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ORIGINAL PAPER

Imbalance of Microglial TLR4/TREM2 in LPS-Treated APP/PS1 Transgenic Mice: A Potential Link Between Alzheimer's Disease and Systemic Inflammation

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Abstract

Clinically, superimposed systemic inflammation generally has significant deleterious effects on the Alzheimer's disease (AD) progression. However, the related molecular mechanisms remain poorly understood. Microglial toll-like receptor 4 (TLR4) and triggering receptor expressed on myeloid cells 2 (TREM2) are two key regulators of inflammation that may play an essential role in this complex pathophysiological process. In this study, intraperitoneal injection of lipopolysaccharide (LPS) into APP/PS1 transgenic AD model was used to mimic systemic inflammation in the development of AD. Initial results from the cortex showed that compared with wild-type mice, APP/PS1 mice exhibited elevated gene and protein expression levels of both TLR4 and TREM2 with different degree. Interestingly, after LPS treatment, TLR4 expression was persistently up-regulated, while TREM2 expression was significantly down-regulated in APP/PS1 mice, suggesting that the negative regulatory effect of TREM2 on inflammation might be suppressed by LPS-induced hyperactive TLR4. This imbalance of TLR4/TREM2 contributed to microglial over-activation, followed by increased neuronal apoptosis in the cortex of APP/PS1 mice; these changes did not alter the expression level of $A\beta_{1-42}$. Similar alterations were observed in our in vitro experiment with β-amyloid$_{1-42}$ ($A\beta_{1-42}$)-treated N9 microglia. Further, Morris water maze (MWM) testing data indicated that LPS administration acutely aggravated cognitive impairment in APP/PS1 mice, suggesting that the addition of systemic inflammation can potentially accelerate the progression of AD. Collectively, we conclude that an imbalance of TLR4/TREM2 may be a potential link between AD and systemic inflammation. TREM2 can serve as a potential therapeutic target for treating systemic inflammation in AD progression.

Keywords Alzheimer's disease · Microglia · Systemic inflammation · TLR4 · TREM2

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by amyloid β (Aβ) aggregation [1–3], tau hyperphosphorylation [4, 5], genetic mutations [6], and neuron loss [7]. Relevant hypotheses have been reported as potential mechanisms of AD pathophysiology [8]. Recently, neuroinflammation has emerged as a significant contributor to AD pathogenesis [9–11] since elevations of inflammatory molecules and cytokines were observed at different stages of AD [12, 13]. Clinically, several age-related inflammation and chronic infection and inflammatory diseases (such as viral infection, atherosclerosis and its ischemic consequences, type 2 diabetes and metabolic syndrome, coronary heart disease, rheumatic diseases and obesity) were proposed as deleterious factors for the progression of AD [13]. These systemic inflammatory events are related

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with the more rapid cognitive decline of AD patients [14]. It is well recognized that AD patients constantly deteriorate after systemic inflammation, suffering long-term cognitive impairment [12, 15]. Some evidence from animal models of AD also indicates that systemic inflammation leads to more serious outcomes in individuals with prior pathology in the central nervous system (CNS) [12, 16, 17]. However, the corresponding potential links between AD and systemic inflammation currently remain unclear [12, 13].

In AD patients and animal models, Aβ can induce microglial activation through interacting with some Toll-like receptors (TLRs), such as TLR4 [18, 19]. Microglia are the innate immune cells in the brain [20]. Activated microglia secrete more pro-inflammatory cytokines and thereby contribute to neurotoxicity and loss of neuronal function [21]. This significant pathological feature is an early event in the AD pathogenesis. As the binding receptor of Aβ, TLR4, localized to the surface of microglia, is a significant mediator in this pathophysiological process [22–24]. Several studies have demonstrated that Aβ deposition enhances the expression and activation of TLR4 in activated microglia cells and AD brain tissues and elicits the activation of MAPK and NF-κB signaling pathways and the subsequent release of pro-inflammatory cytokines [i.e., interleukin (IL)-1β, interferon (IFN)-γ and tumor necrosis factor (TNF)-α] [25–27]. Recent data indicated that TLR4-mediated inflammation was negatively regulated by triggering receptor expressed on myeloid cells 2 (TREM2) [28]. TREM2 is a cell surface receptor of the Ig superfamily that is mainly expressed in microglial cells within the brain [29]. TREM2 mediates crucial functions of microglia, such as suppression of pro-inflammatory responses and stimulation of engulfment of apoptotic neurons and Aβ deposits [30–32]. Mounting evidence now indicates that TREM2 is a key regulator of immune responses within microglia, and its hypomorphic variants are closely associated with an increased risk of AD [33–39]. Concentrations of cerebrospinal fluid-soluble TREM2 were found to be higher in AD than in controls [40].

Despite the fact that TLR4 and TREM2 have received a great deal of attention as two key regulators of inflammation in AD pathogenesis, their roles are still relatively ambiguous, which is probably due to protein functional diversities in various pathological and physiological conditions [23, 41, 42]. In the initial stage, the activation of TLR4 signaling is associated with increased uptake and clearance of Aβ [43]. In contrast to its benefits, in the later stages, chronic long-term activation of TLR4 elicits neurotoxic effects via overactivated microglia [23, 44]. Overactivated microglia may progressively release neurotoxic products, followed by damage to neighboring neurons, thereby persistently aggravating AD neuroinflammation and subsequent neurodegeneration [45]. The persistent activation of TLR4 may not be conducive to the clearance of Aβ in the brain. Based on clinical manifestations, these important biological events may occur in the later stage of AD, which is generally induced by the aforementioned systemic inflammatory stimulation [12, 13]. As a negative regulator of inflammation, TREM2 has been suggested to inhibit TLR signaling during peripheral inflammation by regulating the phagocytosis and inflammatory capacity of microglia [39, 46]. With excessive inflammation mediated by hyperactive TLR4 [47], the negative feedback action of TREM2 could be affected to some degree [48]. This possibility prompted us to investigate microglial TREM2 dysregulation in the pathophysiology of AD with systemic inflammation.

In the present study, we first administered an intraperitoneal injection of lipopolysaccharide (LPS) to APP/PS1 transgenic mice to mimic the systemic inflammation that occurs in AD development [12]. Subsequently, the gene and protein expression levels of TLR4 and TREM2 in LPS-treated APP/PS1 transgenic mice were examined. Further, the changes in microglial activity, neuronal apoptosis, $A\beta_{1-42}$ levels, and cognitive function were investigated to uncover possible molecular mechanisms.

Materials and Methods

Mice and LPS Treatment

Seven-month-old APP/PS1 male transgenic mice (Jackson Laboratory, stock NO.004462) were chosen as the experimental model for the initial stage of AD, and age-matched wild-type (WT) littermates were used as controls. Mice were purchased from the Model Animal Research Center of Nanjing University, and the mutations of the *APP* and *PS1* genes were confirmed in APP/PS1 mice using polymerase chain reaction (PCR), as previously described [49]. All mice were bred and maintained under specific pathogen-free conditions at 26 °C under a 12-h light/12-h dark cycle and were provided unrestricted access to food and water during the experiment.

According to mouse body weight, one intraperitoneal injection of 5 mg/kg LPS [50, 51] (LPS power was dissolved in sterile endotoxin-free 0.9% saline at a concentration of 10 mg/ml, *E. coli*, serotype 055:B5, L2880, Sigma-Aldrich, USA) or an equal dose of saline was administered, and the mice were divided into four subgroups—WT, APP/PS1, WT + LPS and APP/PS1 + LPS—with five mice in each group. This study was carried out in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and the protocol was approved by the Ethics Committee of Chongqing Medical University.

Microglial Cell Culture

The N9 microglial cell line (N9MG) was derived from ICR/CD1 mice and stored at − 80 °C [52]. Frozen N9MG cells were thawed and distributed at a density of 1×10^6 cells per 250-ml culture flask. Then, N9MG cells were cultured in fresh complete medium [DMEM/F12 medium (Gibco) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin] in a humidified 95% air and 5% CO_2 incubator at 37 °C. On the third day after cell recovery, $A\beta_{1-42}$ or/and LPS was added to the complete medium to continue culturing the N9MG cells for 24 h as follows: normal group (complete medium with PBS), $A\beta_{1-42}$ group (complete medium with 1 μM $A\beta_{1-42}$, synthetized by Shanghai Shenggong Company), LPS group (complete medium with 3 μg/ml LPS) [53], and $A\beta_{1-42}$ + LPS group (complete medium with 1 μM $A\beta_{1-42}$ and 3 μg/ml LPS). All in vitro experiments were repeated 3 times.

Morris Water Maze (MWM)

The MWM test was conducted for 7 consecutive days according to a previously described protocol with minor adaptations [52]. Briefly, the MWM test in our experiment included the two following components: a 6-day consecutive place navigation test and a 1-day spatial probe test. LPS administration was conducted after the day 5 test and the duration of the treatment was 24 h. The MWM task was resumed on the final day (day 7) and the escape latency was recorded. Then, the platform was moved, and the mice were gently released into the quadrant opposite that which had previously contained the platform. The mice were allowed to swim for 60 s, and their duration in the target quadrant (quadrant that contained the platform) and the number of platform crosses were recorded. The escape latencies determined during the place navigation test and the spatial probe test (days 1–5 and day 7, respectively) were used to assess visual spatial and learning abilities, respectively. The spatial memory ability of the mice was assessed based on the time spent in the target quadrant and the number of platform crossings during the spatial probe test (on day 7).

Tissue Processing

All APP/PS1 and WT mice were sacrificed under anesthesia with an intraperitoneal injection 50 mg/kg sodium pentobarbital the next day after the MWM test. The brains and N9MG cells were handled as follows.

For Western blotting analysis, quantitative real time polymerase chain reaction (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA), the mice were perfused with 0.9% saline (pH 7.4), the brains were then removed rapidly and stored under liquid nitrogen, and the cortex was chosen for experiments. N9MG cells and the supernatant were separately collected after $A\beta_{1-42}$ and/or LPS administration and were immediately frozen at − 80 °C.

For immunohistochemistry, double-immunofluorescence staining and TUNEL analysis, the mice were perfused with 0.9% saline (pH 7.4). The rapidly removed brains were soaked in 4% paraformaldehyde in 0.9% saline (pH 7.4) and stored at 4 °C, and the cortex of the brains was chosen for experiments. N9MG cells were distributed on glass slides at a density of 1×10^5 cells per ml and cultured for 1–2 days in the above used medium and incubator, followed by the administration of $A\beta_{1-42}$ and/or LPS. Then, the cells were fixed with 4% paraformaldehyde in 0.9% saline (pH 7.4) and stored at 4 °C.

QRT-PCR

Total RNA was extracted from the cortex and N9MG cells using RNAiso Plus (Takara, Japan), according to the manufacturer's protocol. The designed primers of TLR4 and TREM2 were synthesized by Sangon Biotech (Shanghai, China), and β-actin served as an internal control. The following primers were used: TLR4: 5′-GCTCTGGCATCA TCTTCATTG-3′ (forward) and 5′-GCCGTTTCTTGTTCT TCCTCT-3′ (reverse); TREM2: 5′-GCACCAACTTCAGAT CCTCACT-3′ (forward) and 5′-GCAAAAGTAGCAGAA ACAGAAGTC-3′ (reverse); and β-actin: 5′-GTCCCTCAC CCTCCCAAAAG-3′ (forward) and 5′-GCTGCCTCAACA CCTCAACCC-3′ (reverse). qRT-PCR was performed using cycling conditions of 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The relative changes in TLR4 and TREM2 gene expression levels were calculated with the $2(-\Delta\Delta Ct)$ method [52].

Western Blotting

Western blotting was conducted as previously described [52, 54]. Pre-stained standards (Beyotime, Shanghai, China) were used as molecular weight markers. The cortical and N9MG cell samples were homogenized and lysed with RIPA lysis buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, containing protease inhibitors) (Beyotime, China). The proteins in the detergent-soluble fractions were quantified with the bicinchoninic acid assay (Pierce, USA). For each sample, 15 μg of protein was separated via SDS–PAGE and electrophoretically transferred to nitrocellulose membranes. Then, the membranes were incubated with the following primary antibodies: rabbit anti-TLR4 (1:800, ab13556; Abcam, USA), rabbit anti-TREM2 (1:300, sc-48765, Santa Cruz, USA) and mouse anti-β-actin (1:1500; CWBIO, China). The membranes were washed and incubated for 2 h at room temperature with species-specific peroxidase-conjugated secondary antibodies,

and specific bands were detected using an ECL system (Amersham) and a Bio-Rad electrophoresis image analyzer (Quantity One Software). TLR4-immunoreactive bands were ~100 kDa, TREM2-immunoreactive bands were ~36 kDa, and β-actin-immunoreactive bands (loading control) were ~43 kDa. The expression levels of TLR4 and TREM2 obtained by Western blotting were normalized by calculating the TLR4/β-actin and TREM2/β-actin optical density ratios.

Immunohistochemistry and Immunofluorescence Double-Labeling

As previously described [52], paraffin-embedded brain tissue sections were incubated with the primary rabbit anti-TLR4 (1:80, ab13556; Abcam, USA), rabbit anti-TREM2 (1:100, sc-48765, Santa Cruz, USA) or rabbit anti-ionized calcium-binding adaptor molecule 1 (IBA1) antibodies (1:50, sc-98468, Santa Cruz, USA). Then, the sections were sequentially incubated with a biotinylated secondary antibody and avidin–biotin complexes. Diaminobenzidine and hematoxylin were used for color development and counterstaining respectively. The expression levels of TLR4, TREM2 and IBA1 in the cortex of the brain, as determined by immunohistochemistry, were normalized using Image-Pro Plus (IPP) 6.0 software by calculating the mean IOD/area (in 10 random and equidistant areas of each section in five sections for each mouse). For immunofluorescence, tissue sections were incubated in a mixture of primary goat anti-TREM2 (1:50, sc-22634; Santa Cruz, USA) and rabbit anti-TLR4 antibodies (1:50, ab13556; Abcam, USA) followed by donkey anti-goat FITC-conjugated IgG (1:100, bs-0294d; Bioss, China) and mouse anti-rabbit Cy3-conjugated IgG (1:100, bs-0295m; Bioss, China). The sections were imaged using laser scanning confocal microscopy (Leica).

ELISA

As previously described [52], homogenized brain cortical samples and N9MG cell supernatants were collected. The expression levels of $A\beta_{1–42}$ in the tissue and cell supernatant were assayed using a Mouse $A\beta_{1–42}$ ELISA kit (CEA946Mu, USCN, China) according to the manufacturer's instructions. The level of $A\beta_{1–42}$ was calculated in accordance with the methods specified in the ELISA kit. Each experiment was performed in triplicate. $A\beta_{1–42}$ levels are presented as pg per ml of cell supernatant or pg per mg of total tissue protein (means ± SD).

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling (TUNEL)

Double-immunofluorescence staining for TUNEL and microtubule-associated protein 2 (MAP2) was used to detect neuronal apoptosis. Tissue sections were continuously incubated in a primary rabbit anti-MAP2 antibody (1:50, bs-1369R; Bioss, China) followed by incubation in goat anti-rabbit FITC-conjugated IgG (1:100, bs-0295G; Bioss, China) and then in TUNEL solution, according to the manufacturer's instructions (C1089; Beyotime, China).

Statistics

Statistical analysis of all data was carried out using SPSS 17.0 software [52]. The results are reported as the means ± SD. The escape latency of MWM data was analyzed using two-way analysis of variance (ANOVA). One-way ANOVA (comparisons among four groups) and Student's t tests (to compare any two groups) were used to analyze the data from the MWM spatial probe test, qRT-PCR, Western blotting, immunohistochemistry, ELISA and TUNEL. Student's t test was used to analyze the relative increases in TLR4 and TREM2 expression levels. The relative increase = expression level in the APP/PS1 group minus the level in the WT group divided by the level in the WT group. In all cases, $p < 0.05$ was considered significant.

Results

TLR4 and TREM2 Elevations in APP/PS1 Transgenic Mice and $A\beta_{1–42}$-Treated N9MG Cells

It has been reported that both TLR4 and TREM2 are mainly expressed in the surface of microglia in the brain [24, 29]. In the present study, the localizations of TLR4 and TREM2 in the cortex of WT mice were firstly determined using immunofluorescence double-labeling. Co-expression of these two proteins was shown in Supplementary Figure S1, indicating that they were co-localized to the surface of microglia. As Aβ may induce neuroinflammation by interacting with microglial TLR4 [19], we first determined the levels of two key regulators, TLR4 and TREM2, in APP/PS1 transgenic mice and $A\beta_{1–42}$-treated N9MG cells. For the in vivo experiment, Western blotting analysis showed that the expression levels of TLR4 and TREM2 proteins were increased in APP/PS1 mice compared with WT mice (1.69 ± 0.12 vs. 0.48 ± 0.04 and 1.90 ± 0.10 vs. 1.52 ± 0.04, respectively; $p < 0.05$, Fig. 1a). However, the increased extent of TREM2 expression was relatively smaller than that of TLR4 expression in APP/PS1 mice. Similar results were observed in the immunohistochemical analysis (Fig. 2a). These results from AD model mice were further verified by our in vitro experiment. As shown in Fig. 1b, TLR4 and TREM2 levels determined by Western blotting were increased in the $A\beta_{1–42}$ group compared with the control group (1.59 ± 0.05 vs. 0.56 ± 0.04 and 1.88 ± 0.16 vs. 1.36 ± 0.12; $p < 0.05$), but the

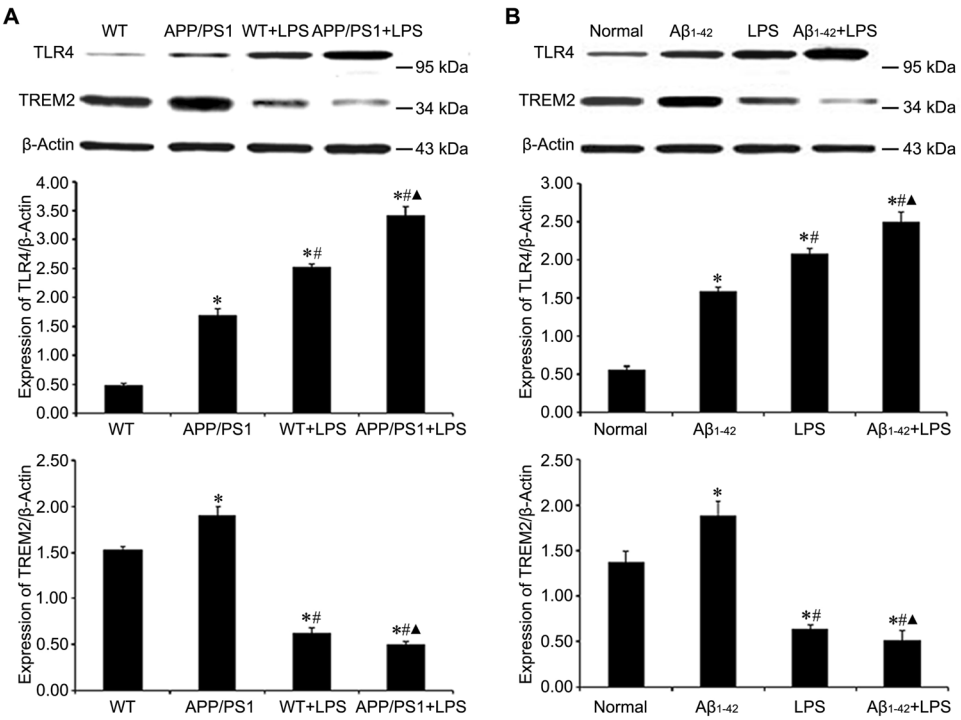

Fig. 1 Western blotting analysis of changes in TLR4 and TREM2 protein levels in APP/PS1 transgenic mice (**a** n = 5) and in $A\beta_{1-42}$-treated N9MG cells (**b** n = 3) before and after LPS treatment. β-Actin was used as an internal control. *Significant compared with the WT or normal group ($p < 0.05$); #Significant compared with the APP/PS1 or $A\beta_{1-42}$ group ($p < 0.05$); ▲Significant compared with the WT + LPS or LPS group ($p < 0.05$)

relative increase in TLR4 production was greater than that in TREM2 production in $A\beta_{1-42}$-treated N9MG cells. Additionally, the immunohistochemical and qRT-PCR results from the in vivo and in vitro experiments showed the same trends (Figs. 2, 3). Changes in TLR4 and TREM2 at the gene and protein levels were also consistent. We speculated that the same trend with a different extent in the changes of TLR4 and TREM2 may represent a compensatory mechanism in the initial stage of AD [47].

Imbalance of TLR4/TREM2 in APP/PS1 Transgenic Mice and $A\beta_{1-42}$-Treated N9MG Cells After LPS Treatment

To investigate the effects of superimposed systemic inflammation on AD development, we treated APP/PS1 mice and $A\beta_{1-42}$-treated N9MG cells with LPS [12]. As shown in Fig. 1a, TLR4 expression determined by Western blotting was persistently up-regulated (3.41 ± 0.16 vs. 1.69 ± 0.12; $p < 0.05$), while interestingly, TREM2 expression was significantly down-regulated (0.50 ± 0.03 vs. 1.90 ± 0.10; $p < 0.05$) in LPS-treated APP/PS1 mice compared with untreated APP/PS1 mice. Similar trends in TLR4 and TREM2 protein expression levels were observed in WT mice treated with or without LPS (2.53 ± 0.05 vs. 0.48 ± 0.04 and 0.63 ± 0.06 vs. 1.52 ± 0.04, respectively; $p < 0.05$). As expected, the expression level of TLR4 was also higher in APP/PS1 + LPS mice than in WT + LPS mice (3.41 ± 0.16 vs. 2.53 ± 0.05; $p < 0.05$), while TREM2 was lower in APP/PS1 + LPS mice than in WT + LPS mice (0.50 ± 0.03 vs. 0.63 ± 0.06; $p < 0.05$).

Similarly, in our vitro experiment, Western blotting data showed that TLR4 was markedly increased but that TREM2 was decreased in the $A\beta_{1-42}$ + LPS group compared with the untreated $A\beta_{1-42}$ group (2.50 ± 0.13 vs. 1.59 ± 0.05 and 0.51 ± 0.11 vs. 1.88 ± 0.16, respectively; $p < 0.05$, Fig. 1b). Subsequently, the changes in TLR4 and TREM2 at the protein level identified by Western blotting were further verified with immunohistochemistry (Fig. 2b). In addition, their changes at the gene level tested with qRT-PCR displayed a similar trend (Fig. 3b). Together, these data revealed a LPS-induced imbalance of TLR4/TREM2 that probably contributed to the over-activation of microglia in the cortex of APP/PS1 mice [55].

Microglial Over-activation and Subsequently Increased Neuronal Apoptosis

To investigate the possible effect of LPS-induced dysfunction of TLR4/TREM2 on microglial activity, we used IBA1 to label activated microglia. We found that microglia were resting under quiescent conditions in the WT subgroup; however, APP/PS1-derived microglia showed more IBA1 staining with a ramified morphology. Following LPS treatment, the number of activated microglia (characterized by IBA1 expression) was significantly increased in LPS-treated APP/PS1 mice compared with untreated APP/PS1 mice (0.79 ± 0.03 vs. 0.53 ± 0.02; $p < 0.05$). Moreover, compared with non-LPS treatment, LPS treatment increased the

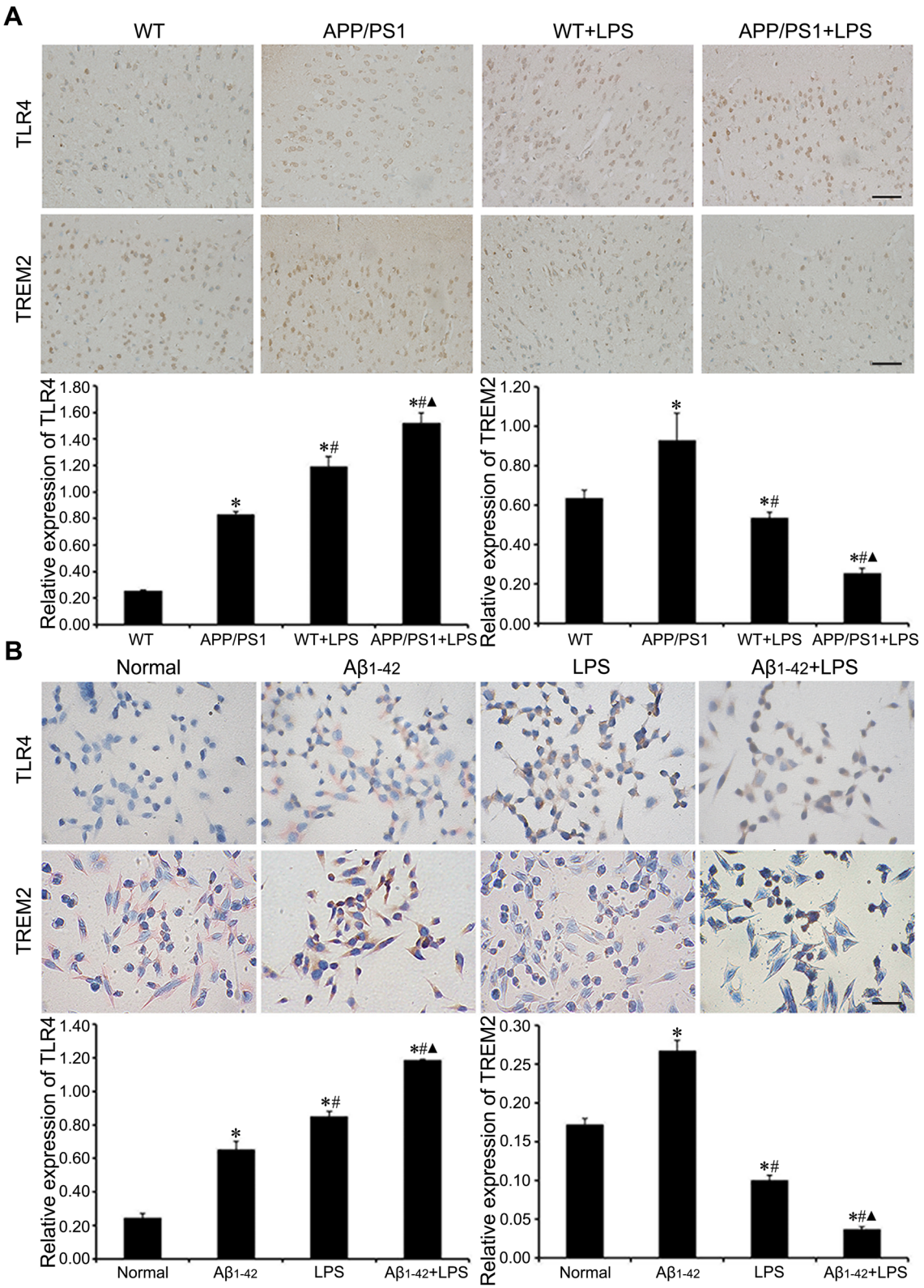

Fig. 2 Immunohistochemistry analysis of changes in TLR4 and TREM2 protein levels in APP/PS1 transgenic mice (**a** n = 5) and in $A\beta_{1–42}$-treated N9MG cells (**b** n = 3) before and after LPS treatment. Scale bar, 50 μm. *Significant compared with the WT or normal group ($p < 0.05$); #Significant compared with the APP/PS1 or $A\beta_{1–42}$ group ($p < 0.05$); ▲Significant compared with the WT + LPS or LPS group ($p < 0.05$)

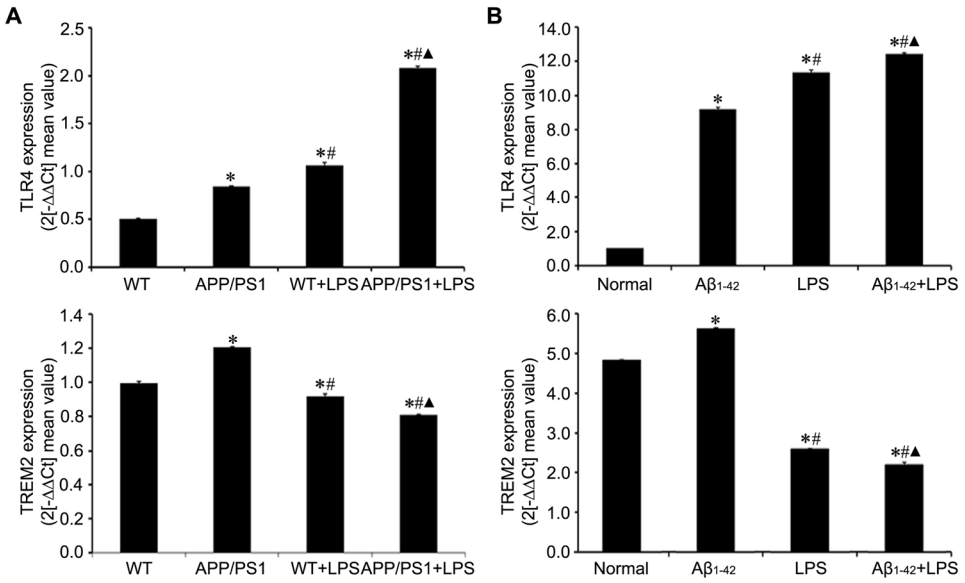

Fig. 3 qRT-PCR analysis of changes in TLR4 and TREM2 gene levels in APP/PS1 transgenic mice (**a** n = 5) and in $A\beta_{1–42}$-treated N9MG cells (**b** n = 3) before and after LPS treatment. β-Actin served as an internal control. *Significant compared with the WT or normal group ($p < 0.05$); #Significant compared with the APP/PS1 or $A\beta_{1–42}$ group ($p < 0.05$); ▲Significant compared with the WT + LPS or LPS group ($p < 0.05$)

number of activated microglia in WT mice (0.67 ± 0.02 vs. 0.37 ± 0.02; $p < 0.05$, Fig. 4). The result indicated that microglia in LPS-treated APP/PS1 mice were overactivated, which probably resulted in increased production of neurotoxic compounds. To determine the possible neurotoxic effect of microglia on neighboring neurons, we next performed immunofluorescence double-labeling of MAP2 (green) and TUNEL (red). The data showed that neuronal apoptosis in the cortex was increased in APP/PS1 mice compared to WT mice (19.00 ± 1.32 vs. 6.33 ± 0.87; $p < 0.05$). In addition, the number of apoptotic neurons in every 100 μm^2 of tissue was increased following LPS treatment in the WT + LPS group compared with the APP/PS1 group (34.78 ± 0.83 vs. 19.00 ± 1.32; $p < 0.05$), and the number of apoptotic neurons in the APP/PS1 + LPS group was higher than that in the WT + LPS group (51.67 ± 1.22 vs. 34.78 ± 0.83; $p < 0.05$, Fig. 5). Together, the results demonstrated that a LPS-induced imbalance of TLR4/TREM2 contributed to microglial over-activation and subsequently greater neuronal injury.

Aggravated Cognitive Impairment in LPS-Treated APP/PS1 Transgenic Mice

To further assess the effect of LPS administration on the cognitive impairment of APP/PS1 transgenic mice, we next performed MWM testing. As shown in Fig. 6a and Supplementary Figure S2, the escape latency of the APP/PS1 group was longer than that of the age-matched WT group on day 1 (57.89 ± 5.23 vs. 45.02 ± 5.75; $p < 0.05$), day 2 (58.64 ± 3.00 vs. 49.62 ± 5.9; $p < 0.05$), day 3 (52.57 ± 9.90 vs. 43.38 ± 7.15; $p < 0.05$), day 4 (46.30 ± 9.80 vs. 36.31 ± 2.34; $p < 0.05$) and day 5 (42.55 ± 8.40 vs. 32.98 ± 2.87; $p < 0.05$). Following LPS administration, compared with the escape latencies of the APP/PS1 group, the escape latencies of the APP/PS1 + LPS group were markedly increased on day 7 (59.98 ± 0.01 vs. 40.36 ± 1.63; $p < 0.05$), and a similar change was also observed between the WT + LPS and WT groups (59.89 ± 0.23 vs. 30.66 ± 1.46; $p < 0.05$). Furthermore, we observed that APP/PS1 + LPS group spent a smaller percentage of time in the target quadrant and crossed the platform fewer times than the APP/PS1 group on day 7 (6.62 ± 1.22 vs. 32.94 ± 1.03 and 0.27 ± 0.46 vs. 2.20 ± 0.56, respectively; $p < 0.05$) (Fig. 6b, c). Similar results were obtained between the WT + LPS and WT groups (21.74 ± 1.80 vs. 48.33 ± 3.18 and 0.33 ± 0.49 vs. 3.80 ± 0.56, respectively; $p < 0.05$). These results indicated that LPS treatment led to a rapid decline in the spatial memory ability of APP/PS1 and WT mice despite the same degree of change. It is also clear that the exacerbated cognitive impairment in APP/PS1 mice can be specifically attributed to LPS-induced systemic inflammation.

Unaltered $A\beta_{1–42}$ Level Despite LPS-Induced Microglial Over-activation

Given the effect of over-activation of microglia on Aβ clearance [56, 57], we next tested whether there were alterations in the expression level of $A\beta_{1–42}$ before and after LPS treatment in vivo and in vitro. In vivo ELISA data indicated that the $A\beta_{1–42}$ level was increased in APP/PS1 mice compared with WT mice (1495.939 ± 67.854 vs. 1209.343 ± 57.069; $p < 0.05$); however, the $A\beta_{1–42}$ level did not differ significantly with or without LPS treatment in APP/PS1 mice (1521.342 ± 58.206 vs. 1495.939 ± 67.854; $p = 0.373$) or in WT mice (1250.344 ± 37.404 vs. 1209.343 ± 57.069; $p = 0.155$). Additionally, there was no significant change in the $A\beta_{1–42}$ level in the normal, $A\beta_{1–42,}$ LPS and $A\beta_{1–42}$ + LPS

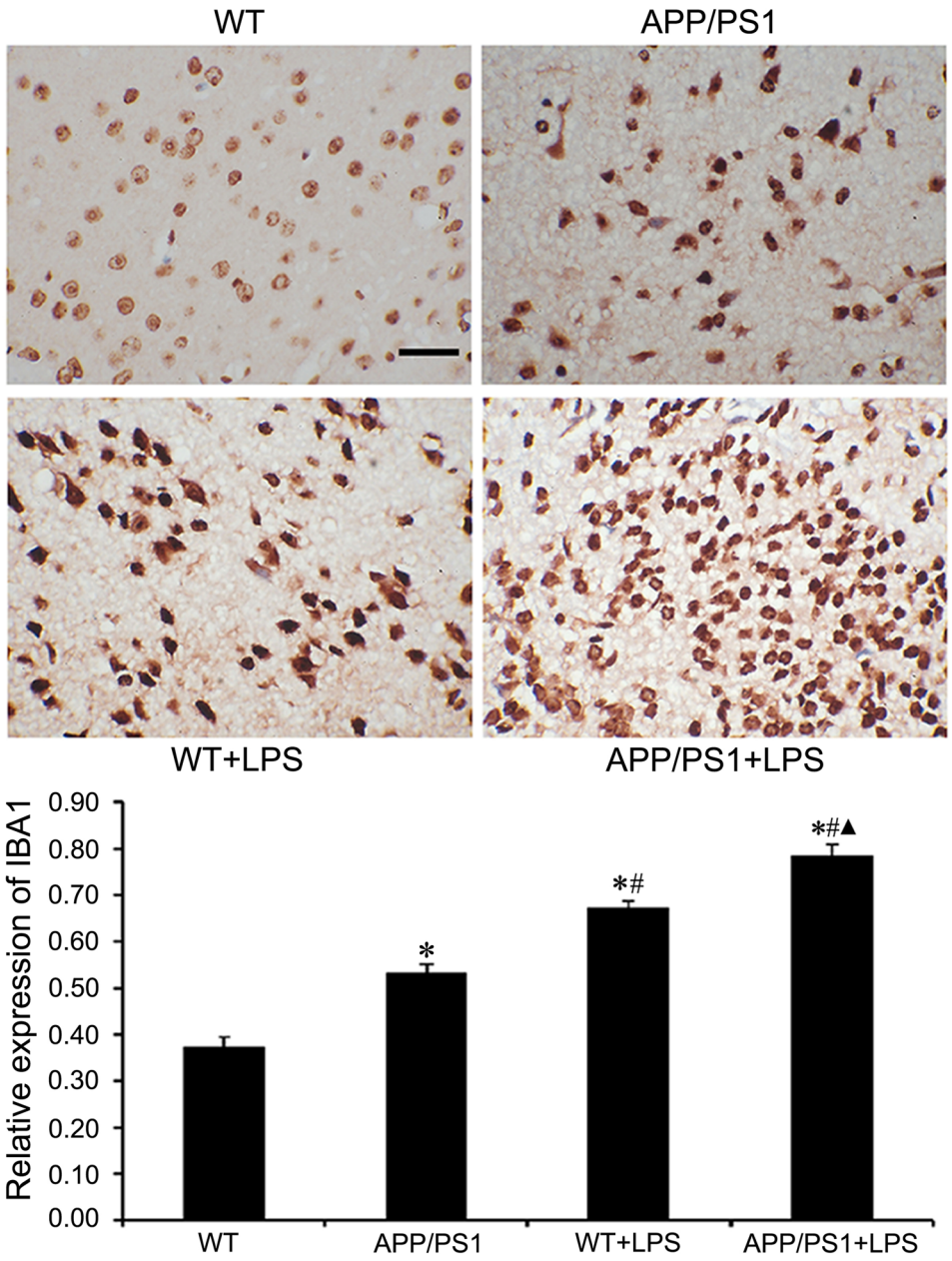

Fig. 4 Evaluation of microglial activation in the cortex of mice (n = 5) by detecting IBA1 expression. Scale bar, 50 μm. *Significant compared with the WT group ($p < 0.05$); #Significant compared with the APP/PS1 group ($p < 0.05$); ▲Significant compared with the WT + LPS group ($p < 0.05$)

N9MG cell treatment groups ($p = 0.757$, Fig. 7). It should be noted that, the $A\beta_{1–42}$ particularly those added $A\beta_{1–42}$ in the cell culture media might form amyloid deposits attached to the cells and/or the culture vessels. In the case, the concentration of $A\beta_{1–42}$ in vitro experiment was tested to be much less than 1 μM. Together, these data indicated that there was no effect of LPS treatment on the $A\beta_{1–42}$ level even if microglia were overactivated.

Discussion

Previous studies have shown that AD is generally accompanied by uncontrolled systemic inflammation during its development [12, 13, 15]. Superimposed inflammation may result in microglial over-activation in the brain and cause deleterious changes to exacerbate the preexisting

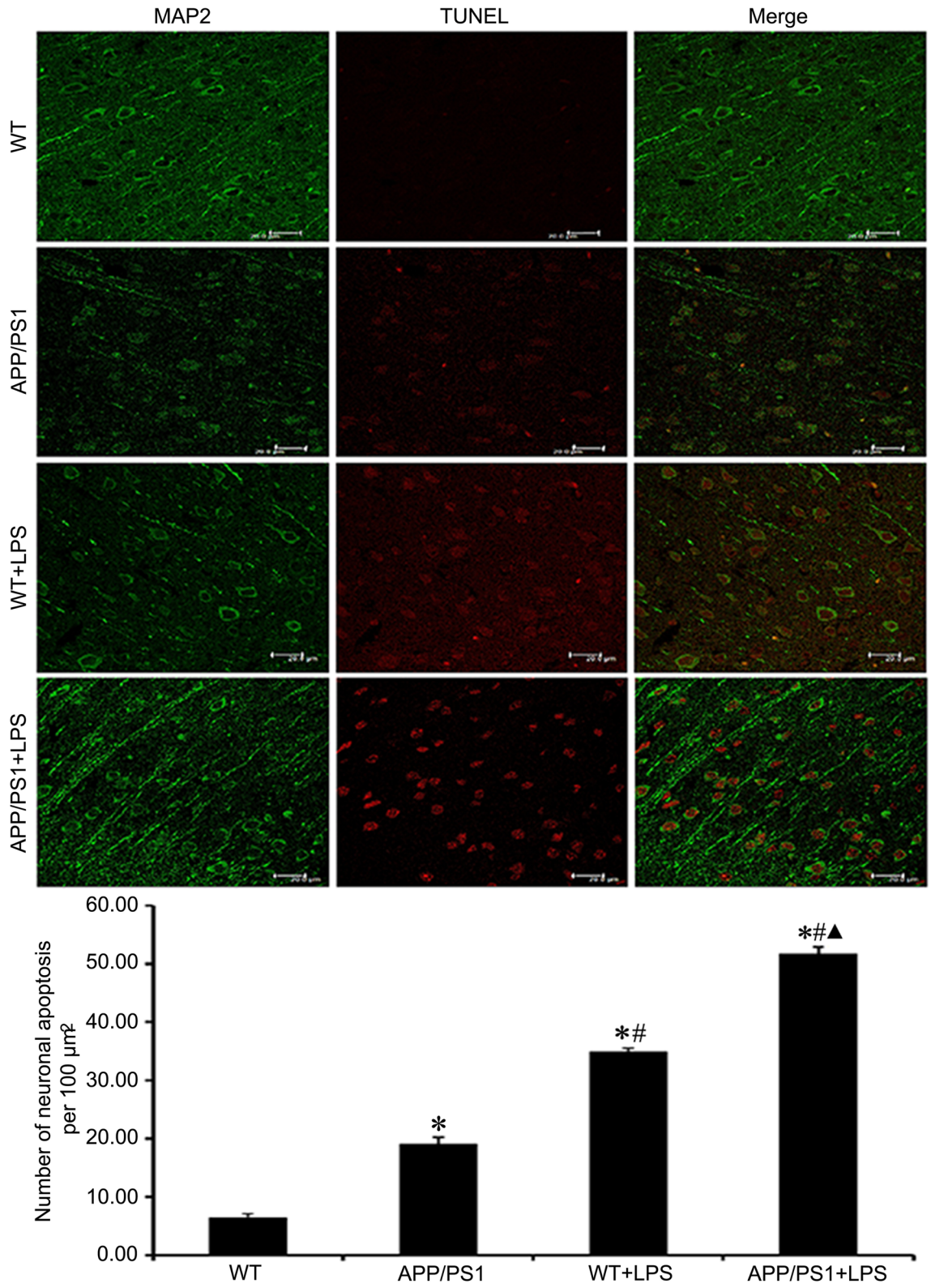

Fig. 5 Evaluation of neuronal apoptosis in the cortex of mice (n = 5) by immunofluorescence double-labeling for MAP2 (green) and TUNEL (red) (800×). Scale bar, 20.0 μm. *Significant compared with the WT group ($p < 0.05$); #Significant compared with the APP/PS1 group ($p < 0.05$); ▲Significant compared with the WT + LPS group ($p < 0.05$). (Color figure online)

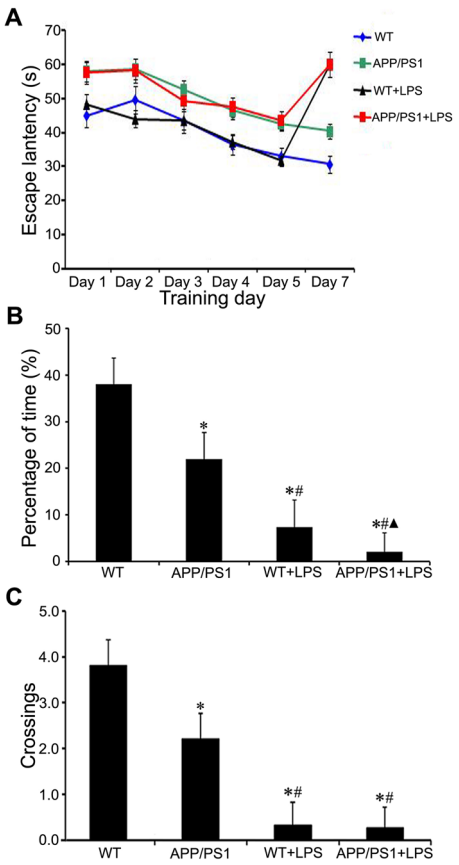

Fig. 6 MWM test for the four experimental groups (n = 5). **a** Comparison of the escape latency on the navigation test on days 1, 2, 3, 4, 5 and 7. **b** Comparison of the time spent in the target quadrant after removal of the platform on day 7. **c** Comparison of the number of platform crossings after removal of the platform on day 7. *Significant compared with the WT group ($p < 0.05$); #Significant compared with the APP/PS1 group ($p < 0.05$); ▲Significant compared with the WT + LPS group ($p < 0.05$)

neuroinflammation of AD and subsequently induce rapid neurodegeneration [12, 13]. However, the potential biological mechanisms have remained unclear and need to be elucidated as an important priority [12]. In the present study, intraperitoneal injection of LPS into the APP/PS1 transgenic AD model was used to mimic the systemic inflammation of AD. LPS can access the circum-ventricular organs, choroid plexus and leptomeninges [55]. Administration of LPS activates peripheral macrophages to move into the CNS and provoke central microglia to chronically express pro-inflammatory cytokines, which in turn cause neuroinflammation [16, 58, 59].

It has been reported that the protein expression of the innate immune receptor TLR4 was increased in the brain of AD patients compared to normal controls, and the TREM2 expression level was also markedly increased [45, 60]. Similarly, in the present study, TLR4 could be activated in the APP/PS1 transgenic mice based on its overexpression relative to the WT. Meanwhile, elevated levels of TREM2 were also observed, suggesting that anti- and pro-inflammatory effects coexist in the initial stage of AD. Up-regulated TREM2 in amyloid plaque-associated microglia can reduce the transcription of inflammatory cytokine genes in microglia to control inflammation and promote microglial phagocytosis [32, 34, 42, 61–63]. Nevertheless, the relative increase in TLR4 expression was higher than that in TREM2 expression. This result was also confirmed to some extent in our in vitro experiment (i.e., $A\beta_{1-42}$-treated N9MG). We hypothesize that the negative regulatory effect of TREM2 on inflammation was subordinate to the pro-inflammatory effect of TLR4 [42, 47].

After LPS treatment, the expression of TLR4 exhibited higher levels in APP/PS1 transgenic mice and $A\beta_{1-42}$-treated N9MG cells. TLR4 has been reported to regulate the microglial immune response by converting microglia to a pro-inflammatory state, which promotes the release of pro-inflammatory cytokines and inhibits the production of anti-inflammatory factors [23, 24, 64]. LPS-induced systemic inflammation evoked a much larger up-regulation of TLR4, suggesting that microglia became overactivated in LPS-treated APP/PS1 transgenic mice [20]. As previously

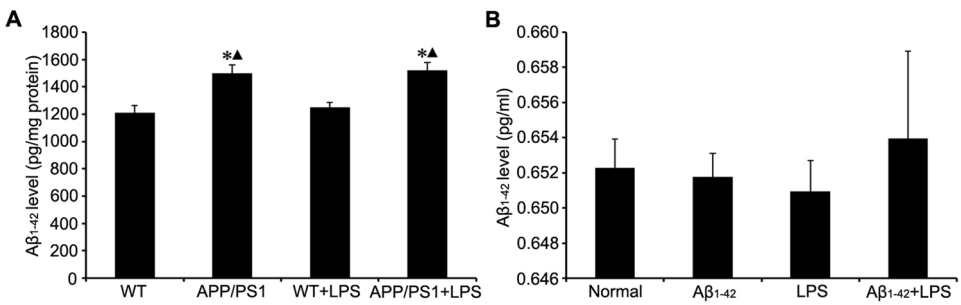

Fig. 7 ELISA detection of the $A\beta_{1-42}$ level in APP/PS1 transgenic mice (**a** n = 5) and in $A\beta_{1-42}$-treated N9MG cells (**b** n = 3) before and after LPS treatment. There was no significant difference between the four groups of N9MG cells. *Significant compared with the WT group ($p < 0.05$); ▲Significant compared with the LPS group ($p < 0.05$)

reported, LPS treatment did not induce microglial proliferation [55]. However, LPS-induced microglial over-activation could lead to more neurotoxicity through the release of multiple pro-inflammatory factors and the generation of excess oxygen/nitrogen free radicals [10]. Unfortunately, TREM2 was significantly down-regulated and failed to compensate for this negative effect through preventing the increased release of pro-inflammatory factors in both APP/PS1 transgenic mice and $A\beta_{1-42}$-treated N9MG cells. The remarkable reduction in the negative regulatory action of TREM2 signaling contributed to the suppression of microglial phagocytosis, probably resulting in irreversible injury to neurons [30, 65]. Our following data indicated that neuronal apoptosis was further increased in APP/PS1 transgenic mice after LPS treatment. Based on this finding, we further investigated the effect of LPS administration on the cognitive impairment of APP/PS1 transgenic mice with MWM testing [52]. We observed that LPS-treated APP/PS1 transgenic mice displayed greater cognitive impairment than the untreated groups. Additionally, it is interesting to note that similar cognitive impairment was observed in LPS-treated WT mice.

We suspected that the suppression of TREM2 via TLR4 activation contributed to the progression of AD, likely via microglial proliferation and neuronal apoptosis. Concretely, LPS activated TLR4 on the surface of microglia and introduced an inflammatory signal to the cell in a MyD88-dependent manner, ultimately initiated to promote the transcription and translation of pro-inflammatory factors. Timely, the CARFD9/Malt1/Bcl10 protein complex also regulated the interaction between non-T cell activation linker and growth factor receptor-bound protein 2 to inhibit tyrosine kinase phosphorylation, which led to further TREM2/DAP12 dysfunction [26, 66–68]. Therefore, suppression of TREM2 hampered the phagocytic ability of microglia and failed to prevent the release of pro-inflammatory factors, which ultimately led to neuronal apoptosis and cognitive impairment. Together, systemic inflammation evoked by LPS would result in more serious deficits in cognitive function attributed to increased neuronal apoptosis in the AD model and thereby potentially exacerbating AD progression. A recent study indicated that TREM2 overexpression in the brain of APPswe/PS1dE9 mice could ameliorate AD-associated neuropathology and improve cognitive functions [69]. It may be tempting to speculate that the aggravated AD-related neuropathology and cognitive decline in the LPS-treated AD model mice could be rescued by overexpressing TREM2. However, the exact function of TREM2 in AD is largely unknown, and its potential as a target for the disease still remains unexplored [41, 70].

It has been reported that Aβ promotes the release of inflammatory factors from immune cells and that inflammation can in turn affect the Aβ level [71]. In the brain, the uptake and clearance of Aβ is mainly dependent on microglial phagocytosis. However, in this study, we did not observe any changes in the Aβ level with or without LPS treatment both in vivo and in vitro, suggesting that microglial phagocytosis was not affected in the short term. Theoretically, microglial phagocytosis of Aβ in the brain of APP/PS1 transgenic mice should be enhanced by persistent up-regulation of the corresponding receptor TLR4 [43]. However, as LPS exposure induced sustained and excessive inflammation, TREM2 suppression potentially led to a reduction in the ability of microglia to engulf Aβ [31, 72]. In this case, the opposing effects caused by the imbalance of TLR4 and TREM2 might counteract each other to some extent, resulting in no significant changes in the Aβ level in LPS-treated APP/PS1 transgenic mice. Thus, LPS-induced systemic inflammation might not be involved in the Aβ mechanism of the AD model mice.

Conclusions

Our in vitro and in vivo studies with LPS treatment demonstrated that the negative regulatory effect of TREM2 on inflammation might be potentially suppressed by LPS-induced hyperactive TLR4. This imbalance of TLR4/TREM2 contributes to microglial over-activation, followed by increased neuronal apoptosis in the cortex and consequently, acutely exacerbates the cognitive dysfunction of APP/PS1 transgenic mice. These results suggested that the superimposition of systemic inflammation with LPS could accelerate the progression of AD. Correspondingly, the imbalance of these two key regulators may be a potential link between AD and systemic inflammation. The finding provided the foundation for further in-depth mechanistic investigation. In a sense, TREM2 represents a potential therapeutic target for controlling systemic inflammation in AD progression, and related drugs need to be further explored.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no competing interests.

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