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ORIGINAL PAPER

TREM2 Attenuates Aβ1-42-Mediated Neuroinflammation in BV-2 Cells by Downregulating TLR Signaling

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Abstract

The pathogenesis of late-onset Alzheimer's disease (LOAD) mainly involves abnormal accumulation of extracellular β-amyloid (Aβ) and the consequent neurotoxic effects. The triggering receptor expressed on myeloid cells 2 (*TREM2*) gene is associated with the pathogenesis of LOAD and plays important roles in mediating the phagocytosis of Aβ by microglia and regulating inflammation in central nervous system. However, the exact mechanisms of these processes have not yet been clarified. In this study, we investigated the mechanism by which TREM2 regulates neuroinflammation and promotes Aβ1-42 clearance by BV-2 cells and further elucidated the underlying molecular mechanisms. We either silenced or overexpressed *TREM2* in BV-2 cells and evaluated the cell viability, Aβ1-42 content, and expression of inflammatory markers (IL-1β, IL-6, and TNF-α). *TREM2* overexpression up-regulated cell activity, promoted clearance of Aβ1-42 by BV-2 cells, and down-regulated expression of the inflammatory factors. In addition, *TREM2* overexpression downregulation the expression of the TLR family (TLR2, TLR4 and TLR6) in BV-2 cells. Moreover, LPS, as an agonist of the TLR family, up-regulated the expression of inflammatory cytokines (IL-1β, TNF-α, and IL-6) in BV-2 cells overexpressing *TREM2*. In conclusion, *TREM2* promoted clearance of Aβ1-42 by BV-2 cells and restored BV-2 cell viability from Aβ1-42-mediated neuroinflammation by downregulating TLRs. These findings suggest that TREM2 may be a target for LOAD therapy.

Keywords Alzheimer's disease · TREM2 · TLR signaling · Neuroinflammation

Introduction

Alzheimer's disease (AD) is the most common form of dementia, responsible for more than 50% of all dementia cases. The incidence of AD is 1–5% in the population over 65 years of age and as high as 20–25% in the population over 80 years of age [1]. One of the major pathological features of AD is extra-neuronal accumulation of β-amyloid (Aβ) [2]. According to the amyloid cascade hypothesis, the neurofibrillary tangles and neuroinflammation that are invariably observed in AD are downstream events caused by Aβ accumulation [3, 4]. The accumulation of Aβ plaques in the brain occurs as much as 10 years or more prior to the onset of AD, usually leading to an increase in the number of tangles and decline in brain cognitive abilities [5, 6]. Some scholars believe that strengthening or promoting Aβ phagocytosis and metabolic degradation are potential AD therapies. However, there is currently no effective treatment in clinical practice.

In the pathogenesis of AD, inflammation is considered to be an important pathological event during the early stages of AD. Microglia are a type of phagocytic cell in the brain and are widely distributed in the hippocampus, olfactory lobes, and basal ganglia tissues and serve as an important line of immune defense in the central nervous system [7]. The role of microglia is to remove damaged nerves, plaques, and infectious agents in the central nervous system [8]. However, microglia play a dual role. In the AD pathological process, microglia can release a large number of inflammatory cytotoxic mediators such as tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), and IL-6 and oxidative stress related indicators such as reactive oxygen species (ROS),

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reactive nitrogen, and nitric oxide (NO) [9, 10]. Therefore, preventing the pathological accumulation of microglia and re-establishing their immune barrier function may be of great significance for alleviating AD.

The triggering receptor expressed on myeloid cells 2 (TREM2) protein is a transmembrane receptor belonging to the TREM family of proteins, which are encoded by genes clustered on human chromosome 6p21.1 and mouse chromosome 17 [11, 12]. As an important innate immune receptor in the brain. TREM2 is primarily expressed on microglia and couples with DAP12 for signaling [13]. The TREM-2 receptor not only pairs with DAP12 to inhibit TLR-induced inflammatory cytokine production in macrophages [14], but also specifically pairs with DAP12 to inhibit TLR responses in BMDCs [15]. Although the extracellular ligand of TREM2 is still unknown, the pathway can be specifically regulated and is activated by interleukin-4 (IL-4)/interleukin-13 (IL-13) [16]. Recent research showed that a rare missense mutation (rs75932628-T) at the original R47H site of the heterozygous *TREM2* gene results in a significantly increased risk of late-onset Alzheimer's disease (LOAD) [17]. *TREM2* mutation was identified as an important risk factor for AD, and its effect size was comparable to that of the E4 allele of the apolipoprotein E (*APOE*) gene [17, 18]. Related studies on the expression of TREM2 in the human brain indicate that TREM2-positive cells are mainly restricted to the blood circulation, promoting the role of systemic inflammation in the development and progression of AD [19]. In animal models of autoimmune diseases, the silencing of *TREM2* aggravates inflammatory symptoms [20, 21]. Therefore, *TREM2* may be associated with some of the processes involved in the AD inflammatory response. In addition, clinical studies have shown significant downregulation of the *TREM2* gene in AD patients [17], and the *TREM2* mutation causes a threefold higher risk of developing LOAD [22]. Moreover, Wang et al. recently pointed out that the lack of *TREM2* increases the accumulation of Aβ [23]. This indicates that *TREM2* is involved in the maintenance of the normal neuro-endogenic environment and in the alleviation and/or reversal of AD pathology via the coordination or regulation of other pathways.

Toll-like receptors (TLRs) are a family of natural receptors of the immune system involved in the sensing and response to pathogen-associated molecular patterns (PAMPs) and endogenous ligands known as damage-associated molecular patterns (DAMPs), which are released upon cell damage and necrosis [24, 25]. Elevation in levels of TLR4 are found in peripheral blood monocytes of AD patients [26]. Studies have also indicated that TLR4 is involved in mediating the immune response of microglia to Aβ and in promoting the release of inflammatory mediators from microglia [27]. In a study of intestinal mucosal injury repair mechanisms, it was confirmed that the TREM2 pathway and TLR4 pathway are mutually inhibited, that is, the anti-inflammatory effect of TREM2 can antagonize the pro-inflammatory damage of TLR4 and then promote the repair of intestinal mucosal damage [28]. However, the interactions between the TREMs and TLRs signaling pathways are currently unknown in AD.

In the current study, we aimed to clarify the role TREM2 plays in the pathogenesis of AD and what role the TLRs may play. Our results may provide in-depth theoretical support for the consideration of *TREM2* as a target in the therapeutic treatment of LOAD.

Materials and Methods

Cell Lines and Reagents

The murine microglial cell line BV-2 was cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) in a humidified incubator with 5% CO_2 at 37 °C. Lipopolysaccharide (LPS) from *Escherichia coli* 055:B5 and Aβ1-42 peptide were purchased from Sigma-Aldrich (St. Louis, MO, USA). For western blotting (WB) and immunofluorescence assays, antibodies specific for TLR2, TLR4, TLR6, and p-p38 obtained from Abcam (Cambridge, UK) and antibodies specific for p-ERK, p-p65, IL-1β, IL-6, TNF-α β-actin, and GAPDH were purchased from Cell Signaling Technology (Beverly, MA, USA). Mouse enzyme-linked immunosorbent assay (ELISA) kits were acquired from Elabscience Biotechnology Co., Ltd (Wuhan, China).

Preparation of Aβ1-42 Solution

To generate soluble oligomers, Aβ1-42 peptide was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP; Sigma-Aldrich) to a concentration of 1 mM and then incubated for 24 h under a fume hood [22]. The residual peptide film was dissolved to a concentration of 5 mM in dry dimethyl sulfoxide (DMSO). For oligomeric conditions, the peptide was dissolved in phenol red-free Ham's F-12 medium to a final concentration of 100 uM and kept at 4 °C for 24 h.

Lentivirus Transduction

Lentivirus containing *TREM2* (LV-TREM2) at 5×10^8 transduction units (TU)/ml and TREM2 shRNA (LV-shTREM2) at 4×10^8 TU/ml were purchased from Genechem (Shanghai, China). The lentivirus encoded the *TREM2* shRNA sequence 5′-AGCGGAATGGGAGCACAGTCA-3′. The optimal multiplicity of infection (MOI) was determined to be 100 by performing transductions with MOIs of 1, 10, and 100.

BV-2 cells were plated into 6-well plates (1×10^5 cells/well) and incubated overnight. The TREM2 lentiviral particles were used to infect the cells at an MOI of 100. After 24 h of lentiviral adsorption and infection, the medium was

removed. The lentivirus transduction efficiency was quantified using flow cytometry and the expression levels of TREM2 were validated using real-time quantitative PCR (RT-qPCR).

Flow Cytometry Analyses

To determine lentivirus transduction efficiency, about 5×10^5 cells were harvested and washed with phosphate-buffered saline (PBS). Flow cytometry analyses were performed on the cells using a FACSCanto analyzer (BD Biosciences, San Diego, CA, USA) equipped with DIVA software.

qRT-PCR Analysis

qRT-PCR was performed to measure the mRNA levels of *TREM2, TNF-α, IL-1β*, *IL-6*, and *GAPDH*. After the cells were treated with Aβ1-42 (1 uM) for 24 h, total RNA was extracted from the BV-2 cells and isolated using an E.Z.N.A. Total RNA Kit (Omega Bio-tek, Norcross, GA, USA) and complementary DNA (cDNA) was synthesized using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). qRT-PCR was performed using an ABI 7500 Real-Time PCR detection system (Applied Biosystems, Carlsbad, CA, USA) using SYBR Green Master Mix (Roche, Basel, Switzerland). The cycling conditions included 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The melting curve of each sample was analyzed to determine the specificity of each gene. The relative mRNA expression of each gene was normalized to the expression of the housekeeping gene *GAPDH*. The nucleotide sequences of each primer used are shown in Table 1.

Cell Viability Assay

BV-2 cell viability was determined using a Cell Counting Kit (CCK-8, KeyGEN BioTECH, China) according to the manufacturer's protocol. Briefly, after 24 h of treatment of cells with Aβ1-42 (1 uM), 10 µl CCK-8 dye was added, after which the cells were incubated for 2 h in 37 °C in darkness. The absorbance value was measured using a microplate reader (Thermo Fisher Scientific, USA) at a wavelength of 450 nm. The experiment was performed in triplicate.

Aβ1-42 Treatment and ELISA Analysis

One day prior to treatment with Aβ1-42, BV-2 cells were inoculated into 6-well plates. The BV-2 cells were then treated with Aβ1-42 (1uM) for 24 h and the culture supernatants and cells were harvested and centrifuged at 1000 rpm for 20 min using a bench-top centrifuge. The supernatants were used to evaluate TNF-α, IL-1β, IL-6, and Aβ1-42 concentrations, and the cell pellets were used to assess the concentration of Aβ1-42 in cells using ELISA kits from Elabscience Biotechnology Co., Ltd. in accordance with the manufacturers' protocols.

Immunofluorescence Assay

BV-2 cells were applied onto poly-D-lysine-coated glass coverslips in 24-well (2×10^5 cells/well) culture plates and incubated with or without Aβ1-42 (1 µM) for 24 h. The cells were washed twice with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature (25–30 °C) followed by permeabilization with 0.3% Triton X-100 for 10 min. Cells were then blocked with goat serum (Beyotime, Shanghai, China) for 1 h followed by incubation with rabbit anti-TLR2 (1:200), anti-TLR4 (1:500), and anti-TLR6 (1:500) antibodies at 4 °C overnight. On the following day, after three washes with PBS, the cells were incubated with goat anti-rabbit IgG at room temperature in the dark for 1 h. The cellular nuclei were counterstained with 1 mg/ml 4′,6-diamidino-2-phenylindole (DAPI; Roche) for 5 min. After a final wash with PBS, fluorescence images were acquired using an Eclipse 80i fluorescence microscope (Nikon, Tokyo, Japan). The quantification of the fluorescence intensity was performed by analyzing the fluorescence images using the ImageJ software.

WB Analysis

WB analysis was used to quantify the protein levels of TLR2, TLR4, TLR6, p65, and p38/ERK after stimulating BV-2 cells with Aβ1-42 (1 uM) for 24 h. WB analysis was also used to quantify the protein levels of p65, TLR4, IL-1β, IL-6, and TNF-α after stimulating BV-2 cells with Aβ1-42 (1 uM) and LPS (100 ng). Cells were washed twice with PBS and then resuspended in

Table 1 Primer sequences used in qRT-PCR experiments

Gene name	Forward primer	Reverse primer
TREM2	5′-TGAAGAAGCGGAATGGGAGC-3′	5′-CACCGGCTTGGAGGTTCTTC-3′
TNF-a	5′-TCAGAGGGCCTGTACCTCAT-3′	5′-GGAGGTTGACCTTGGTCTGG-3′
IL-1β	5′-TGAGCTCGCCAGTGAAATGA-3′	5′-CATGGCCACAACAACTGACG-3′
IL-6	5′-TCTGCGCAGCTTTAAGGAGT-3′	5′-CCCAGTGGACAGGTTTCTGA-3′
GAPDH	5′-AGGGCCCTGACAACTCTTTT-3′	5′-AGGGGTCTACATGGCAACTG-3′

radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Haimen, China) containing phosphatase inhibitors and protease inhibitor to extract total protein. The protein concentration was determined using a bicinchoninic acid assay (BCA) protein assay kit (Beyotime). Equal amounts of protein were separated via 8% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a Bio-Rad electrophoresis apparatus (Bio-Rad Co., USA). The separated proteins were then transferred to polyvinylidene difluoride (PVDF) membranes. After blocking with 5% bovine serum albumin (BSA) for 1 h, the PVDF membranes were incubated with the specific antibodies overnight at 4 °C on a shaker. On the following day, the membranes were incubated with goat anti-rabbit IgG antibody for 1 h at room temperature. The blots were then washed in Tris-buffered saline Tween (TBST) and imaged/quantified using a LI-COR Odyssey CLx imaging system (LI-COR Biosciences, Lincoln, NE, USA).

Statistical Analysis

Statistical analysis was performed using SPSS software 22.0 (IBM, Inc., Armonk, NY, USA). One-way analysis of variance (ANOVA) was used to assess the statistical significance of differences between groups followed with the Bonferroni post hoc test. All data are expressed as mean ± standard deviation (SD) and differences were considered statistically significant at $p < 0.05$. All graphs were generated using GraphPad Prism Version 6.0 software.

Results

TREM2 Improved BV-2 Cell Viability

The efficiency of lentiviral infection was determined by flow cytometry (Fig. 1a). The percentage of cells successfully transduced with lentivirus in each group was > 83%. The infection efficiency in the LV-TREM2 group was 83.15% and that in the LV-shTREM2 group was 83.31%. The expression

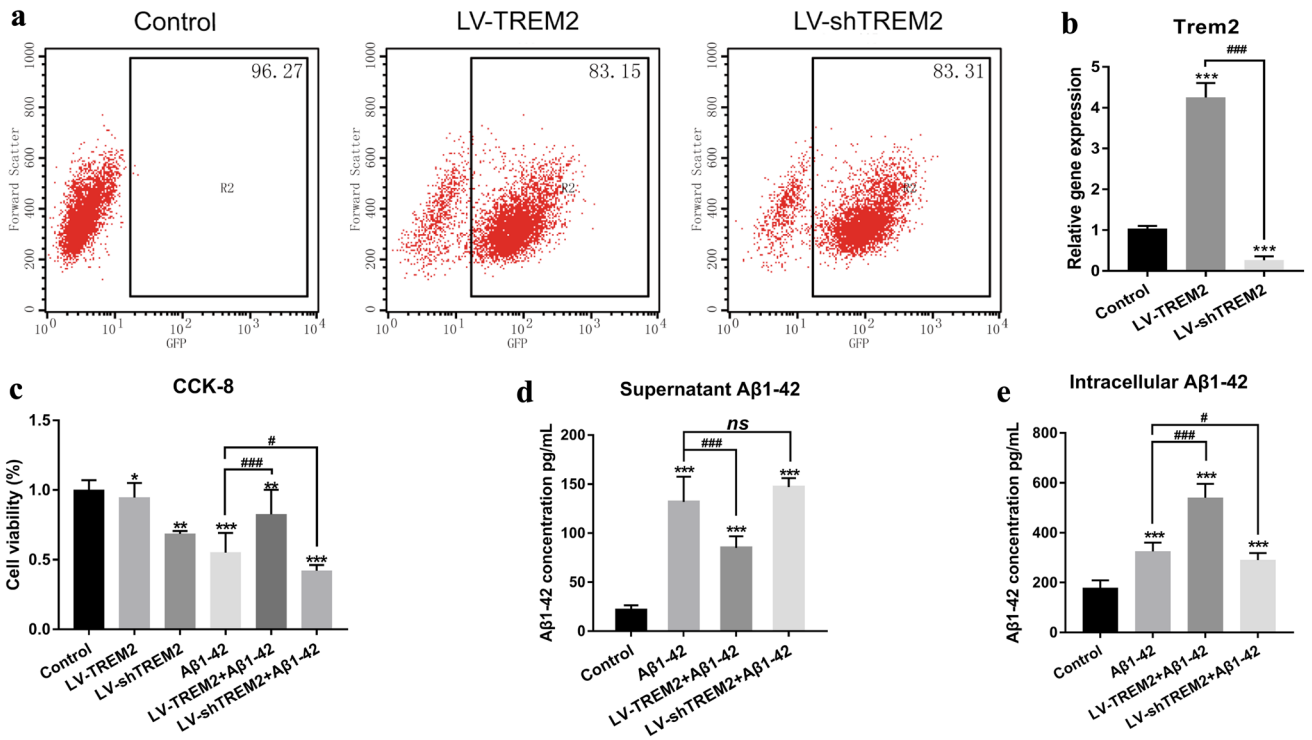

Fig. 1 TREM2 promoted phagocytosis of Aβ1-42 by BV-2 cells and increased cell viability. **a** The lentivirus transduction efficiency was determined by flow cytometry. **b** After cells were transduced with lentivirus, the expression levels of *TREM2* were determined in BV-2 cells using qRT-PCR. Values are presented as the means ± SD, n = 4, ***p < 0.001 vs. Control group; ###p < 0.001 vs. LV-TREM2 group. **c** The CCK8 assay was used to detect the effect of *TREM2* on BV-2 cells activity. Values are presented as the means ± SD, n = 4, *p < 0.05, **p < 0.01, ***p < 0.001 vs. control group; #p < 0.05, ###p < 0.001 vs. Aβ1-42 group. **d** After 24 h of treatment with Aβ1-42, the concentration of Aβ1-42 in the supernatant was determined by ELISA to evaluate the phagocytosis effect of BV-2 cells on Aβ1-42. Values are presented as the means ± SD, n = 4, ***p < 0.001 vs. control group; ###p < 0.001 vs. Aβ1-42 group; ns not significant vs. Aβ1-42 group. **e** The content of Aβ1-42 in cells was determined by ELISA to evaluate the phagocytosis effect of BV-2 cells on Aβ1-42. Values are presented as the means ± SD, n = 4, ***p < 0.001 vs. control group; #p < 0.05, ###p < 0.001 vs. Aβ1-42 group

levels of *TREM2* were determined by qRT-PCR analysis. Compared with that in the control group, the expression level of *TREM2* gene was significantly increased in the LV-TREM2 group, while the expression in the LV-shTREM2 group was significantly decreased. (Fig. 1b). To examine the effect of *TREM2* on the cell viability, BV-2 cells were evaluated using the CCK8 assay (Fig. 1c). CCK-8 results showed that cell viability in the LV-TREM2 group increased slightly by 4% compared with that in the control group, whereas the cell viability in the LV-shTREM2 group decreased significantly by 31.5%. In the Aβ1-42 group, the viability of BV-2 cells was greatly inhibited and decreased by 51.21%. Overexpression of *TREM2* in BV-2 cells reversed Aβ1-42-mediated decrease in cell viability, as evidenced by a 50.13% increase in cell viability in the LV-TREM2 + Aβ1-42 group relative to that in the Aβ1-42 group. In contrast, silencing of *TREM2* (LV-shTREM2 + Aβ1-42 group) further aggravated the activity of BV-2 cells, which was 23.63% lower than that of the Aβ1-42 group. Therefore, our results indicated that *TREM2* helped improve BV-2 cell viability.

TREM2 was Beneficial for the Phagocytosis of Aβ1-42 by BV-2 Cells

After 24 h of Aβ1-42 treatment BV-2 cells, the supernatant and cells were collected and the content of Aβ1-42 was detected by ELISA (Fig. 1d, e). The results showed that the level of Aβ1-42 in the supernatant of Aβ1-42 group was significantly higher than that in the control group. The concentration of Aβ1-42 in the supernatant of the LV-TREM2 + Aβ1-42 group was significantly lower than that in the Aβ1-42 group, which was reduced by 37.5%. However, the content of Aβ1-42 in the supernatant of the BV-2 cells silenced for *TREM2* (LV-shTREM2 + Aβ1-42 group) was not different from that in the Aβ1-42 group. However, as shown in Fig. 1e, the concentration of Aβ1-42 in the cells of the Aβ1-42 group was higher than that in the control group. The content of Aβ1-42 in the LV-TREM2 + Aβ1-42 group was significantly increased compared with the content of Aβ1-42 in the Aβ1-42 group. In addition, the concentration of Aβ1-42 in the LV-shTREM2 + Aβ1-42 group was slightly lower than that in the Aβ1-42 group. ELISA results showed that *TREM2* enhanced the phagocytosis of Aβ1-42 by BV-2 cells.

TREM2 Downregulated the Expression of Inflammatory Mediators

To assess the effect of TREM2 on the levels of inflammatory factors in the AD cell model, BV-2 cells were incubated with Aβ1-42 for 24 h. The mRNA and protein expression levels of inflammation factors (IL-6, IL-1β, and TNF-α) were determined by qRT-PCR and ELISA, respectively. As shown in Fig. 2a, in the Aβ1-42 group, inflammatory factor levels were greatly increased compared with those in the control group. This demonstrates that Aβ1-42 successfully mediated cellular inflammatory responses in BV-2 cells. Inflammatory factor levels were reduced in the LV-TREM2 group compared with that in the control group and increased in the LV-shTREM2 group. In addition, the expression levels of *IL-6*, *IL-1β*, and *TNF-α* were obviously down-regulated in the LV-TREM2 + Aβ1-42 group compared with that in the Aβ1-42 group. However, TREM2 silencing up-regulated Aβ1-42-mediated inflammatory factor soaring in the LV-shTREM2 + Aβ1-42 group (Fig. 2b). Overall, the results demonstrated that Aβ1-42 treatment led to increased expression of TNF-α, IL-6, and IL-1β in the supernatant, and that *TREM2* overexpression downregulated inflammatory factors while *TREM2* silencing upregulated the inflammatory factors.

TREM2 Mediates Phagocytosis of Aβ1-42, Attenuating Neuronal Inflammation by Inhibiting the TLR Pathway

To examine the effect of *TREM2* on the expression of TLRs, protein levels of TLR2, TLR4, and TLR6 were detected by WB. The results demonstrated that in the Aβ1-42-mediated AD model, the expression of the TLR family was increased. As evidence, TLR2, TLR4 and TLR6 protein expression in BV-2 cells was significantly higher in the Aβ1-42 group than in the control group, respectively 94.12%, 136.67% and 52.17% higher (Fig. 3a). Overexpression of TREM2 significantly decreased the expression of TLRs (TLR2, TLR4 and TLR6) in BV-2 cells in the LV-TREM2 + Aβ1-42 group. However, in the LV-shTREM2 + Aβ1-42 group, the expression levels of these proteins were significantly up-regulated, and the increase was even greater than that in the Aβ1-42 group. At the same time, the downstream messengers of the TLR family (REK, P38 and P65) also showed synchronous changes in the WB results, that is, they were significantly downregulated in the LV-TREM2 + Aβ1-42 group and significantly up-regulated in the LV-shTREM2 + Aβ1-42 group. The results of immunofluorescence staining of TLR2, TLR4 and TLR6 also confirmed this conclusion (Fig. 3b). Overall, the experimental results showed that *TREM2* overexpression inhibited the expression of TLR2, TLR4, and TLR6.

To further confirm the molecular mechanism by which TREM2 inhibits neurogenic inflammation, LPS was used as an agonist of the TLR receptor. The expression levels of TLR4 and p-p65 were significantly increased in the Aβ1-42 + LPS group compared with that in the Aβ1-42 group, indicating that LPS successfully activated TLR4 and its downstream messengers. The levels of inflammatory factors were lower in the LV-TREM2 + Aβ1-42 and LV-TREM2 + LPS groups compared to those in the

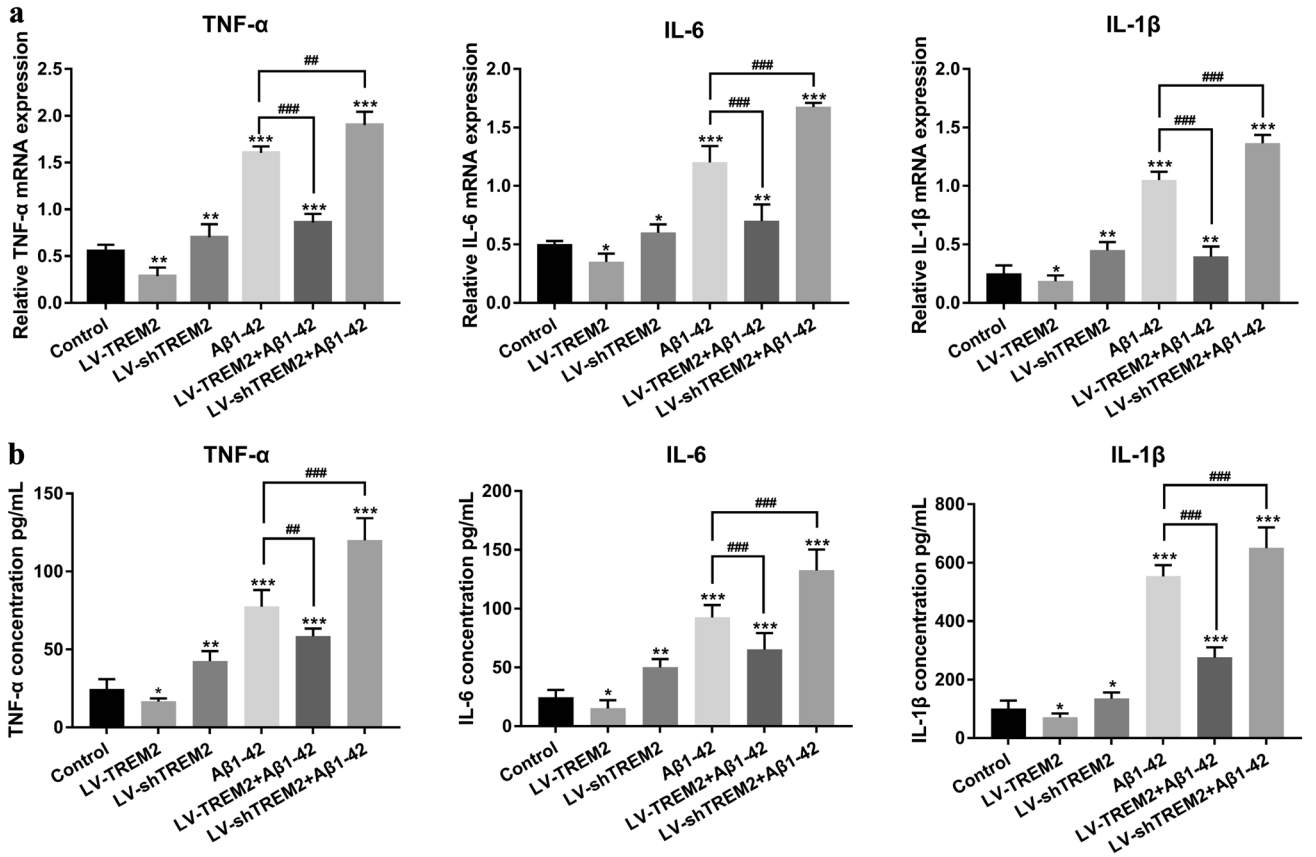

Fig. 2 *TREM2* overexpression downregulated the level of inflammatory factors in the AD cell model. **a** After BV-2 cells were incubated with Aβ1-42 for 24 h, qRT-PCR was performed to determine the gene expression levels of *IL-6, IL-1β*, and *TNF-α*. Values are presented as the means ± SD, n = 4, *p < 0.05, **p < 0.01, ***p < 0.001 vs. Control group; $^{\#\#}$p < 0.01, $^{\#\#\#}$p < 0.001 vs. Aβ1-42 group. **b** ELISA analysis was performed to determine the expression level of IL-6, IL-1β, and TNF-α. Values are presented as the means ± SD, n = 4, *p < 0.05, **p < 0.01, ***p < 0.001 vs. control group; $^{\#\#}$p < 0.01, $^{\#\#\#}$p < 0.001 vs. Aβ1-42 group

control group. However, compared with those in the LV-TREM2 + Aβ1-42 and LV-TREM2 + LPS groups, the levels of inflammatory factors in the Aβ1-42 + LPS group were significantly increased, while those in the LV-TREM2 + Aβ1-42 + LPS group was also increased but still significantly lower than the levels in the Aβ1-42 + LPS group. (Fig. 3c). The above results indicate that the anti-inflammatory effects of the nervous system exerted by TREM2 are achieved through the TLR pathway.

Discussion

To date, there have been a number of studies on the effects of TREM2 on neuroinflammation. However, due to the widespread prevalence of AD, further research into the mechanisms are necessary. It has been reported that the loss of one copy of *TREM2* exacerbates the pathological accumulation of Aβ in the brain, especially in the hippocampus [22]. TREM2 acts on microglia in conjunction with Aβ-mediated microglial immune proliferation with the activation and expansion of this process resulting in microglia clustering around the Aβ plaques and the subsequent removal of Aβ [29].

TLR4-deficient mice display increases in diffuse Aβ and fibrillar Aβ deposits and their levels of inflammatory factors (TNF-α, IL-1β, etc.) are reduced compared with those in control mice [30, 31]. Research has shown that microglia deficient in TLR2, TLR4, or the co-receptor CD14 are not activated by Aβ and do not show a phagocytic response [32]. In addition, microglia deficient in TLR4, TLR6, or the co-receptor CD36 decreased Aβ neurotoxicity and reduced inflammatory factor production [33]. CD14-TLR2-TLR4 mediates Aβ phagocytosis, while CD36-TLR4-TLR6 is involved in mediating inflammatory factor production and inflammatory regulation. TLR4 is not only related to inflammatory regulation, but also related to Aβ phagocytosis, which is considered to be the key to AD inflammation and Aβ phagocytosis imbalance [14]. The *TREM2* gene can negatively regulate the TLR pathway and TREM2 negatively

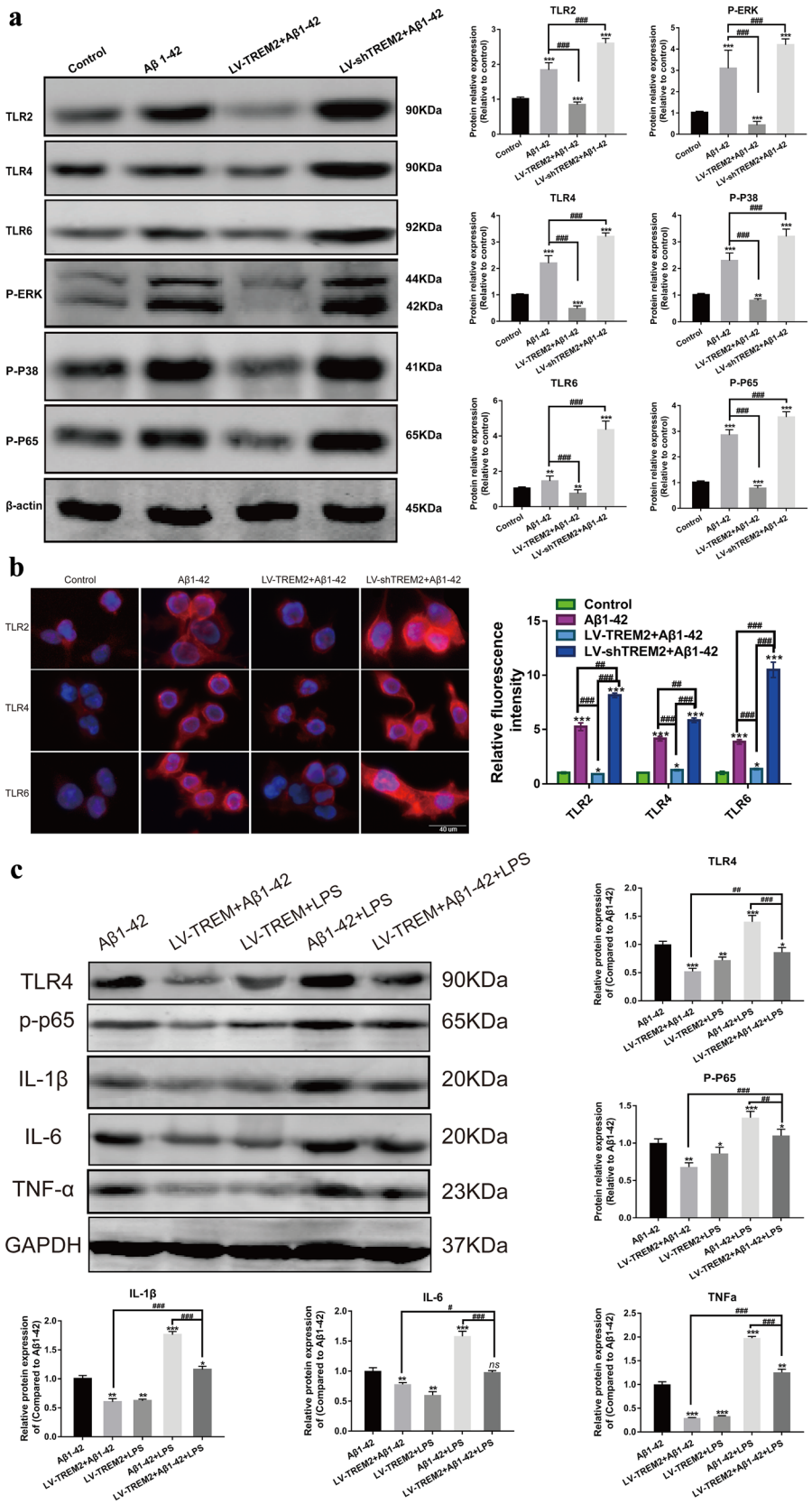

Fig. 3 *TREM2* attenuates neuronal inflammation by inhibiting TLR pathway. BV-2 cells were treated with Aβ1-42 for 24 h. **a** Western blotting was performed to determine the protein expression levels of TLR2, TLR4, TLR6, P-ERK, P-P65, and P-P38. Values are presented as the means ± SD, n = 4, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. Control group; $^{###}p < 0.001$ vs. Aβ1-42 group. **b** Immunofluorescence staining for TLR2, TLR4, and TLR6. BV-2 cells were stained with DyLight 680 (red) and DAPI (blue). Scale bar represents 40 μm. Values are presented as the means ± SD, n = 4, $^{*}p < 0.05$, $^{***}p < 0.001$ vs. Control group; $^{##}p < 0.01$, $^{###}p < 0.001$. **c** Western blotting was performed to determine the protein expression levels of TLR4, P-P65, IL-6, IL-1β, and TNF-α. Values are presented as the means ± SD, n = 4, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. Aβ1-42 group; $^{#}p < 0.05$, $^{##}p < 0.01$, $^{###}p < 0.001$, ns not significant (Color figure online)

regulates TLR2, TLR4 and TLR6 (Fig. 3a), showing that TREM2 is related with the TLR pathway. Therefore, we chose to study the relationship between TREM2 and Aβ1-42 phagocytosis, as well as the relationship between neuroinflammation in AD cells.

As an immune guard of brain tissue against foreign invasions, microglia activation is extremely complex and is rooted in the induction of multiple phenotypes in order to cope with different environments, activating ligands, and genetic backgrounds [26, 34, 35]. TREM2 is a receptor for β-amyloid which mediates microglial function and directly binds to β-amyloid (Aβ) oligomers with nanomolar affinity, whereas AD-associated *TREM2* mutations reduce Aβ binding [36]. TREM2 occupies a critical position in the clearance of Aβ1-42 by BV-2 cells in vitro, as shown by the fact that overexpression of *TREM2* increases clearance of Aβ1-42 by BV-2 cells (Fig. 1d, e) and silencing of *TREM2* significantly attenuates the defense of cells as a result of a decrease in BV-2 cell viability and the deterioration of neurological inflammation (Figs. 1c, Fig. 2a, b). Consistent with this idea, knockdown of *TREM-2* in microglia has been shown to cause an increase in TNF-α and IL-1β transcription and dysregulates microglial phagocytosis [30]. TREM2 binds Aβ and promotes clearance of Aβ deposits [36]. Co-administration of Aβ1-42 with the TLR ligand LPS to microglial cultures leads to an additive release of nitric oxide and TNF-α from microglia [37].

AD is thought to be involved in the upregulation of pro-inflammatory cytokines. Inflammatory factors are implicated in dementia-related peripheral disease and cerebral nervous system diseases [38]. Cerebral Aβ accumulation is one of the main factors influencing AD [3]. In our current study, we silenced or overexpressed *TREM2* in the AD model using BV-2 cells. Stimulation of BV-2 cells with Aβ1-42 increased the mRNA and protein expression levels of *IL-1β*, *IL-6*, and *TNF-α*, which are three common inflammatory factors; however, *TREM2* overexpression attenuated these changes in expression (Fig. 2a, b). These results confirmed the potential role of TREM2 in attenuating neuroinflammation. Therefore, we suspect that anti-inflammatory responses may be occurring during the onset of AD. Abnormal deposition of Aβ activates the TREM2 receptor on the microglia membrane and upregulates the expression of IL-10, IL-4, and other anti-inflammatory factors, thereby reducing the inflammatory response [39]. On the other hand, microglia secrete and release inflammatory factors such as TNF-α, IL-1β, IL-6, and some chemical chemokines and synthesize and release ROS, thereby damaging the nerves. In turn, meta-injured neurons act on microglia, forming an ever-increasing self-toxicity loop in the brain that increases inflammatory factors [40]. TLR4 is involved in this process as a receptor on the microglial membrane, facilitating the central system to verify the response [41]. In our current study, the expression of TLRs increased in Aβ1-42-treated BV-2 cells (Fig. 3a), which suggested that TLRs were associated with the pathogenesis of AD. Furthermore, the use of LPS as an agonist for TLRs obliterated the therapeutic effect of TREM2 overexpression on AD cells. Therefore, we can draw the conclusion that the anti-inflammatory effect was unable to balance the pro-inflammatory effect and ultimately the neuroinflammation was upregulated by the TLR-related pathway.

P65 is a key subunit of NF-κB signaling pathway and is reported to be widely involved in neuroinflammation [42]. A variety of disease-related studies have found that the expression of *TREM2* is regulated by the nuclear transcription factor P65. The findings suggest that PAF-induced impaired transcriptional activity of the p65 subunit is responsible for the downregulation of pro-inflammatory cytokines and upregulation of IL-10 in macrophages specifically activated by TLRs [43]. The MAPK pathway is also involved in inflammation during the pathological condition of AD. P38 and ERK are widely studied members of the MAPK family. TREM2 may reduce neuroinflammation by downregulating TLRs thus inhibiting the nuclear translocation of NF-κB and suppressing the ERK/p38 MAPK signaling pathway. In our current study, ERK, p38, and p65 were activated and phosphorylated by Aβ1-42. *TREM2* overexpression reduced the expression of p-ERK and p-p38. Therefore, *TREM2* may inhibit the activation of ERK/p38 MAPK in the BV-2 cells by downregulating TLRs.

Thus, our findings provide fundamental evidence that overexpression of TREM2 serves as a protective mechanism of AD and should be advocated. Further studies are needed to clarify the mechanism of TREM2 in alleviating AD-related inflammation through which the overexpression of TREM2 impacts the responses of BV-2 cells to Aβ. Taken together, our findings clearly demonstrate that strategies designed to sustain microglial homeostasis and microglia function may be potential treatments for AD and other neurodegenerative diseases linked to TREM2 deficiency, and microglial dysfunction in general.

To date, some research groups are developing immunotherapies that use antibodies to stimulate TREM2 signaling [44]. Furthermore, regulation of TREM2 expression or protein levels is another strategy of interest. In vivo, the lentiviral approach aims to increase the expression of TREM2 in the mouse brain to attenuate cognitive and neuropathological changes [45]. However, it is important to note that lentiviral strategies cannot be used in humans. In addition,studies have shown that TREM2 is also expressed in peripheral blood, which can be used as an accurate tool to understand the biological effects of TREM2 in the pathogenesis of AD [46]. In the future,the regulatory range of TREM2 may be assessed by peripheral blood in patients.

A limitation of this experiment is the use of mouse-derived microglial cell lines alone. Compared with primary cultured cells, cell lines are easier to obtain, have long survival time, high degree of differentiation, easy to observe, good cell homology, and good microglia and neural cell characteristics [47]. However, experiments should be performed using human-derived cells such as hIPSCs-derived microglia to confirm the clinical relevance of the data presented.

In summary, the findings of our study indicated that *TREM2* promoted clearance of Aβ1-42 by BV-2 cells, restored BV-2 cell viability from Aβ1-42-mediated neurotoxicity, and reduced neuroinflammatory responses by downregulating TLRs. Therefore, our findings may provide better understanding of the inhibitory effects of TREM2 on neuroinflammation in AD. These findings indicate that TREM2 may play a protective role against aging-related neuroinflammation.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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