

A β 25–35 induction of necroptosis through activation of the RIPK1/RIPK3 and RIPK1/ERK1/2 signaling pathways is attenuated with DHA treatment in THP-1 monocytes

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Research

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Abstract

Background: Monocytes play a crucial role in Alzheimer's disease (AD), and docosahexaenoic acid (DHA) treatment has a neuroprotective effect for many neurodegenerative diseases. However, mechanisms that regulate monocyte and A β protein interaction in AD and the effects of DHA on monocytes in the context of AD are not fully understood.

Methods: The experiments were designed to further explore possible mechanisms of interaction between monocytes and A β plaques. Another objective of this study was to investigate a potential mechanism for A β -mediated regulation of necroptosis and the MAPK and NF- κ B signaling pathways, as well as how these pathways might be modulated by DHA in human THP-1 monocytes. We also investigated whether DHA indirectly suppressed THP-1 cell-mediated neuronal activation. We used cell viability and cytotoxicity assays, flow cytometry, transwell migration assays, and Western blotting to perform our study.

Results: Our findings indicate that A β ₂₅₋₃₅ regulates two aspects of THP-1 cells necroptosis. We also observed that increased resistance to apoptosis in THP-1 cells that is correlated with THP-1 monocyte differentiation. Our results also indicated that DHA treatment restored migration of THP-1 monocytes that had been treated with A β ₂₅₋₃₅. Pre-treatment of THP-1 monocytes with DHA effectively inhibited A β -induced activation and markedly suppressed protein expression of TNF- α , IL-1 β and IL-6. We also found that THP-1 cell necroptosis was induced by A β ₂₅₋₃₅ through modulation of RIPK1/RIPK3 and phosphorylation status of ERK1/2 and could be attenuated by DHA treatment.

Conclusion: A β ₂₅₋₃₅-mediated induction of necroptosis through activation of the RIPK1/RIPK3 and RIPK1/ ERK1/2 signaling pathways in THP-1 monocytes can be attenuated by DHA treatment, indicating that DHA treatment could be a promising new therapy for AD management.

Introduction

Alzheimer's disease (AD), the leading cause of dementia, encompasses a range of neurological symptoms, including memory loss and cognitive impairment [1]. The neuropathological changes of Alzheimer disease (AD) consist of abundant amyloid plaques and neurofibrillary tangles, neuropil threads, and dystrophic neurites containing hyperphosphorylated tau [2, 3]. AD is likely caused at least in part by an imbalance between amyloid- β (A β) protein production and clearance, which leads to A β accumulation in the central nervous system (CNS). The main pathways that act to clear excess A β are proteolytic degradation, transcytosis across the blood-brain barrier (BBB), and perivascular lymphatic drainage[4, 5].

Monocytes play a crucial role in AD, given that monocyte-derived perivascular macrophages efficiently phagocytose accumulations of A β [6]. A β accumulation can stimulate the RAGE and PECAM-1 receptors to promote the migration of monocytes along human brain endothelial cells [7]. Using live intravital two-photon microscopy in triple-transgenic APP^{swe}/PS1^{+/-}/Cx3cr1^{gfp/+} mice, Michaud et al. demonstrate that patrolling monocytes are attracted to and crawl onto the luminal walls of A β -positive veins.

Furthermore, they report the presence of crawling monocytes carrying A β in veins that are able to circulate back into the bloodstream [8]. Compared to monocytes, microglia have a limited ability to degrade A β plaques, resulting from the low activity levels of several microglial lysosomal enzymes [9]. Patrolling monocytes may therefore play a more important role in A β clearance, and further increasing vascular A β clearance via patrolling monocytes could have significant impact on AD. Reducing the migration, phagocytosis, or number of mononuclear cells in transgenic AD mice is detrimental, whereas treatments with compounds that increase the number and phagocytic activity of mononuclear cells are generally beneficial [10]. Finding a way to promote effective monocyte-mediated A β protein clearance while avoiding the activation of proinflammatory and neurotoxic pathways is an attractive approach for treating AD [11]. The experiments in this current study were designed to further explore possible mechanisms of interaction between monocytes and A β protein.

Studies have shown that the neurotoxicity directly or indirectly induced by accumulation of A β protein is a primary cause of neuron injury in AD. Kwon et al. have shown drug treatment can directly ameliorate the toxic effects A β accumulation causes in neurons [12, 13]. A β accumulation can also indirectly exert a toxic effect on neurons through targeting microglial cells, so protection of microglial cells is also important for alleviating AD symptoms [14, 15]. Further studies are necessary to understand the mechanism by which A β plaques interact with monocytes to exert neurotoxic effects.

Docosahexaenoic acid (DHA) is an essential omega-3 polyunsaturated fatty acid (PUFA) found predominantly in marine products. Preclinical and clinical studies have demonstrated that DHA exerts a neuroprotective effect in the context of several neurodegenerative diseases, including AD, resulting mostly from the antioxidant and anti-inflammatory properties of DHA and its ability to activate several other signaling pathways [16]. Given that monocytes are involved in the clearance of A β plaques, additional studies are needed to elucidate a potential mechanism for how DHA might be involved in promoting monocyte-mediated clearance of A β plaques.

Necroptosis is a lytic cell death program defined by activation of the receptor interacting protein kinase-1 (RIPK1) and RIPK3 to form an oligomeric "necrosome," which leads to the phosphorylation and activation of the effector protein pseudokinase mixed lineage kinase domain-like (MLKL) [17]. This necroptosis pathway is implicated in several neurodegenerative diseases [18]. NF- κ B plays a key role in modulating immune and inflammatory responses, while mitogen-activated protein kinases (MAPKs) (P38, ERK1/2) are key regulators of a variety of cellular functions including cell survival, apoptosis and cellular responses to inflammation [19, 20]. Upon TNF binding to its receptor to form Complex I, RIPK1 can interact with Complex I, which activates the MAPK signaling cascade and the transcription factor NF- κ B [21]. Therefore, we further investigated a possible mechanism for A β -mediated regulation of necroptosis, MAPK and NF- κ B signaling pathways in monocytes.

The experiments in this study were designed to further explore possible mechanisms by which monocytes and A β protein might interact. Another objective of this study was to investigate a possible mechanism for A β -mediated regulation of necroptosis, MAPK and NF- κ B signaling pathways in vitro in

human THP-1 monocytes. We also investigated the role of DHA in modulating A β -regulated signaling pathways and whether DHA indirectly suppressed THP-1 cell-mediated neuronal activation.

Methods

Materials

cis-4,7,10,13,16,19-docosahexaenoic acid (DHA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Neurobasal medium, B-27 supplement, and penicillin, DMEM medium, 1640 medium, and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). CCK8, Necrostatin-1 (NEC-1), GSK872 were purchased from MedChem Express (New Jersey, USA). Anti-RIPK3 (A5431), MLKL (A5579), TNF (A0277), IL-6(A0286), and IL-1 β (A1112) antibodies were purchased from ABclonal (Wuhan, China). P-p44/42 MAPK (ERK1/2), p44/42 MAPK(ERK1/2), NF- κ B p65, NF- κ B p65, p-p38 MAPK and p38 MAPK were purchased from Cell Signaling Technology Company (Danvers, MA, USA). GAPDH antibody and Horseradish peroxidase-conjugated secondary antibodies were purchased from Proteintech (Chicago, USA). β -Amyloid (25-35) was purchased from GL Biochem (Shanghai China). CD11b-PE-7-AAD, FITC Annexin V Apoptosis Detection Kit I were purchased from BD Pharmingen (San Diego, CA, USA). CytoTox 96 Non-Radioactive Cytotoxicity Assay (LDH) was purchased from Promega (Madison, USA).

Preparation of A β 25-35 peptide

A β 25-35 was dissolved in sterile distilled water at a concentration of 1 nM and then filtered through a 0.22 μ m filter. A β 25-35 was aggregated during a 7 day incubation period at 37°C and then stored in a -20°C freezer for later use.

Preparation of DHA

DHA was dissolved in DMSO at a concentration of 10 nM and then filtered through a 0.22 μ m filter. This DHA solution was stored in a -80°C freezer for later use. The control group was added with the same concentration DMSO.

Cell culture conditions

The human THP-1 cell line was a gift from NanFang Hospital of Southern Medical University and was cultured in RPMI 1640 medium supplemented with 10% FBS at 37°C in a humidified incubator (5% CO₂, 95% air). The THP-1 cells (10 × 10⁴ cells/ml) were pretreated with DHA in 0% FBS for 1, 3, or 5 days. Conditioned media (CM) from each THP-1 plate was then collected and stored at -80°C for later use.

The SY5Y cell line was a gift from The Judicial Identification Center of Southern Medical University and was cultured in the same growth conditions as the THP-1 cell line. SY5Y cells were treated with A β 25-

35 aggregates and the conditioned media (CM) collected from plates of THP-1 cells.

Primary cortical neurons were prepared from embryonic day 16–18 (E16–18) C57BL/6 mice, which were purchased from the Experimental Animal Center of Southern Medical University. All animal experiments were approved by the Institutional Animal Care and Use Committee of Southern Medical University in accordance with the guidelines of the Institutional Animal Care and Use Committee of the National Institutes of Health and the Guide for the Care and Use of Laboratory Animals. The cerebral cortices of E16–18 embryos were dissected under bacterial conditions and placed in sterile PBS. The cerebellum, hippocampus and medulla were removed and the cortex was isolated; meninges and blood vessels were then removed. Brain tissue was minced with a razor blade and then digested in 0.25% trypsin at 37°C for 6 min. Addition of complete 10% FBS medium was used to terminate digestion. Cell suspensions were then filtered using a 70 µm cell filter. The filtered cell suspension was collected in a 15ml conical and centrifuged at 1000 rpm for 5 min. The supernatant was discarded and the cells were seeded onto L-lysine coated plates. Initial administration of a modified neurobasal media (B27 supplement, 1% L-glutamine, 0.05% glutamate, and 1% penicillin/streptomycin) was followed by media changes every other day using a standard neurobasal media (B27 supplement, 1% L-glutamine, 1% penicillin/streptomycin). After 5 days of culture, primary cortical neurons were stimulated with addition of varying concentrations of Aβ25–35 or CM obtained from THP-1 monocytes.

Detection of cell viability using cell counting kit-8 (CCK8)

Cells for each treatment group were counted and seeded in 96-well plates at 100 µl/well in quintuplicate for each treatment group. After treatment for the appropriate time, 10 µl of CCK8 were added and the absorbance at 450 nm was measured using a plate reader.

Detection of cytotoxicity via LDH release

Cytotoxicity was monitored using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (LDH assay) kit, which quantifies the amount of lactate dehydrogenase (LDH) released from cells into the media. The assay was performed according to the manufacturer's protocol. Briefly, following treatment, the culture plate was centrifuged at 250 × g in a high-efficiency multi-purpose centrifuge for 5 min, 50 µl of supernatant was pipetted into a new 96-well plate, and LDH activity determined was based on absorbance readings at 490 nm. The amount of LDH release is used to calculate cytotoxicity. Three replicates were used for each experiment.

Measure of apoptosis/ necrosis and CD11b expression by flow cytometry

Flow cytometry experiments were done using the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen) according to the manufacturer's protocol. After stimulating the various cell groups, 6-well

plates of THP-1 cells were centrifuged at 1000 rpm for 5 min twice with gentle PBS wash steps between spins. 500 µl of binding solution was then added to gently suspend the cells. After waiting 10 min, 20 µl of CD11b- PE, 5 µl of Annexin V-FITC stain, and 5 µl of 7-AAD was added to each well and gently mixed. The plate was then incubated at room temperature (20–25°C) in the dark for 15 min. The cells were then analyzed using a flow cytometer. BD FACSDiva 8.0.1 was used to analyze cell apoptosis/necrosis and CD11b expression.

Transwell migration assays

THP-1 cells were collected by centrifugation at 1,200 × g for 5 min at room temperature and resuspended in RPMI 1640 was seeded into the upper chamber (3450, 5.0 µm porous polycarbonate membrane, Corning, USA), and the lower chamber was coated with 600 µl of RPMI 1640 media with 10% FBS. After 12 h of culture, the transwell chamber membranes were cleaned and fixed with 4% paraformaldehyde for 30 min and then stained with 1% crystal violet for 20 min. Cell counting was carried out using a microscope (100×) and was performed in triplicate.

Western blot analysis

The protein concentration of each sample was determined using a dye-binding protein assay kit (Bioimage, San Diego, USA), and the protein was stored at –20°C for later use. Protein samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), after which the protein was transferred to a PVDF membrane (Millipore Corporation, Billerica, MA 01821, USA). The membrane was blocked with 5% bovine serum albumin (Pharmingen San Diego, CA, USA) for 1 h at room temperature, followed by overnight incubation in primary antibody at 4°C, followed by incubation with a horseradish peroxidase-coupled secondary antibody at room temperature for 1 h. Protein bands were visualized with a chemiluminescence detection kit (Beyotime, Guangzhou, China).

Statistical analysis

Spss23.0 software was used for statistical analysis of all data obtained. For experiments comparing multiple groups, statistical analysis was done using Tukey's method with one-way ANOVA. For experiments comparing two groups, statistical analysis was done using a t test. Data are shown as averages ± SD, and differences are considered to be significant at $p < 0.05$.

Results

Aβ25–35 have two aspects of THP–1 cells viability

To investigate the effect of stimulating THP-1 monocytes with Aβ25–35, we treated cells with varying concentrations of Aβ25–35 for different amounts of time and examined cell viability and cytotoxicity.

These experiments indicated that THP-1 cells increased viability compared to control when A β 25-35 concentrations were lower than 10 μ M. However, after seeing a gradual increase in viability up to 10 μ M concentrations, THP-1 cells had decreased viability with prolonged A β 25-35 stimulation (Fig. 1A-1C).

The CCK8 assay measures cell viability based on intact mitochondrial function, and the LDH release assay determines cytotoxicity based on cell membrane integrity. Therefore, to determine if A β 25-35 stimulation has cytotoxic effects on THP-1 monocytes, we measured the LDH content of cell supernatants under the same conditions used for viability assays (Fig. 2A-2C). The results of the LDH assay followed similar trends to those observed with the CCK8 assay. Therefore, stimulation with A β 25-35 for a relatively short time or with a low concentration has minimal cytotoxic effects on THP-1 cells and promotes viability, and A β 25-35 has two aspects of THP-1 cells viability.

A β 25-35 treatment influences THP-1 cell apoptosis (necrosis) and differentiation

One of the most striking features that macrophages acquire as a result of differentiation is increased resistance to apoptosis[22]. To further explore how A β 25-35 treatment influences THP-1 cells, we wanted to determine whether apoptosis (necrosis) and THP-1 cell differentiation might be involved. Therefore, THP-1 cells were treated with A β 25-35 for 3 days, and then analyzed by flow cytometry with Annexin V-FITC, 7AAD and the antigen CD11b (a surface marker for THP-1 cell differentiation [22]). Figure 3A shows the gating scheme used: normal cells (Q3), early apoptotic cells (Q4), late apoptotic /necrotic cells (Q2), and CD11b PE-A (cell expression after differentiation). Treatment with low concentrations of A β 25-35 (0-10 μ M) significantly protected THP-1 cells from apoptosis (necrosis), but treatment with high concentrations (10-40 μ M) resulted in increased apoptosis (necrosis) (Fig. 3B). Simultaneously, compared with the control group (0 μ M A β), CD11b expression increased with A β 25-35 concentration (Fig. 3C).

Taken together. The increased apoptotic resistance is generated at the same time as the cytotoxic effect. Treated with low concentration of A β 25-35, the increased resistance to apoptosis is dominated, conversely, the advantage of cytotoxic effect is highlighted. These results indicate A β 25-35 regulates two aspects of THP-1 cells necroptosis, and the increased resistance to apoptosis is correlated with THP-1 monocyte differentiation.

DHA inhibits A β -induced activation of THP-1 cells

To investigate whether DHA treatment is cytotoxic to THP-1 cells, we repeated the CCK8 viability assay with THP-1 cells treated with varying concentrations of DHA for 1 d, 3 d and 5 d. This experiment showed that DHA has a positive effect on THP-1 cell viability at a range of concentrations between 0.0625 and 1 μ M (Fig. 4).

To determine how DHA affects viability of A β 25–35 treated THP–1 cells, THP–1 cells were pre-treated with or without various concentrations of DHA in RPMI 1640 with 0% FBS for 24 h followed by treatment with 50 μ M A β 25–35 for 3 days. After 3 days, cell viability was analyzed. As shown in Fig. 5, DHA pretreatment significantly inhibited the activation of THP–1 cells induced by A β 25–35 in a concentration-dependent manner.

DHA indirectly suppresses THP–1 cell-mediated neuronal activation

Substantial evidence suggests that DHA can directly reduce A β -induced activation[13, 23]. We therefore tested whether pre-treatment with low concentrations (0.5, 1 μ M) of DHA in RPMI 1640 with 0% FBS for 24 h could reduce A β 25–35-mediated activation of neurons and SY5Y cells. Using the CCK8 assay as a readout, we observed that when treating neurons and SY5Y cells with 50 μ M A β 25–35 alone for 1 d or 3 d, viability did not significantly decrease until day 3 (Fig. 6, Fig. 7). Furthermore, low concentrations of DHA did not inhibit A β -induced activation of neurons and SY5Y cells, similar to previous findings[23].

We next investigated whether A β aggregates could exert their effect on neuronal cells through THP–1 cells and how DHA might regulate this process. THP–1 cells were pre-treated with or without various concentrations of DHA in RPMI 1640 with 0% FBS for 24 h, followed by 50 μ M A β 25–35 treatment for 3 d (the media was not changed). CM from each THP–1 cell treatment group was collected and used to stimulate neurons for 3 d or 5 d. Using the CCK8 assay as a readout, CM treated with A β alone (A β -CM) induced neuronal toxicity to a much greater extent than the vehicle control (control CM), while CM from THP–1 cells that were pretreated with DHA prior to A β treatment (DHA+A β -CM) induced significantly less cytotoxicity in cortical neurons compared with A β -CM (Fig. 8B). Interestingly, compared to cells directly treated with 50 μ M A β 25–35 for 3 days (Fig. 6B), less cytotoxicity was observed in cortical neurons treated with A β -CM for 3 days (Fig. 8A). Taken together, these findings indicate that THP–1 cells played an important role in A β clearance and DHA treatment can indirectly suppress THP–1 cell-mediated neuronal activation.

DHA restored the THP–1 monocytes migration treated with A β 25–35

To investigate whether DHA treatment could modulate the A β 25–35-induced THP–1 monocytes migration phenotype THP–1 cells were pre-treated with or without low concentrations (0.5, 1 μ M) of DHA in RPMI 1640 with 0% FBS for 24 h, followed by 50 μ M A β 25–35 treatment for 3 days. A transwell system was used to test the migratory ability of THP–1 cells collected from different treatment groups. As shown in Fig. 9, the number of migratory THP–1 monocytes after 12h were significantly decreased in A β group compared to control. In comparison to A β group, the number of migratory cells in the A β +DHA groups was significantly higher. Therefore, the results indicate that DHA pre-treatment inhibits the anti-migratory effect exerted by A β 25–35 on THP–1 monocytes.

DHA suppresses A β -induced expression of pro-inflammatory cytokines in THP-1 cells

TNF- α , IL-1 β , and IL-6 are the primary pro-inflammatory cytokines in the inflammatory response cascade. To investigate how DHA treatment affects A β -induced expression of pro-inflammatory cytokines, the protein expression levels of TNF- α , IL-1 β and IL-6 in DHA and A β 25-35 treated THP-1 cells were quantified. TNF- α , IL-1 β , and IL-6 expression all increased significantly in THP-1 cells after treatment with 50 μ M A β 25-35 alone (Fig. 10). However, when THP-1 cells were treated with DHA prior to treatment with A β 25-35, expression of these pro-inflammatory cytokines was notably decreased compared to 50 μ M A β 25-35 treatment alone. These results demonstrate that DHA treatment in THP-1 cells suppresses A β -induced protein expression of TNF- α , IL-1 β and IL-6.

DHA inhibits A β 25-35-induced necroptosis of THP-1 monocytes

We next investigated whether A β -mediated regulation of necroptosis could be modulated by DHA in human THP-1 monocytes. Western blotting for key necroptosis proteins revealed that cells pre-treated with DHA had lower expression levels of RIPK1, RIPK3, and MLKL compared to cells treated with only A β 25-35 (Fig. 11). Therefore, our results indicate that DHA treatment inhibits A β 25-35-induced necroptosis of THP-1 monocytes.

DHA attenuates A β 25-35-induced necroptosis of THP-1 monocytes via the RIPK1/RIPK3 signaling pathway

Necroptosis is a lytic cell death program defined by activation of the receptor interacting protein kinase-1 (RIPK1) and RIPK3 to form an oligomeric "necrosome," which leads to the phosphorylation and activation of the effector pseudokinase mixed lineage kinase domain-like (MLKL). To further investigate whether DHA could attenuate A β 25-35-induced necroptosis of THP-1 monocytes via the RIPK1/ RIPK3 signaling pathway, we tested whether NEC-1 (RIPK1 inhibitor[24]) could inhibit A β -induced activation of THP-1 cells. Viability of THP-1 cells was determined after cultures were pre-treated with or without various concentrations of NEC-1 in RPMI 1640 with 0% FBS for 24 h followed by 50 μ M A β 25-35 treatment for 3 days. NEC-1 pre-treatment significantly inhibited A β 25-35-induced activation of THP-1 cells in a concentration-dependent manner (Fig. 12A). The 10 μ M NEC-1 concentration was chosen for all subsequent experiments.

THP-1 cells were pre-treated with or without NEC-1 and DHA in RPMI 1640 with 0% FBS for 24 h followed by 50 μ M A β 25-35 treatment for 3 days. Western blot analysis was then used to determine RIPK3 expression levels. Expression of RIPK3 was suppressed by NEC-1 treatment in the presence or absence of DHA compared when compared with A β 25-35 treatment (Fig. 12B-C). This finding indicates that DHA could prevent A β -induced necroptosis of THP-1 cells via the RIPK1/RIPK3 signaling pathway.

DHA suppresses ERK1/2 signaling activated A β 25–35 but does not affect p38 or NF- κ B/p65 signaling

NF- κ B signaling plays a key role in immune and inflammatory responses, while MAPK kinases (p38, ERK1/2) are key regulators of a variety of cellular functions, including cell survival, apoptosis and inflammation response[19, 20]. We tested whether DHA treatment modulated A β -mediated regulation of MAPK and NF- κ B signaling in human THP–1 monocytes. THP–1 cells were pretreated with or without varying concentrations of DHA in RPMI 1640 with 0% for 1 day prior to incubation with 50 μ M A β 25–35 for another 3 days. Compared to the 0 μ M A β 25–35 treatment group, the phosphorylation levels of p38 and ERK1/2 were significantly increased in the 50 μ M A β 25–35 treatment group (Fig. 13). However, compared to the 50 μ M A β 25–35 treatment group, the phosphorylation level of ERK1/2, but not p38 or p65, was decreased in the A β +DHA treatment groups. Taken together, our results show that DHA treatment suppresses A β 25–35-induced ERK1/2 signaling in THP–1 cells, but does not affect p38 or NF- κ B/p65 signaling.

DHA targets RIPK1 to inhibit ERK1/2 phosphorylation in THP–1 cells treated with A β 25–35

Increasing evidence indicates that RIPK1 can regulate cell survival via activation of the ERK1/2 signaling pathway[25, 26]. Therefore, THP–1 cells were pre-treated with or without NEC–1 and DHA in RPMI 1640 with 0% FBS for 24 h, followed by treatment with 50 μ M A β 25–35 for 3 days. Western blotting was used to determine the phosphorylation status of ERK1/2. As shown in Fig. 14, phosphorylation of ERK1/2 was suppressed by the RIPK1 inhibitor NEC–1 in the presence or absence of DHA compared with A β 25–35 treatment. Hence, our results indicate that DHA treatment inhibits ERK1/2 phosphorylation via RIPK1 in THP–1 cells treated with A β 25–35.

Discussion

Monocytes play a crucial role in AD, as monocyte-derived perivascular macrophages efficiently execute A β phagocytosis[6, 27]. The mechanism connecting brain amyloid accumulation and monocyte degeneration is multifactorial. Deposition of A β aggregates in blood vessels can directly damage the vessel wall and allow more monocytes to pass through the parenchyma[28]. Toll-like receptor 2 (TLR2) and Toll-like receptor 4 (TLR4) were shown to be indirectly involved in A β phagocytosis through the formation of a receptor complex with CD14 and the subsequent activation of monocytes[29]. A β -induced migration of monocytes across human brain endothelial cells involves both RAGE and PECAM–1[7]. Increasing vascular A β clearance through the activity of patrolling monocytes could help ameliorate AD symptoms. In this regard, reduction of the migration, phagocytosis, or number of mononuclear cells in transgenic AD mice is detrimental, whereas compounds that increase their number and phagocytic activity are generally beneficial [10]. Oligomeric amyloid- β (1–42) is known to induce THP–1 monocyte adhesion and maturation [30], but these findings have not been further explored. Therefore, our

experiments were designed to further explore possible mechanisms of the interaction between monocytes and A β . We demonstrated that A β 25–35 influences THP–1 cell viability, as confirmed by LDH and CCK8 assays. Macrophages are known to become more resistant to apoptosis as a result of differentiation [22, 31]. Interestingly, by flow cytometry analysis, we observed that increased apoptotic resistance develops in correlation with trends in A β 25–35-mediated THP–1 cell cytotoxicity. When THP–1 cells are treated with low concentrations of A β 25–35, we observed increased resistance to apoptosis, conversely, the advantage of cytotoxic effect is highlighted. Taken together, our results indicate that A β 25–35 had a dual effect on THP–1 cell apoptosis (necrosis), and increased THP–1 cell resistance to apoptosis is associated with THP–1 monocyte differentiation.

Studies have shown that DHA has a neuroprotective effect in many neurodegenerative diseases through its antioxidant and anti-inflammatory properties and its ability to activate various cell signaling pathways[16, 32]. Microglial cells and macrophages are key cells in the immune system, and they play important roles in CNS repair and regeneration[33]. In addition to their neuroprotective roles, microglial cells and macrophages are also the major producers of proinflammatory cytokines, such as TNF- α , IL–1 β , and IL–6, which can greatly inhibit brain repair and neurogenesis[34]. Here, we found that low concentrations of DHA were insufficient to inhibit A β -induced activation of neurons and SY5Y cells but could indirectly suppress THP–1 cell-mediated neuronal activation effectively. Moreover, we found that pre-treatment with DHA effectively attenuated A β -induced activation of THP–1 monocytes and markedly suppressed expression of TNF- α , IL–1 β , and IL–6. Interestingly, our results also indicated that DHA treatment restored migration of THP–1 monocytes that had been treated with A β 25–35.

Necroptosis, a form of regulated necrotic cell death mediated by RIPK1, RIPK3, and MLKL (mixed-lineage kinasedomain-like pseudokinase) [35], can be activated under apoptosis-deficient conditions [17, 36]. Substantial evidence indicates that necroptosis is involved in AD pathogenesis [37, 38]. RIPK1, RIPK3, and MLKL expression were all found to be increased in the brains of individuals with AD individuals as well as in the brains of AD animal models [39]. Therefore, we investigated whether A β aggregates or DHA mediated regulation of necroptosis in human THP–1 monocytes. Our results indicate that A β 25–35 treatment induced necroptosis of THP–1 monocytes that could be attenuated with DHA treatment.

NEC–1(necrostatin–1), a specific inhibitor of RIPK1, prevents cell necroptosis[40, 41]. Our results showed that NEC–1 pre-treatment significantly inhibited the activation of THP–1 cells induced by A β 25–35 in a concentration-dependent manner. Additionally, expression of RIPK3 was suppressed by NEC–1 treatment in the presence or absence of DHA compared with A β 25–35 treatment. This finding suggests that DHA treatment could attenuate A β 25–35-induced necroptosis of THP–1 monocytes via modulation of the RIPK1/RIPK3 signaling pathway.

The mitogen-activated protein kinases (MAPKs) in mammals include c-Jun NH2-terminal kinase (JNK), p38 MAPK, and extracellular signal-regulated kinase (ERK). These enzymes are serine–threonine protein kinases that regulate various cellular activities including proliferation, differentiation, apoptosis or cell survival, inflammation, and innate immunity[42, 43]. Impaired signaling pathways contribute to the

pathology of many human diseases, including cancer and neurodegenerative diseases such as AD, Parkinson's disease, and amyotrophic lateral sclerosis [44, 45]. Subsequent studies have shown that NF- κ B is a ubiquitously expressed dimeric transcription factor involved in cellular processes such as inflammation, adhesion, proliferation, differentiation, apoptosis, and oncogenesis [46]. This family of transcription factors also plays an important role in nervous system development and function [47]. We explored whether A β aggregates or DHA regulate the MAPK and NF- κ B signaling pathways in human THP-1 monocytes. Our findings indicate that A β 25-35 treatment activated the ERK1/2 and p38 signaling pathways, but not NF- κ B/p65 signaling, while pre-treatment with DHA followed by A β 25-35 treatment suppressed only ERK1/2 signaling.

Several studies have shown that RIPK1 activates MAPKs such as p38, JNK, and ERK during apoptosis following TNF- α treatment[21, 25, 48] and that RIPK1 plays an important role at the crossroads of a cell's decision to live or die [49]. Our study revealed that ERK1/2 phosphorylation is suppressed by NEC-1 in the presence or absence of DHA treatment when compared with A β 25-35 treatment. Our findings indicate that DHA treatment could inhibit ERK1/2 phosphorylation via RIPK1 in THP-1 cells treated with A β 25-35. Previous work has demonstrated that ERK activation plays a critical role in necroptosis, and the RIPK1/ERK signaling pathway may present a new therapeutic avenue for treatment of ischemia-reperfusion injury and neurodegenerative diseases where necroptotic cell death is implicated[50]. However, the relationship between necroptotic signaling pathways and ERK1/2 is incompletely understood and requires further exploration. Since this study relied on an in vitro model using THP-1 cells instead of primary monocytes, additional studies with primary monocytes and an in vivo model are needed to confirm and extend our findings.

Conclusion

Our findings indicate that A β 25-35 regulates two aspects of THP-1 cells necroptosis. We also observed that increased resistance to apoptosis in THP-1 cells that is correlated with THP-1 monocyte differentiation. Our results also indicated that DHA treatment restored migration of THP-1 monocytes that had been treated with A β 25-35. We found that pre-treatment with DHA effectively inhibited A β -induced activation of THP-1 monocytes and markedly suppressed protein expression of TNF- α , IL-1 β , and IL-6. DHA treatment can attenuate necroptosis via modulation of the RIPK1/RIPK3 signaling pathway and the phosphorylation status of ERK1/2 in THP-1 monocytes. Our study offers insight into the anti-inflammatory and anti-necroptotic properties of DHA. Therefore, our findings suggest that DHA might could have therapeutic potential for use in AD management.

Abbreviations

AD, Alzheimer's disease; DHA, Docosahexaenoic acid; MAPKs, mitogen-activated protein kinases; NF- κ B, nuclear transcription factor-kappa B; TNF- α , tumor necrosis factor-alpha; IL-6, interleukin-6; IL-1 β , interleukin-1 beta; iNOS, inducible NO synthase; MLKL, mixed lineage kinase domain-like; NEC-1, necrostatin-1.

Declarations

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Tianming Lü conceived and designed the study, Shiqi Yuan and Huan Li performed the experiments and wrote the paper. Wenyi Xie, Yuanyuan Wang, Jiafa Zhang, Zibo cai, and Zhenlin Mao performed part of the experiments. Weibing Xie and Tianming Lü reviewed and edited the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

There is no conflict of interest to declare.

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Figures

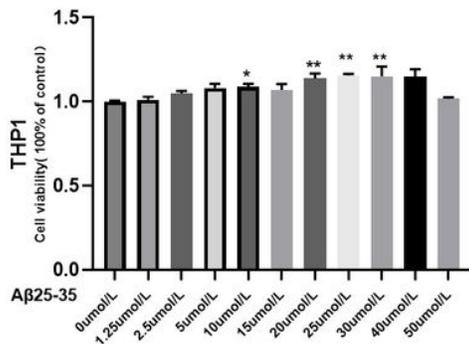


Fig. 1A 1d

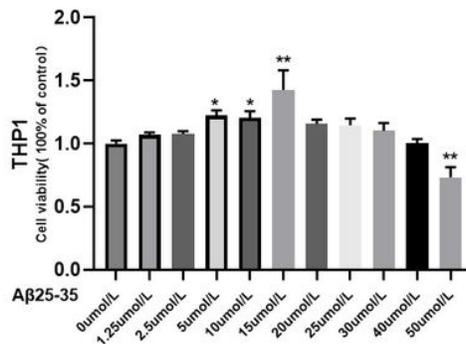


Fig. 1B 3d

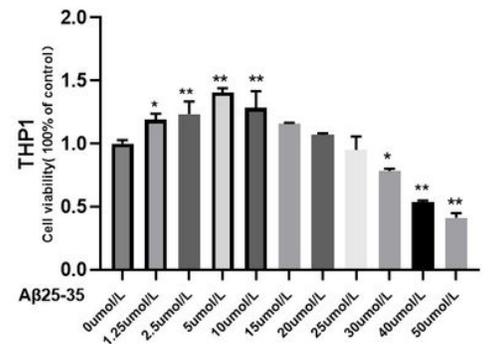


Fig. 1C 5d

Figure 1

THP-1 cell viability following treatment with a concentration gradient of Aβ25-35, with different stimulus times; cell viability was determined using the CCK8 assay (Fig. 1A-1C). All samples compared to control (0 μM Aβ25-35); data are represented as averages ± S.D. * P < 0.05, ** P < 0.01

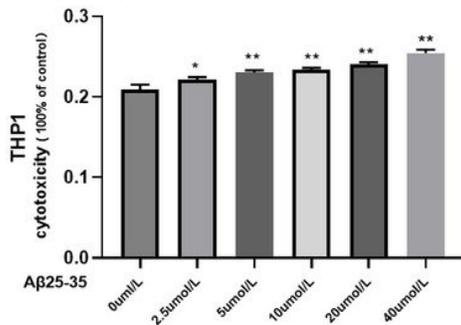


Fig. 2A 1d

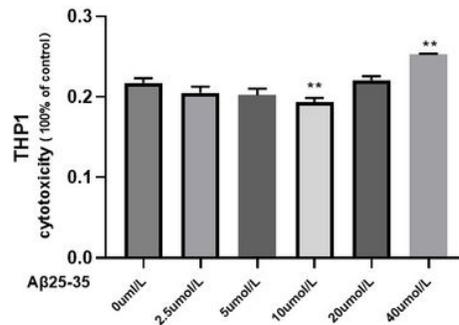


Fig. 2B 3d

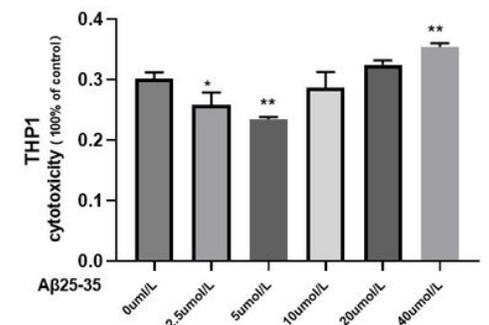


Fig. 2C 5d

Figure 2

Cytotoxicity was determined using an LDH release assay, Fig. 2A-2C. All samples compared to control (0 μM Aβ25-35); data are represented as averages ± S.D. * P < 0.05, ** P < 0.01

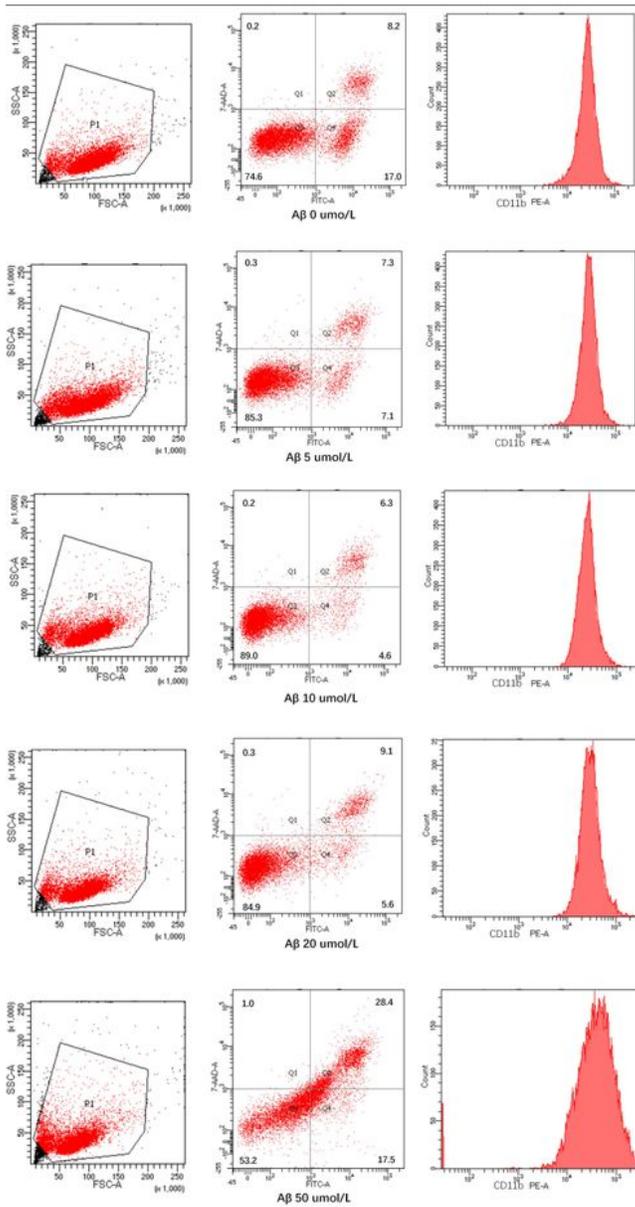


Fig.3A

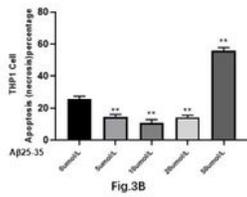


Fig.3B

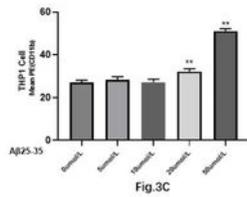


Fig.3C

Figure 3

THP1 cells activity treated with Aβ25-35 whether the apoptosis (necrosis) and differentiation of THP1 cells is involved, after THP1 cells was treated with Aβ25-35 for 3 days, Annexin v-fitc, 7AAD and antigen CD11b (as a surface marker for THP1 cells differentiation were used for flow cytometry analysis. Fig 3A: Normal cells (Q3), Early apoptotic cells (Q4), Late apoptotic /necrosis cell (Q2), CD11b PE-A (cell

expression after differentiation). The statistical results shown as Fig 3B 3C. Data are presented as mean \pm S.D. Compared with Control group (A β 25-35 0uM), * P < 0.05 ** P < 0.01

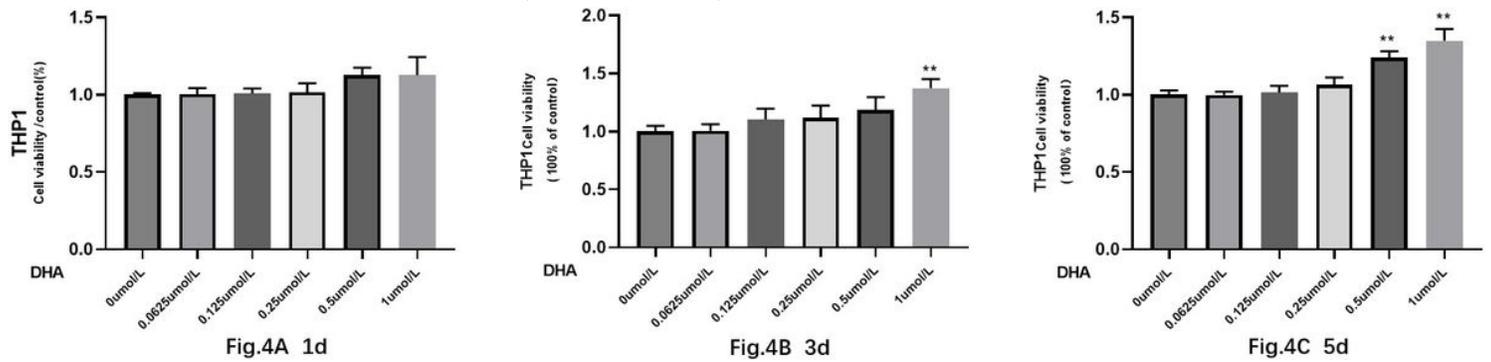


Figure 4

Effect of DHA on viability of THP-1 cells. DHA was not cytotoxic to THP-1 cells at concentrations of up to 1 μ M. THP-1 cells were treated with varying concentrations (0.0625-1 μ M) of DHA for 1 d, 3 d, and 5 d. Data are represented as averages \pm S.D. as a percentage of the control group, * P < 0.05 ** P < 0.01.

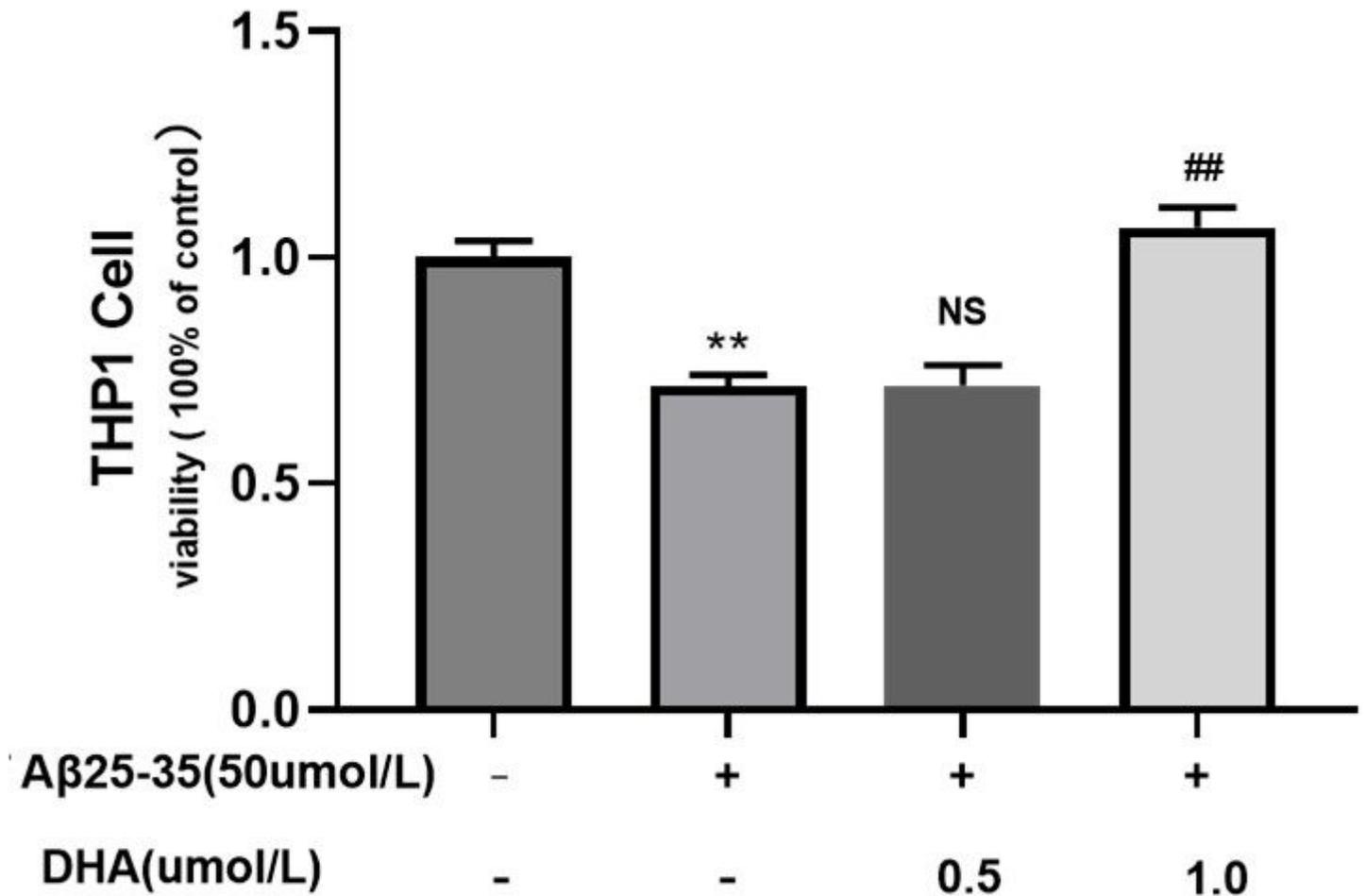


Fig.5

Figure 5

Effect of DHA pre-treatment on viability of THP-1 cells treated with A β 25-35. The viability of THP-1 cells was analyzed after cultures were pre-treated with DHA (0.5, 1 μ M) in RPMI 1640 with 0% FBS for 24 h. followed by 50 μ M A β 25-35 treatment for 3 d. Cell viability was determined using the CCK8 assay. Data are represented as averages \pm S.D. as a percentage of the control group. * p <0.05 and ** p <0.01, compared to 0 μ M A β 25-35 alone; # p <0.05 and ## p <0.01, compared to 50 μ M A β 25-35.

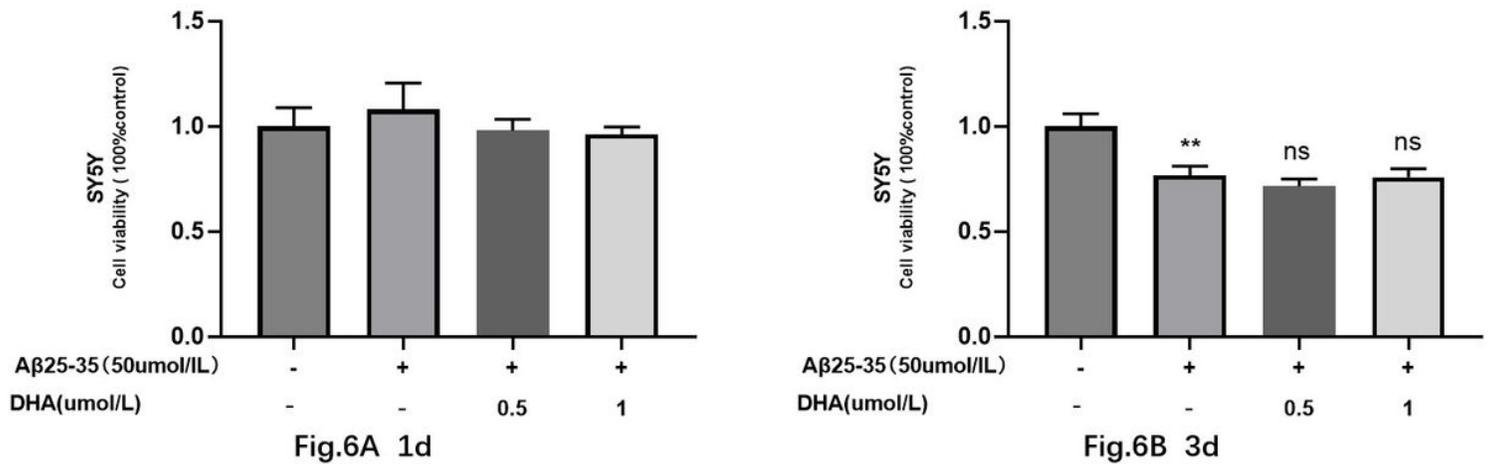


Figure 6

Treatment with low concentrations of DHA did not inhibit A β -induced activation of neurons and SY5Y cells. Following pre-treatment with or without low concentrations (0.5, 1 μ M) of DHA in RPMI 1640 with 0% FBS for 24 h, and subsequent treatment with 50 μ M A β 25-35 for 1 d and 3 d, viability of neurons and SY5Y was determined using the CCK8 assay. Data are represented as averages \pm S.D. as a percentage of the control group. * p <0.05 and ** p <0.01, compared to 0 μ M A β 25-35 alone. # p <0.05, ## p <0.01, compared to 50 μ M A β 25-35.

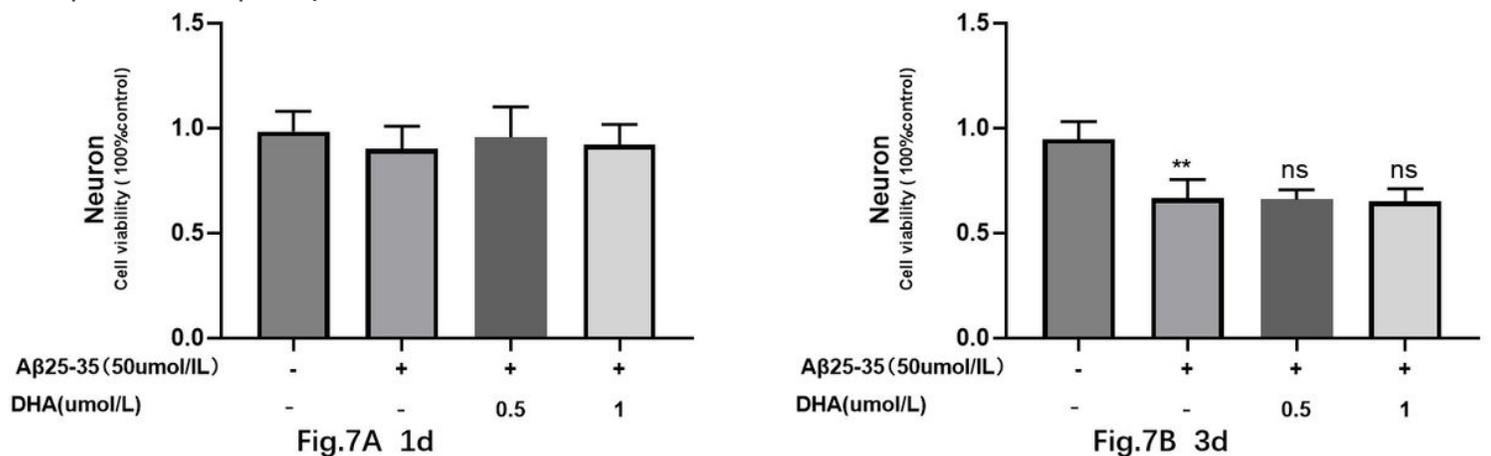


Figure 7

Treatment with low concentrations of DHA did not inhibit A β -induced activation of neurons and SY5Y cells. Following pre-treatment with or without low concentrations (0.5, 1 μ M) of DHA in RPMI 1640 with 0% FBS for 24 h, and subsequent treatment with 50 μ M A β 25-35 for 1 d and 3 d, viability of neurons and SY5Y was determined using the CCK8 assay. Data are represented as averages \pm S.D. as a percentage of

the control group. *p <0.05 and **p <0.01, compared to 0 μ M A β 25-35 alone. #p <0.05, ##p <0.01, compared to 50 μ M A β 25-35.

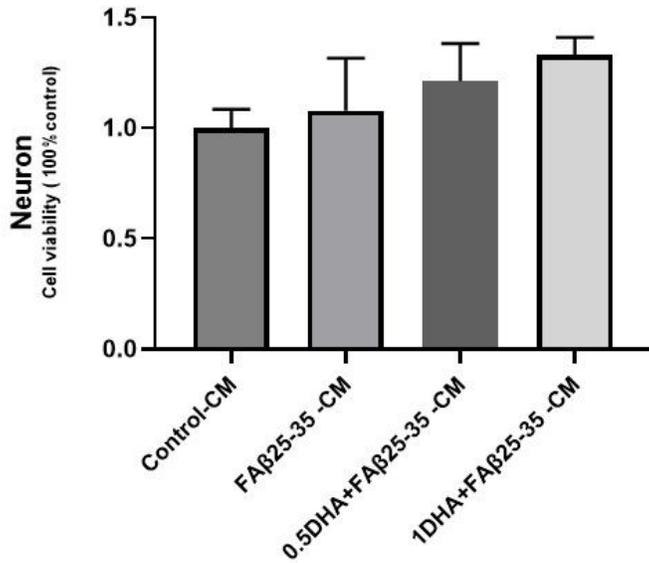


Fig.8A 3d

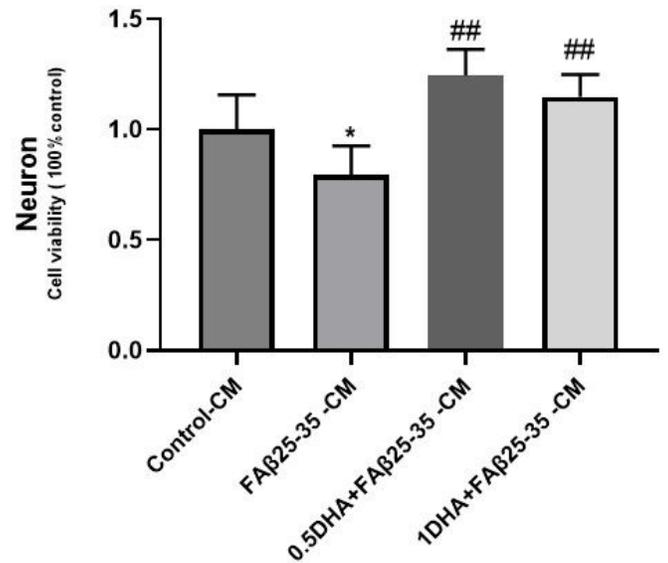


Fig.8B 5d

Figure 8

DHA indirectly suppresses THP-1 cell-mediated neuronal activation. THP-1 cells were pre-treated with or without various concentrations of DHA in RPMI 1640 with 0% FBS for 24 h, followed by 50 μ M A β 25-35 treatment for 3 or 5 d. CM from each THP-1 treatment group was collected and used to stimulate neurons for 3 or 5 d. Cell viability was determined using the CCK8 assay. Data are represented as averages \pm S.D. as a percentage of the control group. *p <0.05 and **p <0.01, compared to control-CM alone. #p <0.05 and ##p <0.01, compared to A β -CM.

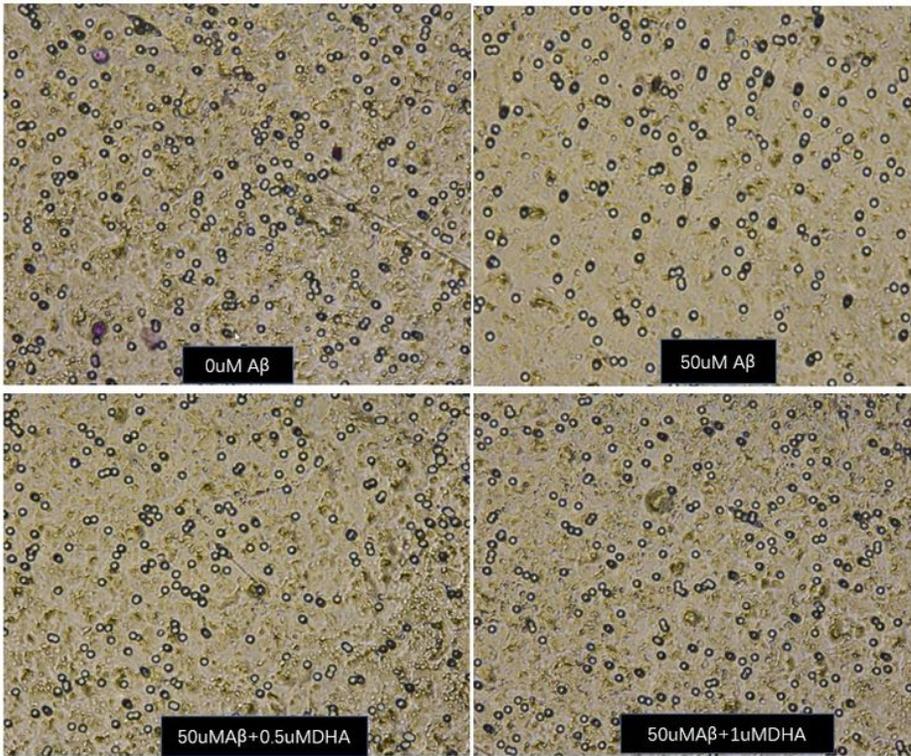


Fig.9A

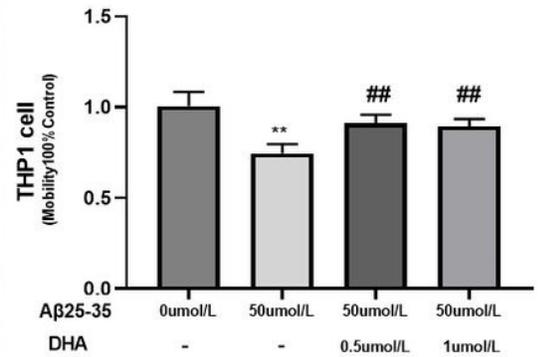


Fig.9B

Figure 9

DHA pre-treatment inhibits the anti-migratory effect exerted by Aβ₂₅₋₃₅ on THP-1 monocytes. THP-1 cells were pre-treated with or without low concentrations (0.5, 1 μM) of DHA in RPMI 1640 with 0% FBS for 24 h, followed by 50 μM Aβ₂₅₋₃₅ treatment for 3 days. A transwell system was used to test the migratory ability of THP-1 cells after 12 h from different treatment groups, Cell counting was carried out using a microscope (100X). Data are represented as averages ± S.D. as a percentage of the control group. *p < 0.05 and **p < 0.01, compared to 0 μM Aβ₂₅₋₃₅ alone. #p < 0.05 and ##p < 0.01, compared to 50 μM Aβ₂₅₋₃₅.

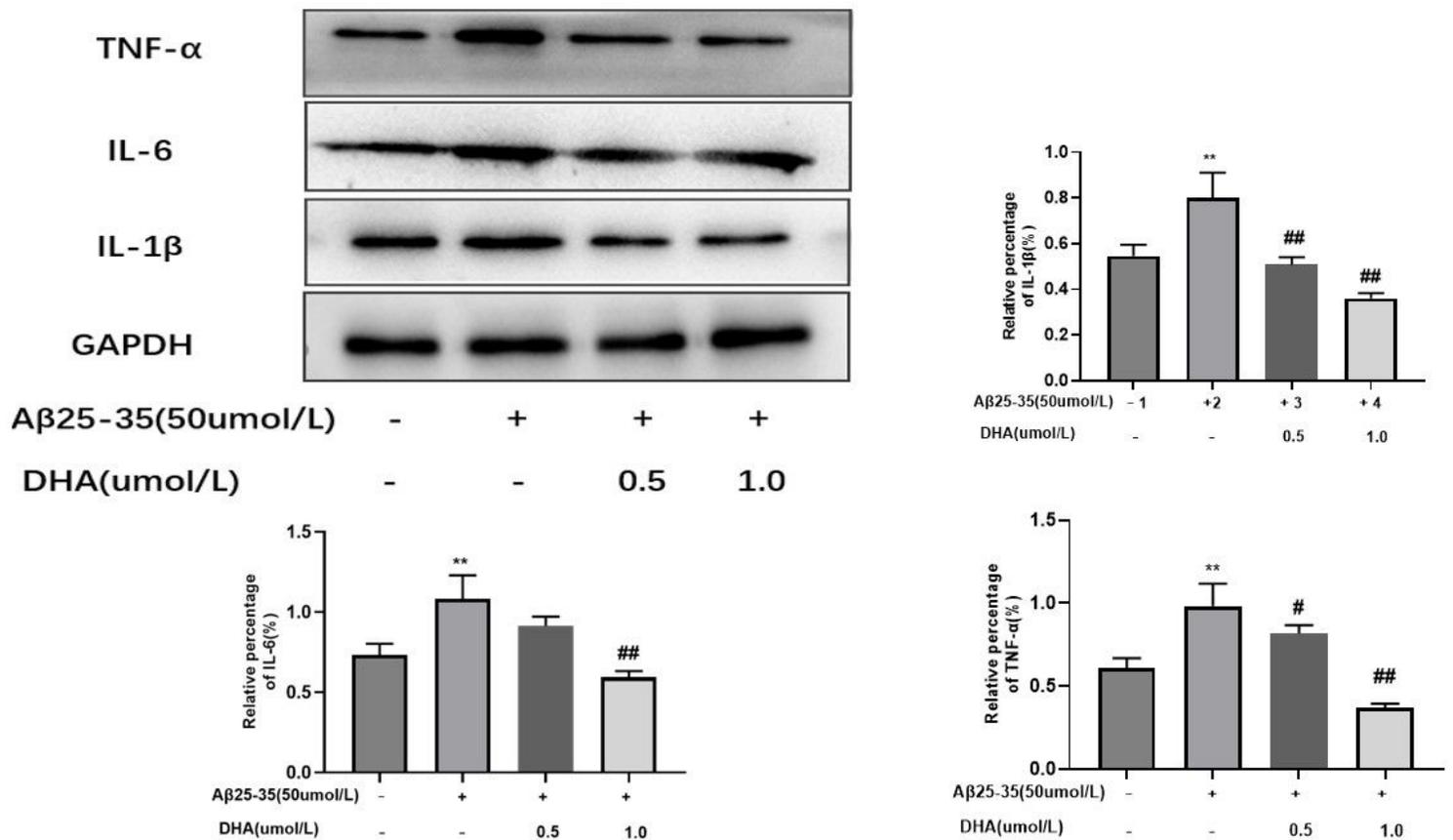


Fig. 10

Figure 10

DHA treatment has an inhibitory effect on expression of pro-inflammatory cytokines in Aβ-stimulated THP-1 cells. THP-1 cells were pretreated with or without varying concentrations of DHA in RPMI 1640 with 0% FBS for 1 day prior to incubation with 50 μM Aβ25-35 for another 3 days. Western blot analysis of protein expression of TNF-α, IL-1β and IL-6, normalized to GAPDH. Data are represented as averages ± S.D. *p < 0.05 and **p < 0.01, compared to 0 μM Aβ25-35 alone. #p < 0.05 and ##p < 0.01, compared to 50 μM Aβ25-35.

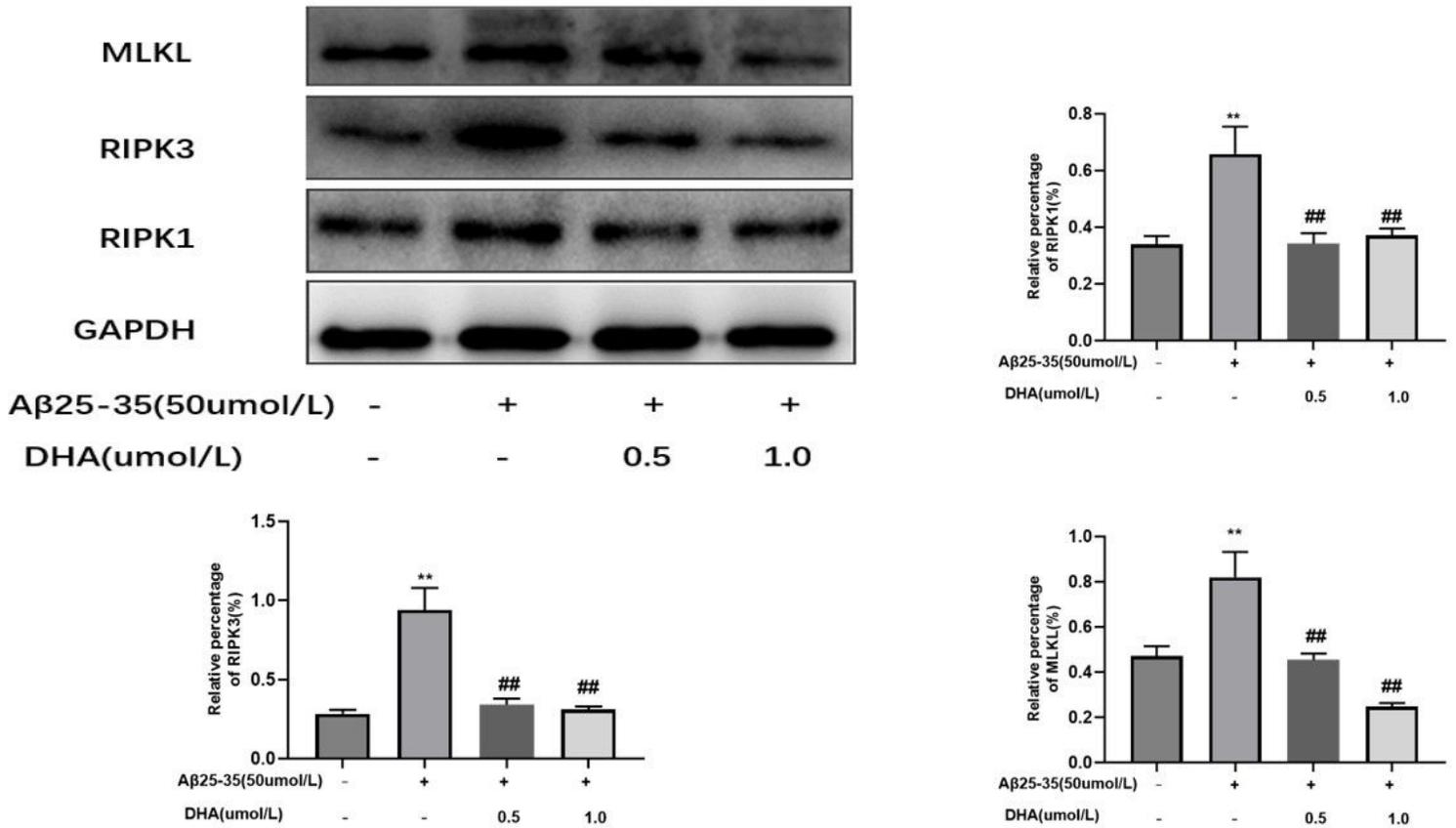


Fig. 11

Figure 11

DHA treatment inhibits Aβ25–35-induced necroptosis of THP-1 monocytes. THP-1 cells were pretreated with or without varying concentrations of DHA in RPMI 1640 with 0% FBS for 1 day prior to incubation with 50 μM Aβ25-35 for another 3 days. Western blot analysis of protein expression of RIPK1, RIPK3 and MLKL, normalized to GAPDH. Data are represented as averages ± S.D. as a percentage of the control group. *p < 0.05 and **p < 0.01, compared to 0 μM Aβ25-35 alone. #p < 0.05 and ##p < 0.01, compared to 50 μM Aβ25-35.

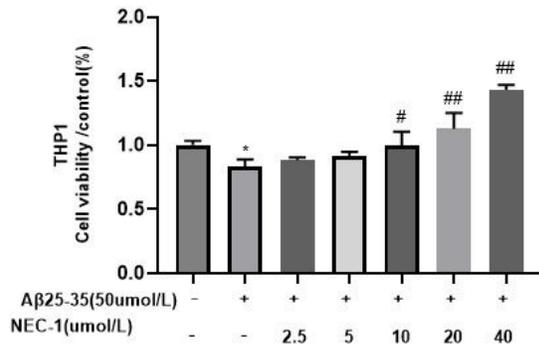


Fig.12A

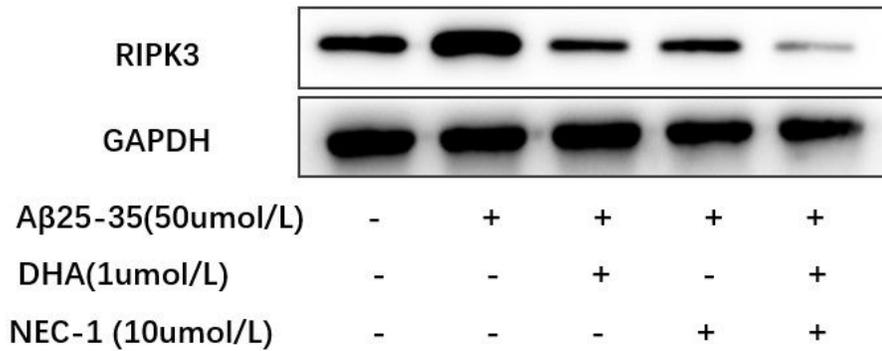


Fig. 12B

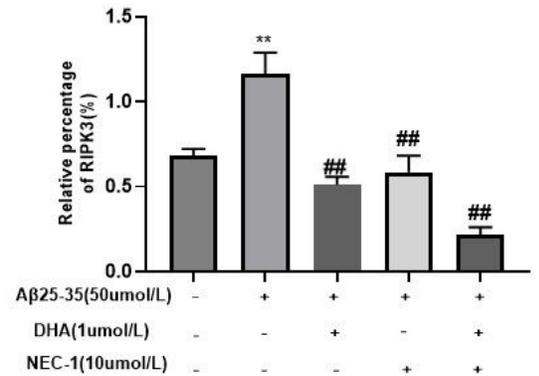


Fig. 12C

Figure 12

DHA treatment attenuates Aβ25–35-induced necroptosis in THP-1 monocytes via the RIPK1/RIPK3 signaling pathway. THP-1 cells were pre-treated with or without various concentrations of NEC-1 in RPMI 1640 with 0% FBS for 24 h, followed by 50 μM Aβ25-35 stimulation for 3 d; cell viability was determined using the CCK8 assay (Fig. 12A). THP-1 cells were pretreated with or without NEC-1 and DHA in RPMI 1640 with 0% FBS for 24 h, followed by 50 μM Aβ25-35 stimulation for 3 d; Western blotting analysis was used to determine expression levels of RIPK3 (normalized to GAPDH) (Fig. 12B–C). Data are represented as averages ± SD. as a percentage of the control group. *p <0.05 and **p <0.01, compared to 0 μM Aβ25-35 alone. #p <0.05 and ##p <0.01, compared to 50 μM Aβ25-35.

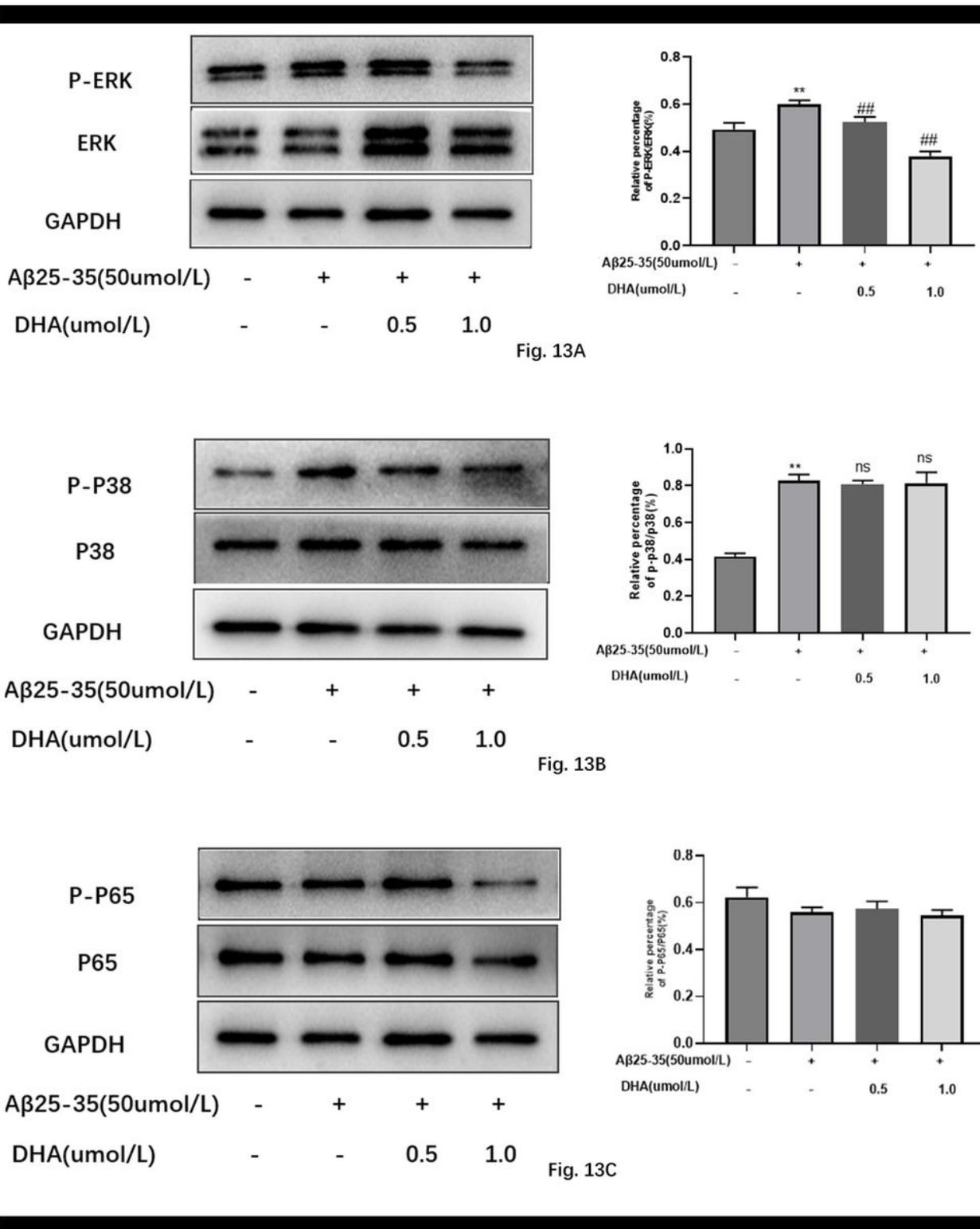


Figure 13

DHA suppresses the ERK1/2 signaling pathway induced by Aβ25–35 in THP-1 cells, but not the p38 or NF-κB/p65 signaling pathways. THP-1 cells were pretreated with or without varying concentrations of DHA in RPMI 1640 with 0% FBS for 1 day prior to incubation with 50 μM Aβ25-35 for another 3 days. The phosphorylation status of ERK1/2 (A), p38 (B), and p65 (C) were determined by Western blot analysis.

Data are represented as averages \pm SD, *p <0.05 and **p <0.01, compared to 0 μ M A β 25-35 alone. #p <0.05 and ##p <0.01, compared to 50 μ M A β 25-35.

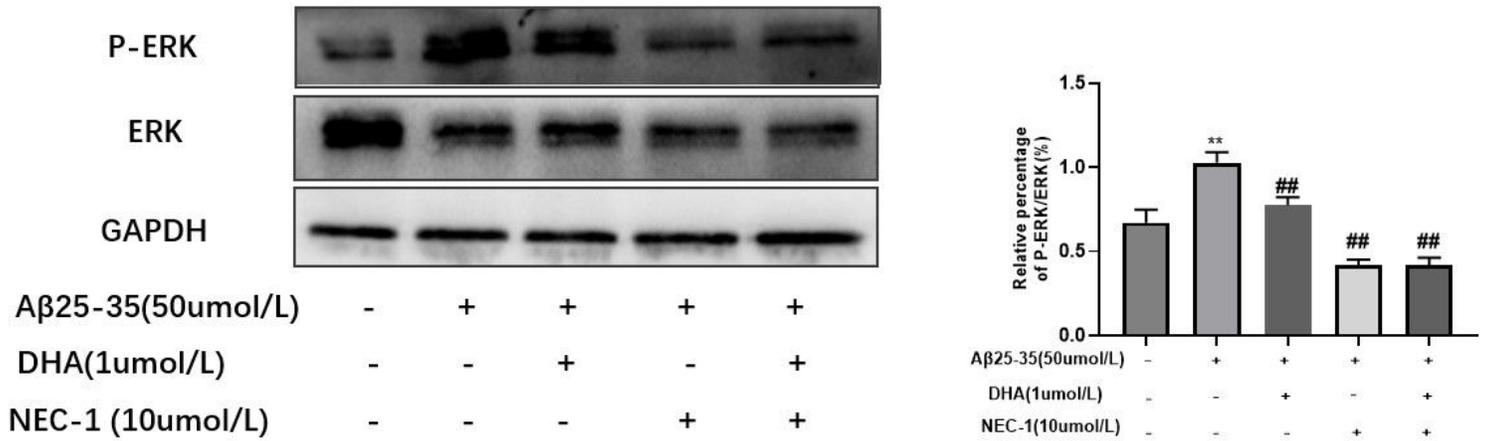


Fig. 14

Figure 14

DHA treatment inhibits ERK1/2 phosphorylation via RIPK1 in THP-1 cells treated with A β 25-35. THP-1 cells were pre-treated with or without NEC-1 and DHA in RPMI 1640 with 0% FBS for 24 h, followed by 50 μ M A β 25-35 treatment for 3 d. Western blotting was used to determine the phosphorylation status of ERK1/2. Data are represented as averages \pm SD, *p <0.05 and **p <0.01, compared to 0 μ M A β 25-35 alone. #p <0.05 and ##p <0.01, compared to 50 μ M A β 25-35.