulation and improve cognition in a transgenic mouse model resembling AD [80].

The membrane receptor CD200R is known to be expressed on microglia and macrophages [81] and to mediate a de-activating effect upon binding its ligand CD200 (OX-2), which is expressed on neurons and astrocytes [82, 83]. Dysfunction of the CD200-CD200R pathway has been shown to increase microglial activation and neurodegeneration in a rat model of Parkinson's disease [84]. Importantly, the expression of CD200 and CD200R has been shown to be decreased in brains from AD patients [85]. We found that only a small proportion of the CHME3 microglia expressed CD200R. The effects of DHA and EPA on CD200R were not significant, with the exception of 1 μM EPA, inducing a decrease after 6 h of incubation. A decrease in CD200R is not desirable from a perspective of treatment of AD, in view of its association with a detrimental neuroinflammation. However, further studies will be required to examine the relevance of this down-regulation in the context of other, more beneficial, effects of EPA.

In studies on the effects of the ω3 FAs on microglial secretion, we found a significant increase in IL-6 by 0.005 and 0.01 μM EPA, an effect that disappeared with higher concentrations. The marked increase in IL-6 secretion by the lowest concentration of EPA was nullified upon co-incubation with $A\beta_{42}$. Most studies suggest an inhibiting effect of EPA (and DHA) on IL-6 production [36]. However, in those studies an activating stimulus was used to induce a robust IL-6 secretion, which was not the case in response to 1 μg/ml $A\beta_{42}$ used in the present study (also reported previously [54]). For example, the increase in IL-6 production by mouse adipocytes induced by bacterial lipopolysaccharide (LPS), an archetypical pro-inflammatory activator, was inhibited by EPA [86]. The toll-like receptor (TLR)-4, a receptor for LPS, needs to be assembled in lipid rafts of the cellular membrane to transduce its pro-inflammatory signal [87]. One of the actions of DHA and EPA is the inhibition of the clustering of immune-related proteins in lipid rafts [65], which may explain the anti-inflammatory effect in conditions where TLR-4 ligands are present. In support of our findings on IL-6, a study on rat lymphocyte function *ex vivo* showed that fish oil, which contains DHA and EPA, significantly increased the levels of IL-6 [88].

The present study is the first to address the influence of DHA and EPA on microglial production of BDNF *in vitro* and to show a stimulatory effect of EPA on BDNF production. Earlier studies have described the positive influence of DHA on BDNF expression *in vitro* in induced pluripotent stem cells [89], and in the CNS [90–92]. Similarly to IL-6, the lowest concentrations of EPA resulted in an increase in BDNF levels that disappeared at higher concentrations. The effect on BDNF was abolished when EPA was co-incubated with $A\beta_{42}$, lending support for the result on depression of BDNF production by $A\beta_{42}$ observed previously [54]. It may be speculated that activation of the CRE motifs present on both the *BDNF* and *IL6* genes are responsible for the stimulation of production of these two mediators, which could explain the synchrony with regard to effective concentrations of EPA. It could also explain the annulment of the stimulatory effect upon co-incubation with $A\beta_{42}$. It has been shown that CREB is inhibited by $A\beta_{42}$ [93], which can account for the lack of effect of EPA on BDNF and IL-6 upon co-incubation. However, there are no data in the literature on the effects of EPA on CREB activation. Considering that DHA was shown to activate CREB in primate hippocampal neurons [94], it is conceivable that EPA may have a similar action. A positive correlation was found between the level of BDNF and the percentage of phagocytic microglia (Supplementary Table 2),

supporting the notion of a concerted restoration of the tissue by phagocytosis and growth factors, in line with the theory on inflammatory resolution.

Incubation of the microglia with DHA resulted in a strong inhibition of the secretion of the pro-inflammatory cytokine TNF-α, whereas the effect of EPA was less prominent. However, these effects of DHA and EPA were only observed upon co-incubation with $A\beta_{42}$. Several studies show an inhibiting effect of these FAs on TNF-α production [95]. Similar to what has been discussed above for IL-6, a strong inducer of TNF-α production, such as LPS, was used in those studies, and the low concentration of $A\beta_{42}$ was not sufficient to affect the production of TNF-α, but may have induced changes that made the microglia more responsive to DHA and EPA.

The level of the anti-inflammatory M2 cytokine IL-10 was unaltered upon incubation with DHA or EPA, except for an increase upon incubation with the lowest concentration of EPA together with $A\beta_{42}$. This is different from responses by peripheral blood mononuclear cells [96] and macrophages [36] showing increased IL-10 secretion upon treatment with DHA.

Conclusions

The study is the first to investigate the effects of DHA and EPA on human microglia. The stimulatory effects of DHA and EPA on microglial phagocytosis of $A\beta_{42}$, together with the down-regulatory effects on pro-inflammatory markers, and stimulatory effects on the neuroprotective factor BDNF, indicate that these ω3 FAs activate a pro-resolving program in human microglia. However, our results also suggest multiple pathways of action of DHA and EPA with different time frames of activities. This creates a complex network of responses that may have different emphasis in different conditions, a hypothesis which has implications for the efficiency of a potential treatment based on ω3 FAs depending on the stage of disease. We also show that low concentrations of $A\beta_{42}$, which do not activate microglia, can still influence responses to other types of stimulation. The present results support the idea that ω3 FAs, and the pathways with which they are associated, are promising treatment targets for AD and other neurodegenerative disorders.

SUPPLEMENTARY MATERIAL

Supplementary material is available here: http://dx.doi.org/10.3233/JAD-130131

ACKNOWLEDGMENTS

Grants supporting this work from the Swedish Research Council (072194), Swedish Brain Power, The Knut and Alice Wallenberg Foundation, Karolinska Institutet Research funds, The Swedish Brain Foundation, Stiftelsen för Gamla Tjänarinnor, The Swedish Alzheimer Foundation, Gun och Bertil Stohnes stiftelse, Petrus och Augusta Hedlunds Stiftelse, and Stockholm County Council, are gratefully acknowledged. The authors thank Professor Charles N. Serhan (Department of Anesthesiology, Perioperative and Pain Medicine, Harvard Medical School, Boston, USA) for helpful discussions.

Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=1681).

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