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n-3 PUFA supplementation benefits
microglial responses to myelin pathologySUBJECT AREAS:
MULTIPLE SCLEROSIS
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Microglia represent rational but challenging targets for improving white matter integrity because of their dualistic protective and toxic roles. The present study examines the effect of Omega-3 polyunsaturated fatty acids (n-3 PUFAs) on microglial responses to myelin pathology in primary cultures and in the cuprizone mouse model of multiple sclerosis (MS), a devastating demyelination disease. Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), the two main forms of n-3 PUFAs in the brain, inhibited the release of nitric oxide and tumor necrosis factor- α from primary microglia upon IFN- γ and myelin stimulation. DHA and EPA also enhanced myelin phagocytosis *in vitro*. Therefore, n-3 PUFAs can inhibit inflammation while at the same time enhancing beneficial immune responses such as microglial phagocytosis. *In vivo* studies demonstrated that n-3 PUFA supplementation reduced cuprizone-induced demyelination and improved motor and cognitive function. The positive effects of n-3 PUFAs were accompanied by a shift in microglial polarization toward the beneficial M2 phenotype both *in vitro* and *in vivo*. These results suggest that n-3 PUFAs may be clinically useful as immunomodulatory agents for demyelinating diseases through a novel mechanism involving microglial phenotype switching.

Chronic inflammation is common in many demyelinating diseases, including multiple sclerosis (MS). Although cytotoxic action of myelin-specific T cells has been largely incriminated for the MS immunopathology, accumulating evidence indicates that microglia-mediated innate immune responses also play an important role in progressive demyelination¹. In demyelinating conditions, microglia may be activated by myelin debris or by the inflammatory cytokines released from activated T cells, such as IFN- γ . Nodules of activated microglia have been observed surrounding the white matter plaques in patients with MS, a phenomenon that is more prominent in progressive MS than in early MS¹. The functions of microglia in MS are quite diverse and still controversial. Initial microglial activation within an active lesion site has been shown to be pro-inflammatory and lead to demyelination and axonal injury. For example, a recent study showed a close association between microglial activation and clinical disability in MS patients². In more advanced lesions, however, the function of microglia and infiltrated macrophages may be more beneficial and involve phagocytosis of myelin debris and enhancement of remyelination and regeneration^{3,4}. Because of these dualistic roles in demyelination/remyelination, microglia represent rational but complex targets for the clinical management of MS.

Depending on the environmental milieu, microglia vary in their phenotype. Whereas the “classically activated” M1 phenotype is associated with increased antigen presentation and the production of toxic cytokines and reactive oxygen species, the “alternatively activated” M2 phenotype is devoted to the clearance of cellular debris, resolution of local inflammation, and the release of trophic factors that foster recovery after neurological injury^{5,6}. Phenotype polarization is known to be affected by the progression of many neurological diseases including MS⁷, stroke⁸, traumatic brain injury⁹, and spinal cord injury^{10,11}, and may therefore represent a potential therapeutic target for neurological disorders.

Omega-3 polyunsaturated fatty acids (n-3 PUFAs) have been studied for their beneficial effects in many animal models of neurodegenerative diseases, such as Alzheimer’s disease^{12–14} and Parkinson’s disease¹⁵. Several mechanisms are known to underlie n-3 PUFA-mediated neuroprotection, such as suppression of cell death, inhibition of inflammation, and promotion of neurogenesis. Recent studies have also examined the effect of n-3 PUFAs on white matter pathology. For example, n-3 PUFA supplementation is linked with fewer white matter lesions and improved executive function in older adults^{16,17}. Furthermore, several epidemiological studies suggest that n-3 PUFA supplementation is associated with improved clinical outcomes in MS patients^{18,19}. Beneficial effects of n-3



PUFA supplementation have also been reported in the experimental autoimmune encephalomyelitis (EAE) animal model of MS and attributed to the modulatory effect of docosahexaenoic acid (DHA) on dendritic cell-dependent T cell activation²⁰. The effect of n-3 PUFAs on microglia responses in MS or other white matter pathology, however, has not been investigated.

In the current study, we used primary microglial cultures and an animal model of cuprizone-induced demyelination to elucidate the action of n-3 PUFAs on microglial responses to myelin pathology. We found that DHA and eicosapentaenoic acid (EPA), the main n-3 PUFAs in the central nervous system, concentration-

dependently inhibited the microglial release of inflammatory mediators in response to myelin and IFN- γ stimulation and boosted myelin phagocytosis *in vitro*. Further mechanistic studies demonstrated that DHA and EPA can shift microglial polarization toward the M2 phenotype under physiological *in vitro* condition and inhibit M1 polarization under inflammatory conditions. *In vivo* studies further confirm that n-3 PUFA supplementation reduced cuprizone-induced demyelination and improved motor and cognitive abilities. These beneficial effects of n-3 PUFAs in the animal model were also accompanied by a shift to the M2 microglial phenotype *in vivo*.

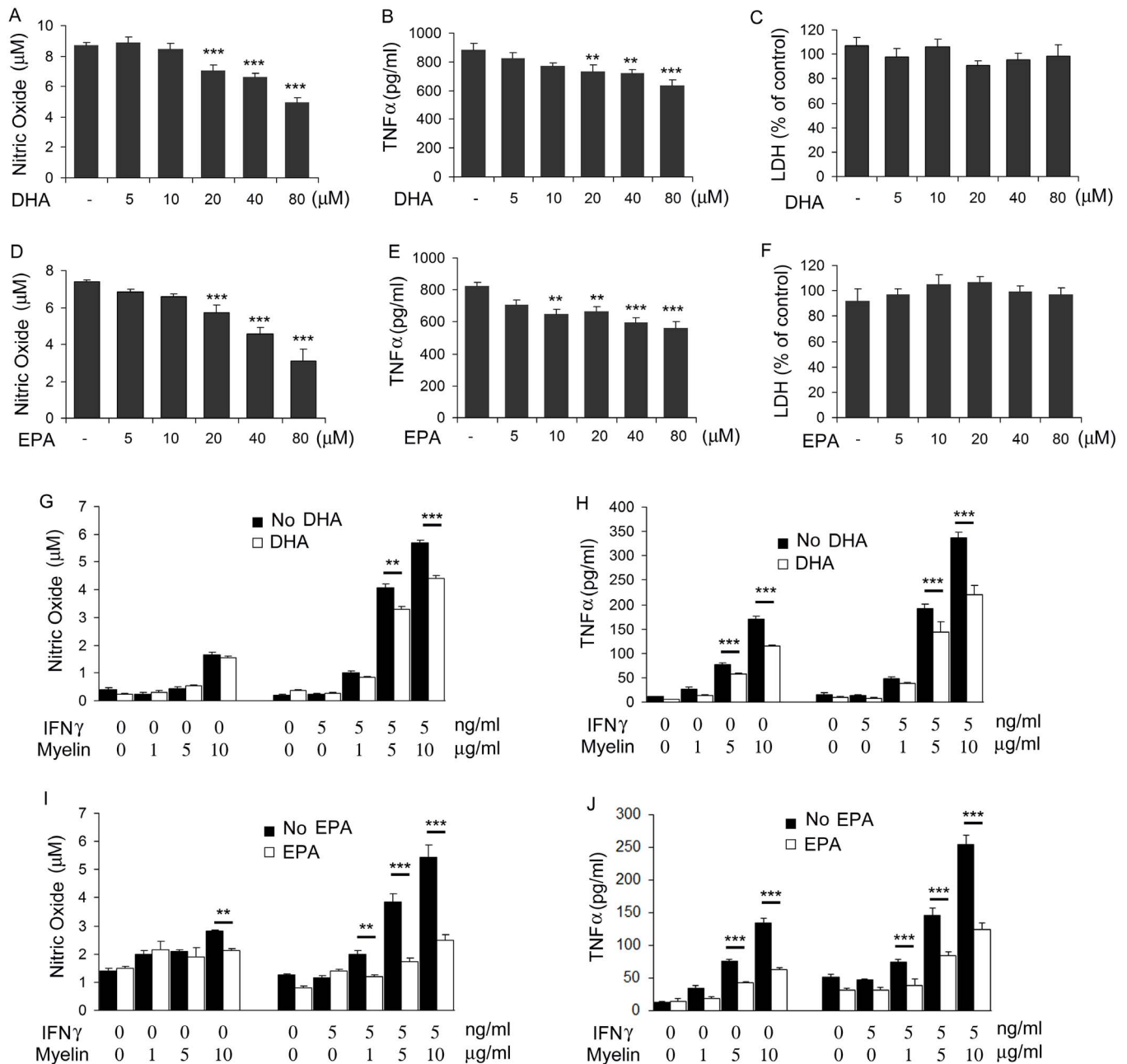


Figure 1 | DHA and EPA inhibit inflammatory responses in primary microglia. (A–F) Primary microglia seeded at 5×10^4 /well were pretreated with varying concentrations of DHA or EPA (5–80 μ M) for 24 hrs followed by LPS (2.5 ng/mL) stimulation. Culture medium was collected at 24 hrs after LPS. The production of nitric oxide (NO) (A, D) and tumor necrosis factor- α (TNF- α) (B, E) was measured as markers of inflammation. Production of lactate dehydrogenase (LDH) (C, F) was measured as a cell death assay. (G–J) Primary microglia seeded at 5×10^4 /well were pretreated with DHA (20 μ M) or EPA (20 μ M) for 24 hrs followed by myelin (1, 5 or 10 μ g/mL) without or with IFN- γ (5 ng/mL) stimulation for an additional 24 hrs. Extracellular NO (G, I) and TNF- α (H, J) were measured in the culture medium. Results are expressed as mean \pm SEM from three to four independent experiments, each performed in triplicate. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ versus vehicle treatment.

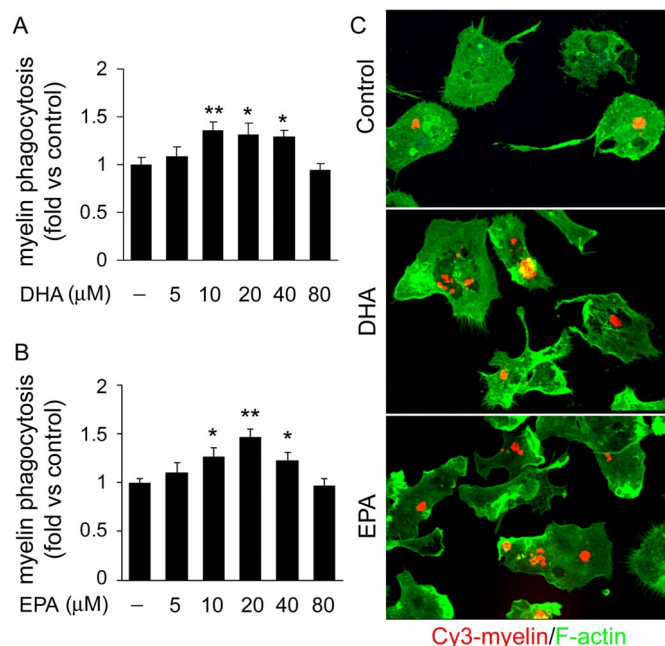


Figure 2 | DHA and EPA enhance myelin phagocytosis in primary microglia. Primary microglia were pretreated with varying concentrations of DHA or EPA (5–80 μM) for 24 hrs, followed by incubation with Cy3-labeled myelin (0.5 $\mu\text{g}/\text{mL}$) for an additional 6 hrs. (A, B) Myelin phagocytosis was quantified as intracellular fluorescence. (C) Representative images showing that DHA (20 μM) or EPA (20 μM) treatment increased myelin phagocytosis. Results are expressed as mean \pm SEM from three to four independent experiments, each performed in triplicate. * $P \leq 0.05$, ** $P \leq 0.01$ versus vehicle treatment.

Results

DHA and EPA inhibit inflammatory responses in microglia. In order to determine whether DHA and EPA can modulate microglial activation, primary microglia were treated with DHA or EPA for 24 hrs followed by LPS stimulation (2.5 ng/mL) for another 24 hrs. Nitric oxide (NO) and $\text{TNF}\alpha$ release in culture media were measured as markers of the inflammatory response. As shown in Figure 1, pretreatment with DHA significantly attenuated LPS-induced NO and $\text{TNF}\alpha$ release in a concentration-dependent manner, beginning at 20 μM (Figure 1A and 1D). The effect of EPA was qualitatively similar to the effect of DHA (Figure 1B and 1E). The lowest concentrations of DHA (20 μM) and EPA (20 μM) that successfully inhibited both NO and $\text{TNF}\alpha$ release were then used for the majority of subsequent experiments. The suppression of NO and $\text{TNF}\alpha$ production could not be explained by cell toxicity associated with DHA and EPA, as neither DHA nor EPA increased extracellular release of LDH into the medium (Figure 1C and 1F).

Next we confirmed the inhibitory effect of DHA and EPA on inflammatory microglial responses in a disease-related *in vitro* model, using myelin and $\text{IFN}\gamma$ as inflammatory stimulators. While the individual activating factors by themselves elicited only a weak microglial response, myelin and $\text{IFN}\gamma$ co-treatment showed strong synergistic and concentration-dependent effects on inflammatory indicators in microglia (Figure 1G–J). Furthermore, pretreatment with DHA (20 μM) or EPA (20 μM) significantly decreased myelin + $\text{IFN}\gamma$ -induced production of NO (Figure 1G and 1I) and $\text{TNF}\alpha$ (Figure 1H and 1J). These data strongly suggest that n-3 PUFAs inhibit microglial responses to MS-related inflammatory stimuli. Furthermore, EPA appeared to exert slightly greater anti-inflammatory effects.

DHA and EPA enhance myelin phagocytosis in microglia. To investigate the effect of n-3 PUFAs on phagocytosis of myelin, microglia were incubated with varying concentrations of DHA or EPA ranging from 5 μM to 80 μM for 24 h. Cy3-labeled myelin was added to

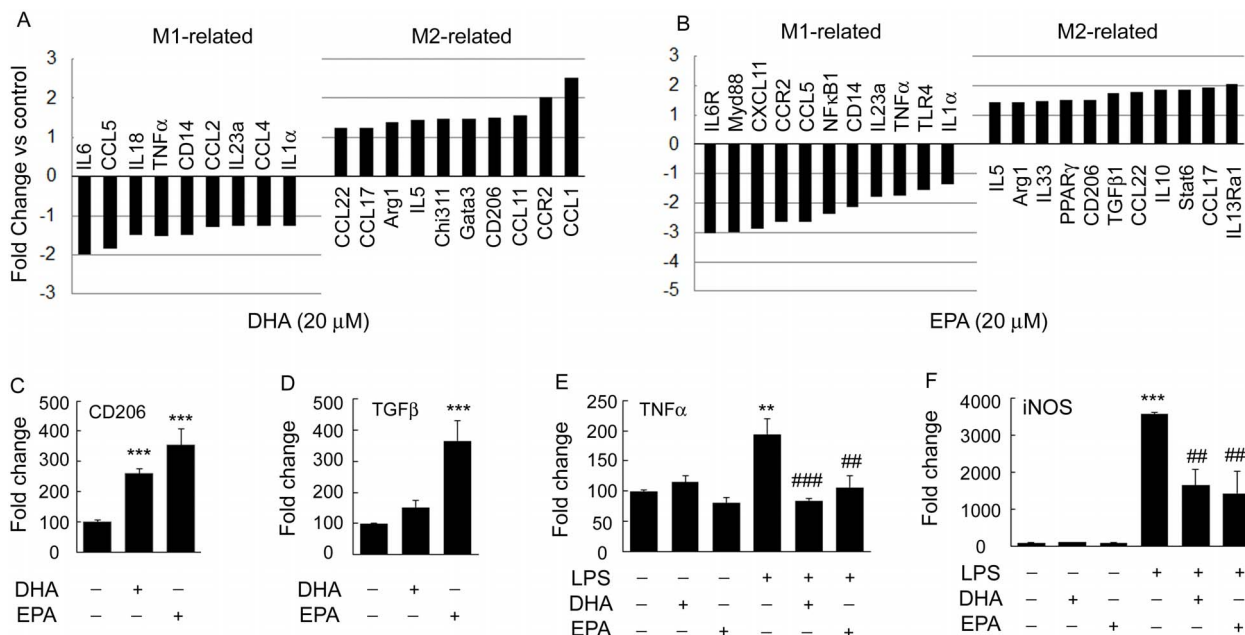


Figure 3 | DHA and EPA induce microglial polarization toward the M2 phenotype. Primary microglia seeded at $5 \times 10^4/\text{well}$ in a 6-well plate were pretreated with DHA or EPA (20 μM each) for 24 hrs. (A–B) Real-time PCR arrays show that DHA (A) and EPA (B) treatment significantly inhibited expression of multiple M1 genes and enhanced expression of M2 genes. (C–F) Real-time PCR showed significant stimulation of M2-related gene CD206 (C) and $\text{TGF}\beta$ (D) after DHA or EPA treatment. In addition, DHA or EPA pretreatment significantly inhibited the expression of M1-related genes $\text{TNF}\alpha$ (E) and iNOS (F) following LPS (2.5 ng/mL) stimulation for 24 hrs. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ versus control; ## $P \leq 0.01$, ### $P \leq 0.001$ versus LPS.

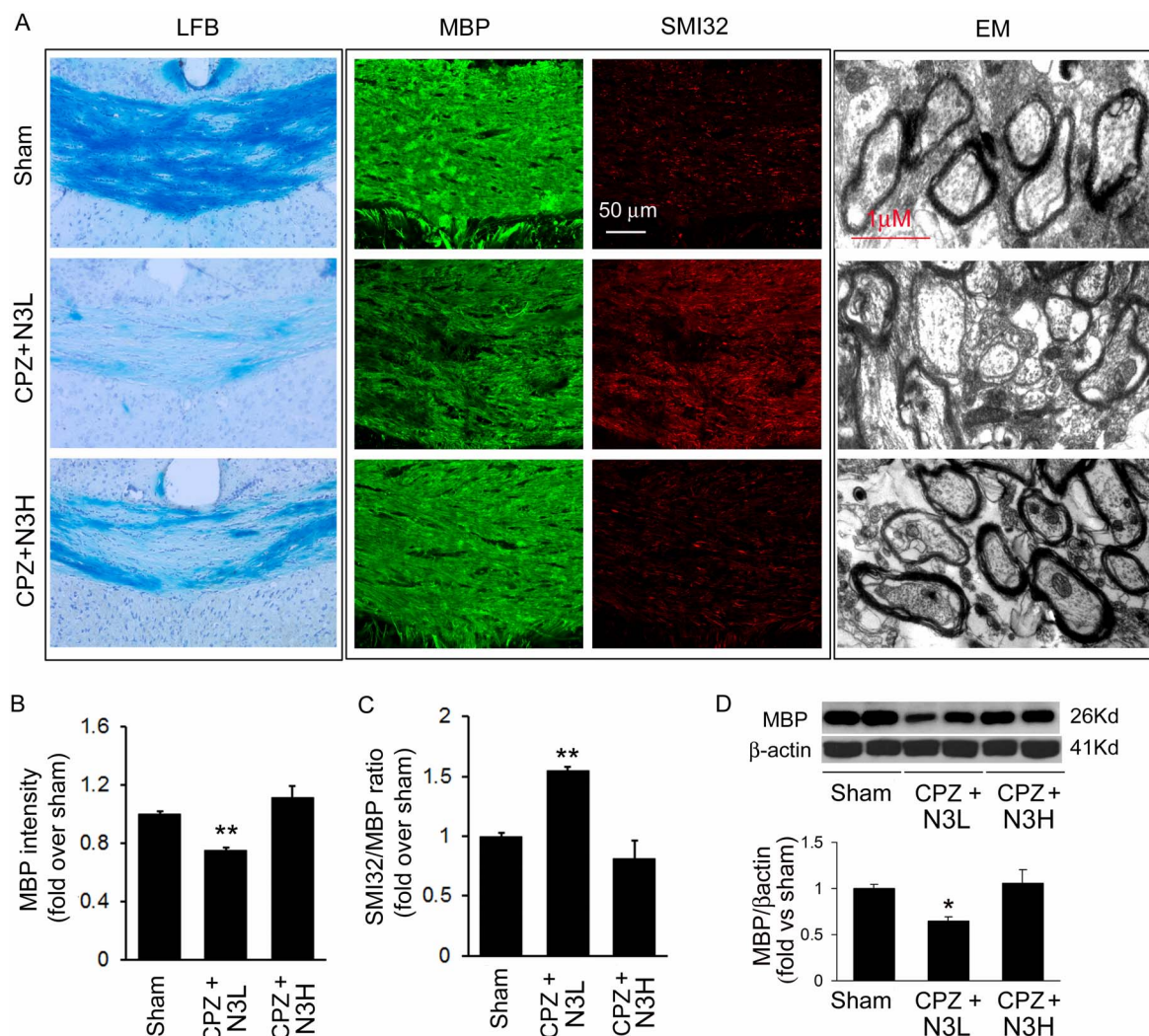


Figure 4 | n-3 PUFA supplementation reduces demyelination in cuprizone model of MS. Mice were fed for 5 weeks with a diet containing 0.2% cuprizone with (N3H) or without (N3L) n-3 PUFA enrichment. (A) Demyelinating lesions in the corpus callosum (CC) were revealed by Luxol fast blue (LFB) staining (left), MBP staining, and electron microscopy (EM, right). Pathological changes in axons were revealed by SMI32 immunostaining. Images are representative of sections from four animals. (B) MBP intensity was measured and calculated as fold change over sham. (C) The ratio of SMI32 to MBP was calculated as an indicator of white matter injury (axonal damage and demyelination). (D) Western blot analysis of MBP expression. $n = 4$ mice for each group. * $P \leq 0.05$, ** $P \leq 0.01$ versus sham controls.

the cultures for an additional 6 hrs. After washing, myelin phagocytosis was quantified by measuring Cy3 fluorescence in cell lysate. Treatment with DHA (Figure 2A) or EPA (Figure 2B) at concentrations of 10–40 μM significantly increased myelin phagocytosis. Confocal imaging confirmed that either DHA (20 μM)- or EPA (20 μM)-treatment boosted microglial phagocytosis (Figures 2C).

DHA and EPA modulate microglial polarization under physiological and inflammatory conditions. As mentioned in the Introduction, microglia are known to assume distinct phenotypes, such as M1 and M2, in response to various external stimuli^{10,21}. Thus, we used a high-throughput PCR array to analyze the expression of multiple M1 and M2 signature genes in DHA or EPA-treated microglia. Both DHA and EPA demonstrated striking trends towards upregulating M2 genes and downregulating M1 genes under physiological conditions (Figure 3A–3B).

Next we used conventional real time-PCR to validate the effects of DHA and EPA on microglial polarization. Primary microglia were treated with DHA (20 μM) or EPA (20 μM) for 24 hrs. Consistent with the PCR array data, DHA and EPA treatment significantly enhanced the expression of CD206 and/or TGF β , two representative

M2 genes, under physiological conditions (Figure 3C–3D). DHA or EPA treatment did not inhibit the expression of TNF α and iNOS, two representative M1 genes, under physiological conditions but significantly reduced the production of these two inflammatory genes following LPS stimulation for 24 hrs (Figure 3E and 3F). These data suggest that DHA and EPA may modulate phenotypic polarization of microglia under physiological as well as pathological conditions.

n-3 PUFA supplementation reduces demyelination in the cuprizone mouse model of MS. In order to validate the effect of n-3 PUFAs on myelin pathology *in vivo*, we used the cuprizone-induced demyelination model of MS. Five weeks of dietary n-3 PUFA supplementation dramatically attenuated the cuprizone-induced reduction in myelin expression in the corpus callosum, as shown by Luxol fast blue staining (Figure 4A, left). Double immunofluorescent staining with anti-MBP and anti-SMI32 antibodies was also performed to further evaluate pathological changes in the corpus callosum (Figure 4A, middle). Under control, non-injured conditions, SMI32 immunostaining for abnormally dephosphorylated neurofilament proteins was very faint in the corpus callosum. However, cuprizone increased the

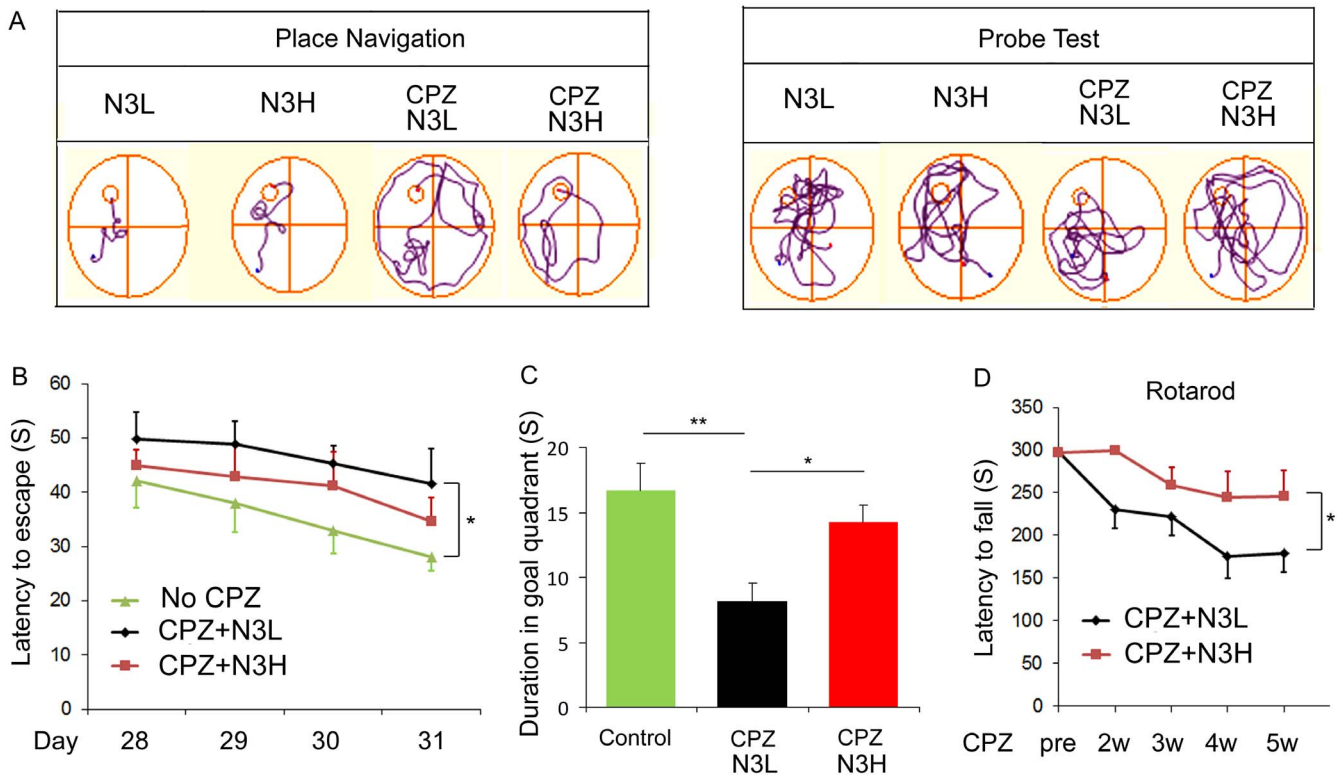


Figure 5 | n-3 PUFA supplementation reduces neurological deficits in cuprizone model of demyelination. Mice were fed for 5 weeks with a diet containing 0.2% cuprizone with (N3H) or without (N3L) n-3 PUFA enrichment. (A–C) The Morris water maze test performed 28–32 days after initiation of the cuprizone diet. (A) Representative images of the swim paths of mice in each group while the platform was present (place navigation; learning phase) and after it was removed (probe test; memory phase). (B) Latency to locate the submerged platform was measured in n-3 PUFA-supplemented and regular diet mice 28–31 days after initiation of the cuprizone diet. (C) Spatial memory of the location of previously submerged platform was measured in n-3 PUFA-supplemented and regular diet mice 32 days after initiation of the cuprizone diet. Spatial memory is expressed as the time spent in the goal quadrant when the platform was removed. (D) Rotarod test before and after 2–5 weeks of cuprizone treatment. $n = 8$ animals/group. * $P \leq 0.05$, ** $P \leq 0.01$ for indicated comparisons.

SMI32 immunostaining and elicited loss of MBP-positive myelin in mice fed with a regular diet with low n-3 PUFAs (N3 low, N3L). In contrast, cuprizone-treated animals on an n-3 PUFA-rich diet (N3 high, N3H) exhibited improved myelin integrity, as shown by enhanced MBP immunostaining and a fall in the SMI32/MBP ratio in the corpus callosum (Figure 4A–C). Western blots for MBP levels confirmed the immunohistochemical findings (Figure 4D). The ultrastructure of myelin sheaths was also observed with transmission electron microscopy (Figure 4A, right). The cuprizone diet elicited demyelination in the corpus callosum, as manifested by lower electron density and less well-defined myelin sheaths. As expected, n-3 PUFA supplementation elicited prominent protection of myelin sheath integrity. These experiments strongly suggest that n-3 PUFAs can ameliorate cuprizone-induced myelin pathology.

n-3 PUFA supplementation reduces neurological behavioral deficits in the cuprizone-treated animal model of demyelination. We also tested the hypothesis that n-3 PUFAs reduce motor and cognitive deficits in cuprizone-treated animals *in vivo*. Cognitive deficits were examined by the Morris water maze test. Cuprizone diet retarded spatial learning and impaired spatial memory (Figure 5A–C), as revealed by significantly increased latency to escape swimming when the platform was present and reduced time spent swimming in the target quadrant when the platform was absent, respectively. Dietary supplementation with n-3 PUFAs greatly improved memory in cuprizone-treated mice. The learning ability in n-3 PUFA-supplemented cuprizone mice showed a trend of improving although did not reach statistical significance as compared to mice without n-3 PUFA supplementation. There is no difference in swimming speed

between N3L and N3H groups (data not shown). The locomotor activity of mice subject to cuprizone with or without n-3 PUFA supplementation was also assessed using the rotarod test. Dietary supplementation with n-3 PUFAs significantly ameliorated cuprizone-induced sensorimotor deficits, as revealed by increased time spent on the rotarod (Figure 5D). Promisingly, we found that n-3 PUFA supplementation starting after cuprizone withdrawal could also promote neurobehavioral performance (data not shown), which suggest a therapeutic potential for n-3 PUFAs.

n-3 PUFA supplementation promotes microglia M2 polarization in the cuprizone-treated animal model of demyelination. To investigate the effects of n-3 PUFAs on microglial polarization in the *in vivo* model of demyelinating disease, we measured the expression of a series of M1 and M2 signature genes using RT-PCR. As expected, the expression of M1 genes (CD16 and iNOS) was significantly increased after 5 weeks of the cuprizone diet. n-3 PUFA supplementation inhibited the elevation of these M1 genes (Figure 6B) and enhanced the expression of three M2 genes (CD206, YM1/2, and Arg1, Figure 6A) in cuprizone-treated mice. Immunofluorescent staining further revealed that the protein expression of both M1 (CD16) and M2 (CD206) markers were elevated after 5 weeks of the cuprizone diet (Figure 6C–F). n-3 PUFA supplementation shifted microglial polarization toward M2 in this model, as shown by enhancement of CD206 expression and inhibition of CD16 expression in the corpus callosum.

Discussion

The results presented here demonstrate a robust beneficial effect of DHA and EPA on microglial responses toward myelin pathology.

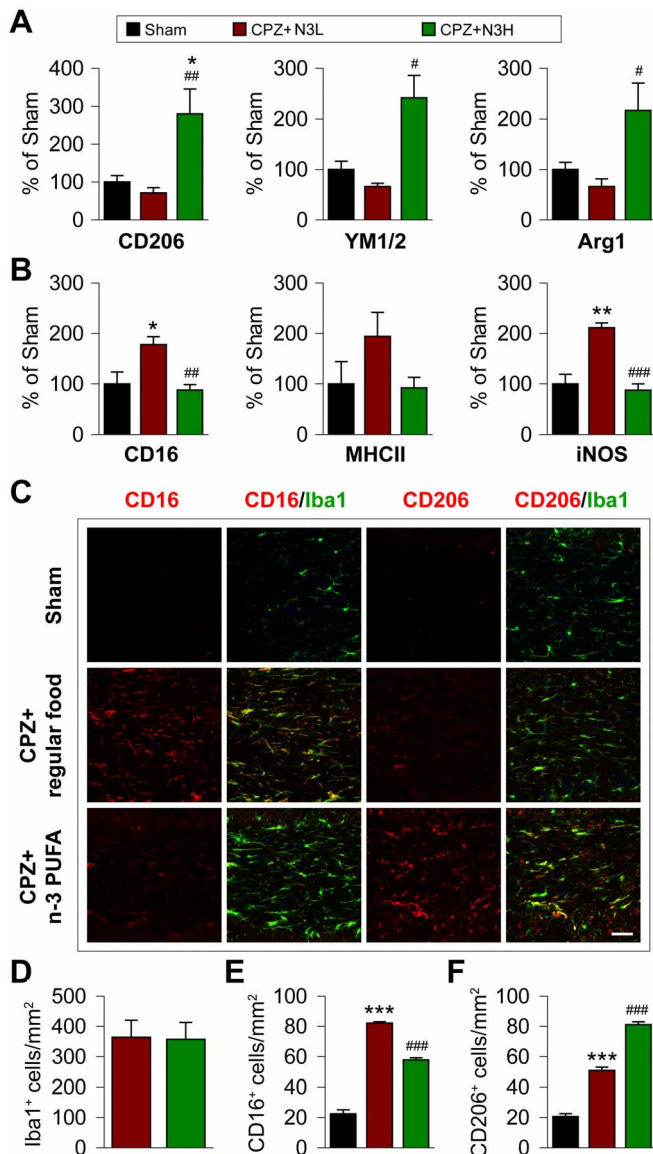


Figure 6 | n-3 PUFA supplementation promotes M2 microglial polarization in cuprizone model of demyelination. Mice were fed for 5 weeks with a diet containing 0.2% cuprizone with (N3H) or without (N3L) n-3 PUFA enrichment. (A–B) Real time-PCR for M1 markers (A) and M2 markers (B) was performed using total RNA extracted from the corpus callosum. (C) Representative double staining immunofluorescence of Iba1 with CD16/32 (M1) or CD206 (M2) in the corpus callosum. Scale bar: 40 μ m. (D–F) Quantification of the Iba1⁺ (D), CD16/32⁺ (E), and CD206⁺ (F) cells in the corpus callosum. n = 4 animals per group. *P \leq 0.05, **P \leq 0.01, ***P \leq 0.001 versus sham; #P \leq 0.05, ##P \leq 0.01, ###P \leq 0.001 versus CPZ + N3L.

The ability of n-3 PUFAs to inhibit inflammation while simultaneously enhancing phagocytosis in myelin-stimulated microglia reveals exciting new possibilities for neurological disease therapies. Furthermore, the discovery of the M1-to-M2 shift points to the beneficial modulation of microglia phenotype by n-3 PUFAs.

Recent studies have increasingly confirmed the importance of microglia in the progression of MS^{2–4}. Microglial function is known to be affected by myelin debris and the cytokines released from other immune cells. However, divergent effects of myelin on cultured microglia have been reported. For example, myelin alone was shown to activate the release of inflammatory cytokines from microglia, which in turn caused neuronal death²². Another study revealed that,

when microglia were pre-activated with IFN γ , myelin treatment induced a biphasic change in microglial release of pro-inflammatory cytokines, with an early elevation lasting up to 6 hrs, followed by a delayed suppression 24 hrs after myelin exposure²³. In contrast, myelin-loaded foam microglia/macrophages have been reported to produce anti-inflammatory cytokines in response to LPS stimulation⁵. In the present study, we demonstrated that simultaneous application of myelin and IFN γ to cultured microglia elicits synergistic inflammatory responses. These studies suggest that myelin is an active modulator of microglia; the microglial response to myelin depends on microglial activation status and, importantly, the presence of other stimulators in the microenvironment. As myelin and IFN γ co-application induced robust inflammatory cytokine release from microglia, we used this model to rigorously test the anti-inflammatory properties of DHA and EPA.

Phenotype polarization is known to be affected by the progression of many neurological diseases including MS⁷. In the present study, we discovered a dramatic increase in M1 phenotypic markers in MS mice fed a cuprizone-rich diet for 5 weeks. The expression of the M2 marker CD206 at the protein level also slightly increased with 5 weeks of cuprizone exposure. However, this elevation may not be sustained for much longer because the mRNA expression of M2 markers exhibited downward trends within 5 weeks of cuprizone treatment. A shift toward the M1 phenotype at this time would be extremely detrimental to the already damaged myelin sheath as we recent showed that M1 microglia-conditioned media exacerbates oligodendrocyte death following noxious stimuli such as oxygen/glucose deprivation⁹ or AMPA (not shown). In line with our results, there is a dramatic increase of M1 microglia/macrophages at the onset of injury in the EAE model of MS⁷. Furthermore, a subsequent decrease in M2 markers is related to the relapse of MS⁷. Intriguingly, intravenous infusions of *ex vivo* activated M2 monocytes significantly elevate the number of M2 microglia/macrophage in the brain and ameliorate the clinical symptoms in EAE mice, suggesting that transplantation of M2 phagocytes might be a promising cell-based therapy for MS. However, one potential concern in using M2 cells as a therapy is that microglia/macrophages are highly sensitive and may encounter unexpected stimulating factors during cell preparation and transplantation, resulting in the reduction or loss of their protective M2 features. Thus, identifying approaches to induce microglia M2 polarization endogenously is a legitimate therapeutic strategy for MS.

Anti-inflammatory effects of DHA and EPA have already been reported in various neurodegenerative diseases, including stroke and Alzheimer's disease^{24–29}. n-3 PUFAs were also shown to enhance microglial phagocytosis of amyloid- β in an *in vitro* model of AD³⁰. However, the roles of DHA and EPA in microglia polarization and in MS pathology have not been previously investigated; the present study is the first to study the role of n-3 PUFAs in microglial polarization in a demyelination disease-related model. We found that n-3 PUFAs exerted beneficial effects on microglial function by differentially inhibiting myelin and IFN- γ induced inflammation and enhancing myelin phagocytosis. These functional effects were associated with microglial polarization toward an M2-dominant phenotype. The phenotypic polarization may foster a microenvironment that resists inflammatory damage and further demyelination in MS, as manifested by improved neurological function *in vivo*.

Some studies suggest that an endogenous shift from M1 to M2 microglial phenotype occurs at the initiation of remyelination in models of MS⁶. Furthermore, selective depletion of M1 or M2 phenotypic cells revealed that the M2 phenotype promoted, while the M1 phenotype impaired, oligodendrocyte regeneration in this model⁶. It is therefore conceivable that n-3 PUFA-afforded M2 polarization may not only reduce demyelination, as shown in this study, but also benefit the remyelination process in MS. Ongoing studies in our lab are therefore examining the effect of n-3 PUFAs on oligodendrocyte differentiation and remyelination.



Table 1 | Primers for Real-Time Polymerase Chain Reaction

Gene	Primer	Gene	Primer
Mouse		Rat	
M1		M1	
iNOS	SENS: CAAGCACCTTGAAGAGGAG REVS: AAGGCCAAACACAGCATACC	iNOS	SENS: CCTGGTGCAAGGGATCTTGG REVS: GAGGGCTTGCCTGAGTGAGC
CD16	SENS: TTGGACACCCAGATGTTTCAG REVS: GTCTTCCTTGAGCACCTGGATC	TNFα	SENS: TTCCCAAATGGGCTCCCTCT REVS: GTGGGCTACGGGCTTGTAC
MHCII	SENS: GACGCTCAACTGTCCCAAAAC REVS: GCAGCCGTGAACCTGTTGAAC		
M2		M2	
CD206	SENS: CAAGGAAGGTTGGCATTGT REVS: CCTTCAGTCCTTTCGAAGC	CD206	SENS: GGTCCGGTTTGTGGAGCAG REVS: TCCGTTTGCATTGCCAGTA
Arg1	SENS: TCACCTGAGCTTTGATGTCG REVS: CTGAAAGGAGCCCTGTCTTG	TGFβ	SENS: TGGCGTTACCTTGGTAACC REVS: GGTGTTGAGCCCTTCCAG
Ym1/2	SENS: CAGGGTAATGAGTGGGTGG REVS: CACGGCACCTCTAAATTGT		

In addition to its beneficial effects on microglial polarization, a recent study of ours also showed a direct protective effect of DHA against excitotoxicity in oligodendrocytes³¹. Thus, both direct (*via* oligodendrocytes) and indirect (*via* microglia) mechanisms may contribute to n-3 PUFA-afforded white matter protection. Interestingly, the optimal dose of DHA for anti-inflammatory effect in microglia and protective effect in oligodendrocytes are both about 20 μM ³¹. DHA or EPA at similar dose range also suppresses the inflammation in human T-helper cells and enhances the secretion of anti-inflammatory factors from monocytes³². Moreover, animal study documented that food supplementation of DHA for several weeks can greatly raise the brain DHA levels to about 10–20 $\mu\text{M/g}$ wet brain tissue³³. Given their ability to target multiple cell types and the ease for food supplementation, n-3 PUFAs should be further investigated as a therapeutic agent for MS patients. If DHA and EPA are able to invoke similar protective effects in humans, these natural compounds would serve as a safe and inexpensive treatment for the 2.3 million people worldwide suffering from MS as well as innumerable other patients with demyelinating diseases.

Methods

Primary microglia-enriched cultures. Primary microglia-enriched cultures were prepared from the whole brains of 1-day-old pups as described previously³⁴. In brief, brain tissues were triturated after removal of the meninges and blood vessels. Cells were then seeded at 5×10^7 cells/150-cm³ culture flask in DMEM/F12 medium containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μM nonessential amino acids, 50 U/ml penicillin, and 50 μg /ml streptomycin. After a confluent monolayer of glial cells was achieved (typically within 12–14 days after initial seeding), microglia were shaken off, collected, and reseeded. Microglia cultures (97% purity) were then treated with DHA or EPA (Sigma, St. Louis, MO) dissolved in media for 24 hours, followed by various concentrations of myelin and IFN- γ for another 24 hours.

Myelin preparation. Myelin was isolated from the brains of 3-month-old mice by sucrose density gradient centrifugation (0.32 M and 0.85 M) at $75000 \times g$, as described previously³⁵. The protein content of myelin was determined by Bradford protein assay (Bio-Rad) with bovine serum albumin as the standard. The endotoxin concentration of myelin debris was <0.01 U/mL, as determined by the Limulus Amebocyte Lysate assay (Pierce). For fluorescent labeling, myelin was first dissolved in 0.1 M NaHCO₃-Na₂CO₃ buffer at 1 mg/mL protein concentration and then labeled using a cyanine 3 (Cy3) mono-reactive dye pack, according to the manufacturer's instructions (GE Healthcare).

Phagocytosis assay. Microglia were plated in 96-well plates (5×10^4 cells/well) and incubated with DHA or EPA for 24 hours. Cells were then incubated in the presence or absence of Cy-3-labeled myelin (0.5 $\mu\text{g}/\text{mL}$) for an additional 6 hours. To quench the signal from extracellular or outer plasma membrane-associated microspheres, the medium was removed and the cells were thoroughly rinsed with 0.25 mg/mL Trypan blue in phosphate-buffered saline (PBS) for 1 min. Cells were lysed using PBST (1% Triton in PBS). Intracellular fluorescence was then measured using a fluorescence plate reader at 535 nm excitation and 575 nm emission. For image analysis of phagocytotic structures, microglia were plated at 1×10^5 cells/well into 8-well chamber slides (Nunc). Cy3-labeled myelin was then

added as described above. Cells were subsequently rinsed with PBS and fixed in 4% paraformaldehyde. AlexaFluor488 phalloidin (1 : 50, Invitrogen) was added to these fixed cultures and incubated at room temperature in the dark for 1 hour. Images were captured with an Olympus confocal microscope by a blinded observer.

Assay for soluble factors in culture media. Extracellular tumor necrosis factor- α (TNF- α) levels were measured with a commercial ELISA kit according to the manufacturer's instructions (R&D Systems). Extracellular lactate dehydrogenase (LDH) release was also measured to assess loss of membrane integrity (Pointe Scientific). Nitric oxide was measured using a colorimetric reaction with the Griess reagent (Invitrogen). Absorbances were read on a SpectraMax microplate reader (Molecular Devices).

Induction of experimental demyelination. All animal experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and carried out in accordance with the *NIH Guide for the Care and Use of Laboratory Animals*. Male 5 week-old C57/BL6 mice (Jackson Labs) were fed for 5 weeks with a diet containing 0.2% cuprizone (Sigma-Aldrich) mixed into a regular laboratory rodent diet (Teklad global 2918, Harlan) with low n-3 PUFAs concentration (0.3%) or into a regular diet supplemented with n-3 PUFAs (DHA + EPA, 15 g/kg, Puritan's Pride).

Morris water maze test. The Morris water maze test was performed between 28 and 32 days after initiation of the cuprizone diet. Briefly, an 11 cm diameter platform is submerged in the second quadrant of a circular pool with a diameter of 109 cm. The aim of hidden platform test is to assess the ability of the mice to find the platform by memorizing external spatial cues. During the test, each mouse was put in the water in 1 of 4 quadrants and was allowed 60 seconds to find the platform. When each trial ends, the mouse was placed on the platform for another 30 seconds to remember the external spatial cues displayed around the room. Four trials were performed per day with platform present from day 28–31 after cuprizone. Data are showed as the time (in seconds) or latency to locate the hidden platform on each day. A single 60 second probe trial on each mouse was performed on Day 32 after the initiation of the cuprizone diet. When the platform was removed, each mouse was placed at the same starting place in the pool and swim for 60 seconds. The time spent in the target quadrant, where the platform previous located, were recorded. These tests were carried out by researchers who were blinded of experimental groups.

Immunohistochemistry. Animals were deeply anesthetized before intracardiac perfusion with 0.9% saline followed by 4% paraformaldehyde. Brains were cryoprotected in 30% sucrose in PBS. Immunohistochemistry was performed on serial coronal sections (4–5 sections per animal per stain). Primary antibodies include the following: goat anti-CD206 (1 : 200, R&D Systems), rat anti-CD16/32 (1 : 500, BD), rabbit anti-myelin basic protein (MBP, 1 : 200, Abcam), mouse anti-non-phosphorylated neurofilament H (SMI-32, 1 : 1000, Calbiochem) and rabbit anti-ionized calcium binding adaptor molecule 1 (Iba1, 1 : 1000, Wako). Sections were also stained for myelin using Luxol fast blue.

Image analysis. For the calculation of cell densities, the number of immunopositive cells in the designated area was divided by the total area as measured by the Image J analysis system. Myelin loss and axon damage were quantified by measuring of the intensity of immunostaining for MBP, which is reduced with white matter injury, and SMI-32, which is increased with white matter injury. The SMI32/MBP ratio thus represents the degree of white matter injury. For each brain section, immunostaining intensity was measured in 5 regions of interest in the corpus callosum per section. A mean intensity value was then calculated for each brain.



Real-time PCR and PCR array. Total RNA was isolated from brains at the end of demyelination using the RNeasy Mini Kit, according to the manufacturer's instructions (Qiagen). Five micrograms of RNA were used to synthesize the first strand of cDNA using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen). Commercial quantitative PCR array plates (SA Biosciences) were used to detect the expression of a series of M1- and M2-associated genes in DHA or EPA-treated microglia. In addition, real-time PCR was performed on the Opticon 2 Real-Time PCR Detection System (Bio-Rad) using corresponding primers (Table 1) and the SYBR green PCR Master Mix (Invitrogen). The cycle time values were normalized to GAPDH levels within the same sample. The expression levels of the mRNAs were then reported as fold changes versus sham control.

Transmission electron microscopy (EM). Samples from the corpus callosum were collected for electron microscopy and fixed in 2.5% glutaraldehyde in 0.1 M PBS (pH 7.4) for 12 hours and 1% osmium tetroxide for 1 hour. After dehydration in a series of alcohols, tissues were embedded in epoxy resin. Ultrathin sections were prepared using a Reichert ultramicrotome, contrasted with uranyl acetate and lead citrate, and examined under a CM120 electron microscope at 80 kv.

Statistical analyses. All data are reported as the mean \pm SEM. Significant differences between means were assessed by ANOVA and *post hoc* LSD tests for multiple comparisons, unless otherwise indicated. $p \leq 0.05$ was considered statistically significant.

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Author contributions

S.C., H.Z. and H.P. contributed equally to this manuscript. J.C., A.K.L. and X.H. designed experiments. S.C., H.Z. and W.L. performed experiments in Figures 1–3 and prepared Figures 1–3. H.Z., H.P. and G.W. performed experiments in Figures 4–6 and prepared Figures 4–6. S.C., R.K.L. and X.H. wrote the manuscript. All authors reviewed the manuscript.

Additional information

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