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Eritoran attenuates neurotoxicity via apoptosis and TLR signaling pathways and modulates T cell responses in Alzheimer's disease model

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ABSTRACT

Objective(s): Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by amyloid-beta (Aβ) accumulation, tau hyperphosphorylation, and neuronal loss. Toll-like receptor (TLR)-mediated neuroinflammation plays an important role in AD pathogenesis by triggering inflammatory responses. This study investigated the neuroprotective effects of eritoran, a TLR4 antagonist, and its role in modulating T-cell responses in an in vitro AD model.

Materials and Methods: SH-SY5Y cells were differentiated into cholinergic neurons and exposed to Aβ to establish an in vitro AD model. The model was validated by measuring p-Tau, α-synuclein, Aβ, and acetylcholinesterase (AChE) levels. Cell viability was assessed using a CCK-8 assay. Apoptosis and TLR pathway gene expression were analyzed by qRT-PCR. In addition to apoptosis-related protein levels, ELISA was used to evaluate IL2, IL4, and IFN-γ concentrations in Jurkat T cells treated with conditioned media.

Results: In the AD model, p-Tau, α-synuclein, Aβ, and AChE levels increased significantly. Eritoran pretreatment significantly reduced these pathological markers. Eritoran pretreatment significantly down-regulated apoptosis-related genes (BAX, CASP3, CASP7, CYCS, CASP8, CASP9, FADD, FAS and P53) and up-regulated anti-apoptotic BCL2. Protein levels of CASP7, BAX, and CYCS were also reduced. Additionally, TLR signaling pathway genes (TLR1–5, TLR7, TLR9, TLR10, and MYD88) were significantly down-regulated. Furthermore, eritoran up-regulated IL2 and IL4 while decreasing IFN-γ levels in T cells.

Conclusion: Eritoran demonstrated neuroprotective effects in an *in vitro* AD model and modulated T-cell cytokine responses. These findings support further investigation of eritoran as a candidate for AD-related research.

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Introduction

Alzheimer's disease (AD) is one of the leading causes of dementia in middle-aged and elderly individuals. It is an irreversible neurodegenerative disorder characterized by an insidious onset and progressive course (1). In the early stages, amyloid beta (Aβ) accumulation becomes prominent and drives the spread of tau pathology. Additionally, α-synuclein increases Aβ and tau pathology and contributes to cognitive decline (2). It has been reported that 60-80% of the risk of AD is due to hereditary factors, and to date, more than 40 genes have been shown to be associated with the disease (3). However, it is well known that inflammatory processes play a significant role in the pathogenesis of neurodegenerative diseases, including AD. Current pharmacological approaches in AD include acetylcholinesterase (AChE) inhibitors, anti-tau, anti-amyloid β, and anti-inflammatory treatment strategies (4).

Toll-like receptors (TLRs) are a family of 10 type I membrane glycoproteins in humans (5). TLR1, 2, 4, 5, and 6, which recognize bacterial and fungal components, are localized on the cell surface. However, the viral and nucleic acid sensors, including TLR3, 7, 8, and 9, are located in endosomal structures (5, 6). In the central nervous system, astrocytes, neurons, microglia, and oligodendrocytes express various TLR family members (7, 8). Neurons express all 10 human TLRs identified to date, and microglia express only nine (9). TLR activity is known to play an important role in neurodegenerative conditions such as AD, Parkinson's disease, amyotrophic lateral sclerosis, and stroke (10). Aberrant TLR activation has been associated with chronic inflammation and disease progression. TLRs have been reported in various studies to be associated with sepsis, asthma, cancer, autoimmune diseases, intestinal disorders, diabetes, and neurodegenerative diseases (5, 11, 12). In AD, specific TLRs and coreceptors can be activated directly or indirectly to induce Aβ uptake or inflammatory responses,

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depending on the disease stage. Fibrillar Aβ can directly interact with TLR2, TLR4, and CD14 to induce microglial Aβ phagocytosis in the initial stages and neuroinflammatory responses in the later stages. Therefore, TLR2 and TLR4, in particular, are considered potential targets for therapeutic intervention. Targeting these receptors may increase Aβ autophagy and phagocytosis or reduce the inflammatory response (13).

Eritoran is a synthetic TLR4 antagonist that binds to the hydrophobic pocket of the MD2 portion of the TLR4 receptor complex (14). Eritoran has been reported to exhibit potential therapeutic effects in various disease models by targeting inflammatory responses associated with excessive TLR4 activation (15, 16). Previous studies have reported that eritoran exhibits anti-inflammatory effects in inflammatory conditions including liver disease (17), brain ischemia (18), sepsis (19) and colon cancer (20). However, the effects of eritoran in neurodegenerative diseases, where inflammation plays a significant role, remain unclear.

While inflammation is important in AD, T cells play a critical role in driving inflammation. T cell activation is a crucial step in forming an immune response. IL2 stimulates the T cell population, thereby enhancing the inflammatory response (21). Another cytokine that plays an important role in regulating the immune response is interferon-gamma (IFN-γ), which is crucial in phagocytic cell activation and increased T cell function (22). In contrast, IL4, which exhibits anti-inflammatory properties, is reported to play a significant role in suppressing the neuroinflammatory process that develops in the brains of individuals with AD (23).

This study hypothesizes that the TLR4 antagonist eritoran may exert a neuroprotective effect in AD by suppressing neuroinflammation, and that this effect may be related to the regulation of T cell response. Although the literature has shown eritoran's effects in various inflammatory disease models, its role in T cell-mediated immune responses in neurodegenerative diseases, particularly AD, remains unclear. Therefore, this study aimed to investigate the neuroprotective effects of eritoran and its potential role in modulating neuro-immune interactions in an *in vitro* AD model.

Materials and Methods

Chemicals and reagents

Amyloid β1-42 (Adooq Bioscience; Cat#A14075), all-trans retinoic acid (ATRA; TargetMol; Cat#T1051), brain-derived neurotrophic factor (BDNF; Elabscience; Cat#PKSH033559) and eritoran (Dayang Chem; Cat#DYN-CD359) were purchased and dissolved in dimethyl sulfoxide (DMSO; AppliChem; Cat#A3672) according to the manufacturer's recommended protocol. CCK-8 (Abbkine; Cat# BMU106-EN), TRIzol reagent (Qiagen; Cat#79306), fetal bovine serum (FBS; Capricorn; Cat# FBS-HI-11A), PBS (Capricorn; Cat# PBS-1A), RPMI-1640 (Biowest; Cat# L0500), DMEM/F-12 (Capricorn; Cat# DMEM-12-A), penicillin/streptomycin (Capricorn; Cat# PS-B) and the first-strand cDNA synthesis kit (Bio-Rad; Cat# 1708891) were commercially obtained.

Cell culture

SH-SY5Y (CRL-2266™) human neuroblastoma cell line and T-cell leukemia Jurkat (TIB-152) cells were obtained from the American Type Culture Collection (ATCC). SH-SY5Y cells and Jurkat T-cell leukemia cells were cultured in an incubator at 37 ˚C under 95% humidity and 5% CO_2 in DMEM/F-12 and RPMI-1640 media supplemented with 10% FBS and 1% penicillin/streptomycin, respectively.

Differentiation of the SH-SY5Y cells

Approximately 3×10^3 SH-SY5Y cells were seeded into 6-well plates using standard medium. To initiate differentiation, the cells were incubated for 5 days in DMEM/F-12 medium supplemented with 1% FBS and 1% penicillin/streptomycin, with 10 μM ATRA added 24 hr after seeding. Subsequently, to support differentiation, 50 ng/ml BDNF was added to the medium, and the cells were incubated for an additional 48 hr, thereby completing the differentiation protocol (24).

CCK-8 analysis

After completing the differentiation protocol, SH-SY5Y cells were transferred to 96-well culture plates for experimental procedures. After overnight incubation, the cells were treated with eritoran at 0.5, 1.5, 2.5, 5, 10, 20, 30, and 50 nM to determine protective doses. After treatment, the cells were incubated for 24 hr to determine the optimal dose range. To evaluate the toxic effect of Aβ peptide on neurons, differentiated SH-SY5Y cells were similarly seeded and cultured in 96-well plates. The cells were then exposed to Aβ at doses of 5, 10, 20, 40, 80, and 100 μM. After the 24 hr incubation period, cell viability was quantified using the CCK-8 cell proliferation kit, and the Aβ dose that resulted in approximately 60% cell viability was selected as the reference concentration for subsequent experiments.

To evaluate eritoran's neuroprotective effects, differentiated SH-SY5Y cells were seeded into a 96-well plate at a cell density of 1×10^3 per well and cultured overnight. Subsequently, cells were pretreated with eritoran (0.5, 1.5, 2.5, and 5 nM) for 24 hr before adding Aβ (20 μM). After pretreatment, the cell culture medium was replaced with medium containing 20 μM Aβ. Following treatment, the cells were incubated for 24 h. Cell viability was assessed at the end of incubation by measuring absorbance at 450 nm using an ELISA reader. Subsequently, conditioned media obtained from the constructed AD cell model and AD model media pretreated with eritoran were applied to the Jurkat T cell line to evaluate the effect of eritoran on T cells (24 hr). Jurkat cell viability was analyzed using the CCK-8 cell proliferation kit.

The percentage of cell viability was calculated using the following formula:

$$\% \text{ Cell Viability} = [(\text{Measured OD value})/(\text{Control OD value})] \times 100$$

Experimental groups

Based on the data obtained from the CCK-8 analysis, the experimental groups were established as follows: Control (differentiated control SH-SY5Y cells), Aβ (differentiated cells exposed to 20 μM Aβ), Eritoran+Aβ (differentiated cells treated with 1.5 nM eritoran for 24 hr followed by 24 hr Aβ exposure), and Eritoran (differentiated cells treated with 1.5 nM eritoran for 24 hr).

RNA isolation, cDNA synthesis, and qRT-PCR analysis

Total RNA was isolated from the experimental groups

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using TRIzol Reagent. cDNA synthesis was performed using the first-strand cDNA synthesis kit according to the manufacturer's instructions. qRT-PCR was used to determine changes in gene expression associated with apoptosis (BAX, BCL2, CASP3,7,8, 9, CYCS, FADD, FAS, and P53) and TLR (TLR1-10 and MYD88). The primer sequences for the apoptosis and TLR pathways and reference genes used in qRT-PCR (Table 1) and the protocol were obtained from our previous studies (25, 26). ACTB was used as an internal control for normalization of qRT-PCR data.

ELISA

p-Tau (Phosphorylated Microtubule-associated Protein Tau ELISA kit, BT Lab; Cat#E5874Hu), Aβ (Amyloid beta aggregated ELISA kit, FineTest; Cat#EH4494), α-Synuclein (Alpha-Synuclein ELISA kit, BT Lab; Cat#E1313Hu), Acetylcholinesterase (AChE ELISA kit, BT Lab; Cat#E0817Hu), caspase 7 (Caspase 7 ELISA kit, BT Lab; Cat#E2257Hu), BAX (Apoptosis Regulator Bax ELISA kit, BT Lab; Cat#E4977Hu) and cytochrome c (Cytochrome c ELISA kit, BT Lab; Cat#E7110Hu), IL2 (Interleukin 2 ELISA kit, BT Lab; Cat#E0094Hu), IL4 (Interleukin 4 ELISA kit, BT Lab; Cat#E0092Hu) and IFN-γ (Interferon gamma ELISA kit, BT Lab; Cat#E0105Hu) protein expression levels were determined according to the manufacturer's instructions. According to the BT Lab protocol, briefly, 10 µl of biotinylated antibody and 50 µl of streptavidin-HRP were added to 40 µl of the sample in the plates, and the plates were incubated for 1 hr at 37 ˚C. After incubation, the plates were washed five times, then 50 µl of substrate A and 50 µl of substrate B solutions were added. After incubating the plates at 37 ˚C for 10 min, 50 µl of stop solution was added, and absorbance was measured using an ELISA plate reader at 450 nm. For the FineTest protocol, 100 µl of sample was added to the wells, incubated at room temperature for 120 min, and then washed. Subsequently, 100 µl of biotinylated antibody was added to the wells, and the wells were incubated at room temperature for 60 min and then washed. Then, 100 µl of SABC solution was added to each well. Following a 30 min incubation, the wells were washed again. Finally, 90 µl of TMB solution was added to the wells and incubated for 20 min; then 50 µl of stop solution was added, and absorbance values were measured at 450 nm.

Statistical analysis

All experiments were repeated three times. qRT-PCR data were analyzed according to the $2^{-(\Delta Ct)}$ method. Comparisons between the groups were evaluated using the SPSS 27.0 statistical analysis program. One-way analysis of variance (ANOVA) followed by a *post hoc* Tukey test was used for comparisons among multiple groups. Data are presented as mean±SEM. In all experiments, $P \leq 0.05$ was considered statistically significant.

Table 1. Primer sequences of apoptosis, toll-like receptor (TLR) pathway, and reference genes used in qRT-PCR analyses

Gene	Forward primer sequence	Reverse primer sequence	PCR product size (bp)	Reference
BAX	GGAGCTGCAGAGGATGATTG	GGCCTTGAGCACCAGTTT	151	(25)
BCL2	GTGGATGACTGAGTACCTGAAC	GAGACAGCCAGGAGAAATCAA	125	(25)
CASP3	GAGCCATGGTGAAGAAGAAATA	TCAATGCCACAGTCCAGTTC	162	(25)
CASP7	CGAAACGGAACAGACAAAGATG	TTAAGAGGATGCAGGCGAAG	169	(25)
CASP8	GCCCAAACTTCACAGCATTAG	GTGGTCCATGAGTTGGTAGATT	160	(25)
CASP9	CCACCTGACTGCCAAGAAA	CATCCATCTGTGCCGTAGAC	153	(25)
CYCS	GGAGATGATACACTGATGGAGTA	GTCTGCCCTTTCTTCCTTCTT	102	(25)
FADD	TGACCGAGCTCAAGTTCCTATG	CCAGGTCGTTCTGCTCCAG	108	(25)
FAS	GTGATGAAGGACATGGCTTAGA	GTGTGCATTCCTTGATGATTCC	156	(25)
P53	GAGATGTTCCGAGAGCTGAATG	TTTATGGCGGGAGGTAGACT	129	(25)
TLR1	CAGCGATGTGTTCGGTTTTCCG	GATGGGCAAAGCATGTGGACCA	111	(26)
TLR2	TTATCCAGCACACGAATACACAG	AGGCATCTGGTAGAGTCATCAA	160	(26)
TLR3	GGCTAGCAGTCATCCAACAGAA	GCAGTCAGCAACTTCATGGC	135	(26)
TLR4	CCCTGAGGCATTTAGGCAGCTA	AGGTAGAGAGGTGGCTTAGGCT	126	(26)
TLR5	CCTTACAGCGAACCTCATCCAC	TCCACTACAGGAGGAGAAGCGA	130	(26)
TLR6	TTCTCCGACGGAAATGAATTTGC	CAGCGGTAGGTCTTTTGGAAC	75	(26)
TLR7	CTTTGGACCTCAGCCACAACCA	CGCAACTGGAAGGCATCTTGTAG	142	(26)
TLR8	ACTCCAGCAGTTTCCTCGTCTC	AAAGCCAGAGGGTAGGTGGGAA	145	(26)
TLR9	TGAGCCACAACTGCATCTCGCA	CAGTCGTGGTAGCTCCGTGAAT	134	(26)
TLR10	GGTTAAAAGACGTTCATCTCCACG	CCTAGCATCCTGAGATACCAGG	145	(26)
MYD88	GAGGCTGAGAAGCCTTTACAGG	GCAGATGAAGGCATCGAAACGC	129	(26)
ACTB	TGGCTGGGGTGTTGAAGGTCT	AGCACGGCATCGTCACCAACT	179	(25)

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Results

Eritoran exhibits neuroprotective effects and modulates neuro-immune interactions

CCK-8 analysis was conducted to investigate the effect of eritoran on the viability of differentiated SH-SY5Y cells, and it was observed that eritoran was not toxic to the differentiated cells at 0.5 and 1.5 nM (Figure 1A). Therefore, a non-toxic dose of 1.5 nM eritoran was used for further analyses. A 24 hr cell viability assay showed that 20 µM Aβ reduced the viability of differentiated SH-SY5Y cells by approximately 40% (Figure 1B). In subsequent experiments, a dose of 20 µM Aβ was used to establish the AD model. Differentiated cells were treated with eritoran before Aβ exposure to assess its neuroprotective potential. Eritoran pretreatment (0.5, 1.5, 2.5, and 5 nM) attenuated Aβ-induced toxicity compared with the Aβ-exposed group (Figure 1C). To assess eritoran's effect, Jurkat T-cells were treated with conditioned media, and a cell viability assay was performed. Conditioned medium from eritoran-pretreated SH-SY5Y cells (Eritoran+Aβ) enhanced T-cell viability compared with the AD model group (Aβ-only)(Figure 1D).

Eritoran regulates the expression of apoptosis and TLR signaling pathway genes in AD

qRT-PCR analyses revealed a statistically significant increase in the expression levels of BAX, CASP3, CASP7, CASP8, CASP9, CYCS, FAS, and P53 in the Aβ group compared to the control group. Conversely, eritoran pretreatment significantly down-regulated these genes and FADD relative to the Aβ group. Eritoran pretreatment significantly reversed Aβ-induced down-regulation of the anti-apoptotic gene BCL2 (Figure 2). Collectively, these findings suggest that eritoran exerts neuroprotective effects by suppressing apoptotic pathways.

In the gene expression analyses of the TLR signaling pathway, the Aβ group showed markedly increased expression of TLR1, TLR2, TLR3, TLR4, TLR6, TLR7, TLR9, TLR10, and MYD88 compared with the control group. Eritoran pretreatment, however, significantly reversed the Aβ-induced up-regulation of these genes (Figure 3).

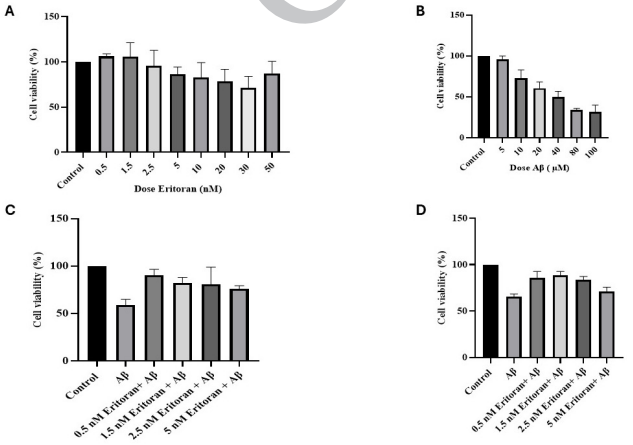


Figure 1. Effects of eritoran on differentiated SH-SY5Y cell viability and neuro-immune interaction

(A) Effects of various concentrations of eritoran on differentiated SH-SY5Y cell viability were assessed by CCK-8 assay. (B) Impact of Aβ exposure on differentiated SH-SY5Y cell viability. (C) Neuroprotective effect of eritoran pretreatment against Aβ-induced cytotoxicity in differentiated SH-SY5Y cells. (D) Impact of conditioned media (derived from eritoran-pretreated SH-SY5Y cells) on Jurkat T-cell viability. Data are expressed as mean ± SEM (standard error of the mean)

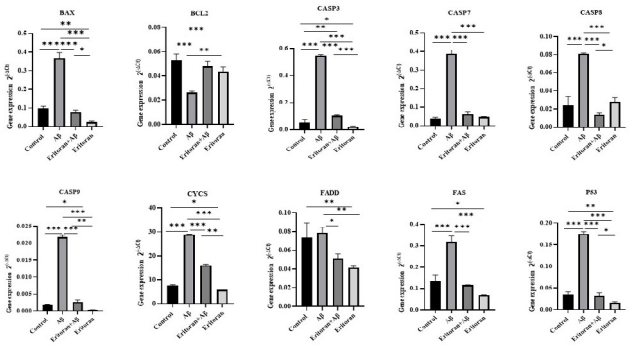


Figure 2. Effect of eritoran on the expression of apoptosis-related genes in differentiated SH- SY5Y cells

The mRNA expression levels of BAX, CASP3, CASP7, CASP8, CASP9, CYCS, FADD, FAS, P53, and BCL2 were analyzed using qRT-PCR. Data are presented as mean±SEM. $^{*}P\leq0.05$, $^{**}P\leq0.01$, and $^{***}P\leq0.001$

ELISA results

Eritoran mitigates AD pathologies

ELISA analyses showed that Aβ exposure significantly up-regulated key neurodegenerative markers compared with the control group. Specifically, p-Tau (from 1348.93±170.94 ng/L to 2254.93±107.89 ng/L), α-Synuclein (from 191.31±26.89 pg/ml to 321.31±12.9 pg/ml), Aβ (from below measurable levels to 1106.83±88.92 pg/ml), and AChE (from 34.79±6.51 mU/ml to 63.02±8.6 mU/ml) levels were markedly elevated in the Aβ-exposed group. Notably, eritoran pretreatment significantly mitigated these pathological increases. It restored p-Tau (1330.93±28.93 ng/L), α-Synuclein (209.74±16.57 pg/ml), Aβ (396±106.27 pg/ml), and AChE (11.96±3.57 mU/ml) levels to values close to those of the control group (Figure 4). Collectively, these findings indicate that eritoran exerts a neuroprotective effect by suppressing the Aβ-induced accumulation of pathological proteins.

Eritoran regulates apoptosis-related protein expression in AD model

ELISA analyses showed that Aβ exposure significantly up-regulated key pro-apoptotic proteins. Specifically, Caspase-7 levels were markedly increased in the Aβ group compared to the control group (from 2512.17±590.28 ng/ml to 9272.17±982.62 ng/L), whereas eritoran pretreatment significantly attenuated this elevation, reducing levels to 3145.5±279.86 ng/ml. Similarly, BAX protein levels were elevated in the Aβ group (from 32.41±2.58 ng/ml to 97.02±6.03 ng/ml) and were restored to near-control levels in the Eritoran+Aβ group (35.74±1.89 ng/ml). Cytochrome

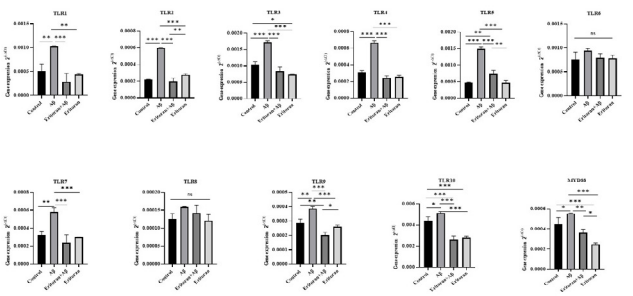


Figure 3. Effect of eritoran on the expression of TLR signaling pathway-related genes in differentiated SH-SY5Y cells

The mRNA expression levels of TLR1-10 and MYD88 were analyzed using qRT-PCR. Data are presented as mean±SEM. $^{*}P\leq0.05$, $^{**}P\leq0.01$ and $^{***}P\leq0.001$

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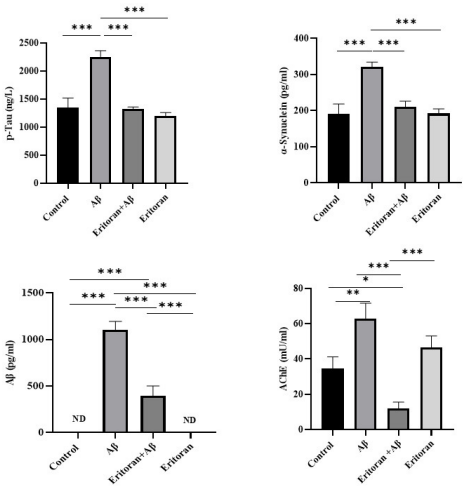


Figure 4. Effect of eritoran on the levels of pathological proteins in Aβ-exposed SH-SY5Y cells
ELISA results show the modulation of p-Tau, α-Synuclein, Aβ, and AChE protein levels among control, Aβ, and Eritoran+Aβ groups. ND: not detected. Data are presented as mean±SEM. *P≤0.05, **P≤0.01 and ***P≤0.001

c levels were significantly higher in the Aβ group than in controls (121.95±8.21 ng/ml vs 227.68±25.16 ng/ml); however, eritoran treatment suppressed this increase, reducing levels to 94.87±4.34 ng/ml. Collectively, these results suggest that eritoran exerts neuroprotective effects by inhibiting the increased levels of Aβ-induced apoptotic proteins (Figure 5).

Eritoran alters T cell response in AD model

ELISA analysis of Jurkat T cells incubated for 24 hr with conditioned media revealed that Aβ exposure decreased IL2 and IL4 levels, while simultaneously increasing IFN-γ levels. Specifically, IL2 levels in the Aβ-conditioned media group (567.52±32.5 ng/L) increased after treatment with eritoran+Aβ conditioned media (830.3±20.3 ng/L). Similarly, IL4 levels increased from 255.9±16.8 ng/l in the Aβ group to 362.3±14.2 ng/L in the eritoran+Aβ group. Conversely, IFN-γ protein levels in the Aβ group (19.22±1.2 ng/ml) were significantly suppressed (6.91±2.4 ng/ml) after eritoran+Aβ treatment. These findings also demonstrate that eritoran treatment reverses the Aβ-induced IL2 and IL4 decreases, while attenuating the increase of IFN-γ (Figure 6).

Discussion

AD is a neurodegenerative disorder characterized by progressive cognitive decline, memory loss, and neurodegeneration; it is the most common cause of dementia in the elderly population (27). In AD pathogenesis, inflammation is considered a fundamental process that contributes to the progression of neuronal damage by aggravating Aβ accumulation and tau pathology (28). TLRs are important immunomodulators that play a critical role in regulating neuroinflammatory responses in AD and exert both protective and detrimental effects in disease pathogenesis (29). This study investigated the effects of eritoran, a TLR4 antagonist, in an AD model. Data on the role of the TLR signaling pathway in AD pathogenesis are complex. In previous studies, it has been reported that TLR4 expression is increased in Alzheimer's patients (30) and in rat brains (31) exposed to Aβ. Furthermore, TLR2 and MyD88 protein levels are elevated in the hippocampus and cortex of Alzheimer's patients (32). It has also been reported that the expression of six TLR genes (1, 2, 4, 5, 6, and 8) is significantly increased in the temporal cortex tissues of Alzheimer's patients (33). However, findings

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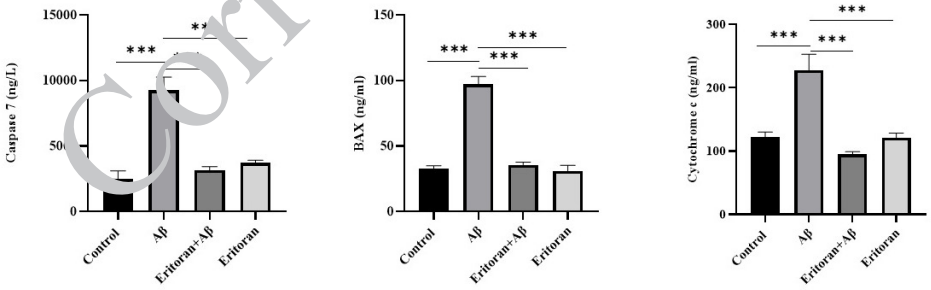


Figure 5. Effect of eritoran on the expression of apoptosis-related proteins in Aβ-exposed SH-SY5Y cells
Protein levels of Caspase-7, BAX, and Cytochrome c were quantified via ELISA. Data are presented as mean±SEM. *P≤0.05, **P≤0.01, and ***P≤0.001

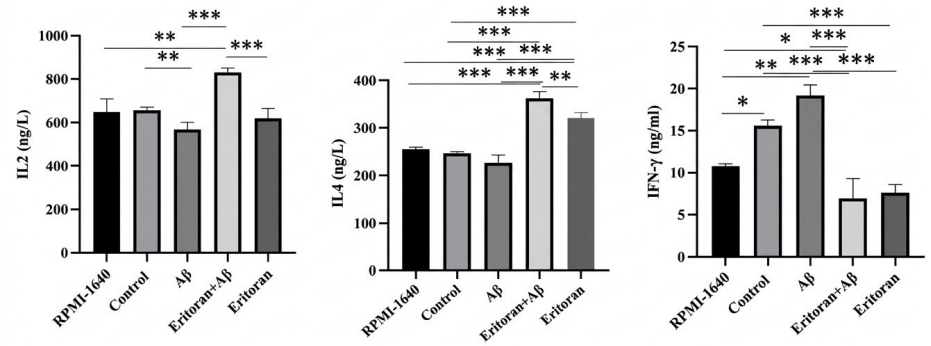


Figure 6. Effect of eritoran on IL2, IL4, and IFN-γ cytokine levels in Aβ-exposed Jurkat T cells
Protein levels were quantified by ELISA. Data are presented as mean±SEM. *P≤0.05, **P≤0.01, and ***P≤0.001

regarding TLR9 expression in Alzheimer's patients are inconsistent. Studies have shown increased TLR9 mRNA levels in the middle temporal gyrus of Alzheimer's patients, but decreased levels in temporal lobe tissues (33, 34). Furthermore, TLR3 mRNA expression has been shown to increase in the middle temporal gyrus of Alzheimer's patients (34). TLR7 mRNA expression has been reported to be increased in plaque-associated brain tissue in an AD animal model (APP23 transgenic mice)(35). In the present study, qRT-PCR analysis revealed that expression levels of TLR signaling pathway genes (TLR1, TLR2, TLR3, TLR4, TLR5, TLR7, TLR9, TLR10, and MYD88) were significantly down-regulated in eritoran-pretreated AD model cells compared with the AD model group (Figure 3).

In a study involving the co-culture of HUVECs with peripheral blood mononuclear cells (PBMCs) from high-stenosis (HS) patients and healthy controls, eritoran treatment suppressed the expression of lipopolysaccharide-induced inflammatory cytokines to a greater extent in HS patients (16). It has been demonstrated that eritoran attenuates liver fibrosis and inflammation by suppressing hepatic stellate cell activation, α-smooth muscle actin (α-SMA), and TGF-β1 expressions (15). Because TLRs are important immunoregulators in AD, further investigation of eritoran as a potential modulator of TLR-associated responses in AD is warranted. In this study, non-cytotoxic doses of eritoran were primarily determined as 0.5 and 1.5 nM for SH-SY5Y cells differentiated into cholinergic neurons using the CCK-8 assay (Figure 1A). Additionally, to investigate eritoran's effect on the T cell response, Jurkat T cells were treated with conditioned media; media from cells pretreated with eritoran increased Jurkat T cell viability compared with the Aβ group (Figure 1D). Therefore, eritoran's reduction in Aβ-induced neuroinflammation suggests that attenuated secretion of pro-inflammatory cytokines likely created a more favorable microenvironment for T cell survival. Taken together, these findings suggest a potential neuro-immune interaction in which eritoran pretreatment of neuronal cells may influence T-cell viability through changes in the conditioned medium. However, the precise mechanisms underlying this interaction require further investigation.

Aβ and tau accumulation are among the key neuropathological hallmarks of AD (36). ELISA analyses to validate the AD model showed that p-Tau, α-synuclein, and Aβ levels were significantly increased in the Aβ group compared with the control group, and that eritoran treatment reversed these increases (Figure 4). In AD, neurodegeneration and loss of cholinergic neurons cause a significant deficiency in acetylcholine (37). Acetylcholinesterase (AChE) catalyzes the hydrolysis of acetylcholine, producing choline and acetate ions. Studies have shown reduced acetylcholine activity in some brain regions of AD patients and that AChE inhibitors can alleviate cognitive impairments and other clinical symptoms in these patients (38). In the present study, AChE levels were elevated in the Aβ-exposed group and significantly reduced by eritoran pretreatment (Figure 4).

Apoptosis, one of the main mechanisms of neuronal loss in AD, is triggered by pathological processes such as amyloid-β accumulation, oxidative stress, and mitochondrial dysfunction, thereby contributing to disease progression and the deepening of cognitive impairment (39). Compared to the AD model established in this study, qRT-PCR analysis revealed that pretreatment of AD-induced cells with eritoran significantly down-regulated the expression of apoptosis pathway genes BAX, CASP3, CASP7, CYCS, CASP8, CASP9, FADD, FAS, and P53, while significantly up-regulating the expression of BCL2, an anti-apoptotic protein (Figure 2). Furthermore, ELISA results showed that eritoran pretreatment significantly reduced CASP7, BAX, and CYCS protein levels in the AD model (Figure 5).

It has been reported that IL2 expression is significantly decreased in the hippocampus of patients with AD. In the same context, intraperitoneal IL2 injection increased IL2 and regulatory T cell levels in the brains of APP/PS1ΔE9 mice, effectively reversing memory impairments (40). Furthermore, it has been reported that IFN-γ levels, a critical cytokine in AD progression, were significantly elevated in altered T lymphocytes of AD patients, showing a strong correlation with both age and the severity of the disease (13, 41). Supporting this, IFN-γ release from Aβ-specific T cells significantly accelerated disease markers in APP/PS1-overexpressing transgenic mice (42). Levels of the anti-inflammatory cytokine IL4, which plays a vital role in suppressing neuroinflammation in AD, were decreased in peripheral mononuclear cells of AD patients (23). Moreover, *in vivo* studies indicated that IL4 treatment alleviates cognitive deficits in AD models (43). Consistent with these clinical and *in vivo* reports, the present study found that Jurkat T cells exposed to Aβ-conditioned media showed significantly reduced IL2 and IL4 levels, accompanied by a marked increase in IFN-γ expression. Notably, eritoran pretreatment reversed these pathological changes by restoring diminished IL2 and IL4 levels and suppressing elevated IFN-γ levels (Figure 6). These results suggest that eritoran pretreatment may modulate the balance between pro- and anti-inflammatory cytokines in T cells exposed to AD model-derived conditioned media, which may contribute to its observed effects on the neuro-immune response.

Conclusion

However, this study is limited by its reliance on *in vitro* models, which do not fully capture the complex *in vivo* environment of AD, and by the use of Jurkat T cells, which may not accurately represent primary human T cell responses. Therefore, future studies should confirm these findings in *in vivo* AD models and in primary human T cells to further assess the biological relevance and potential therapeutic implications of eritoran.

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Authors' Contributions

CE G contributed supervision, review and editing, project management, original drafting, and visualization. EN D handled investigation, methodology, visualization, and original draft writing. FS Ç was involved in investigation, methodology, and original draft writing. S A contributed through investigation, methodology, and original draft writing. E K provided supervision, review, and editing.

Corrected Proof

Conflicts of Interest

The authors declare no conflicts of interest.

Declaration

The authors declare that AI tools (ChatGPT and Gemini by OpenAI) were used solely to enhance clarity and correct grammar in this manuscript. All scientific interpretations and conclusions were independently generated.

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