Resatorvid Modulates Microglial M1/M2 Polarization to Improve Cognitive Impairment after Repetitive Mild Traumatic Brain Injury

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Research

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Abstract

Background

In recent years, more and more attention has been paid to repetitive mild TBI (rmTBI), which can increase the incidence of chronic neurodegenerative disorders. Microglia are the main mediators of the innate immune response in the central nervous system (CNS), and their polarization phenotype plays a dual role in exerting beneficial and detrimental effects on neuroinflammation. This study investigated the mechanism and effect of Resatorvid (TAK242), a specific inhibitor of TLR4, on learning and memory in mice with rmTBI.

Methods

A controlled cortical impact (CCI) method was used to establish the mild TBI model in this study. Mice in the rmTBI model underwent four head impacts with a 24-hour interval between each impact.

Results

TAK242 treatment significantly reduced the expression levels of APP and p-Tau, promoted neurological recovery, and improved learning and memory after rmTBI. Furthermore, TAK242 promoted the polarization of microglia from the M1 to M2 phenotype, accompanied by the upregulation of anti-inflammatory factors and downregulation of pro-inflammatory factors. The inhibition of the TLR4/MyD88/NF-κB signalling pathway might be involved in the protective effect of TAK242 mentioned above.

Conclusions

TAK242 significantly inhibits the neuroinflammatory response by regulating microglial M1/M2 polarization, thereby improving cognitive function after rmTBI.

Background

Traumatic brain injury (TBI) is an important public health concern worldwide. According to the degree of brain injury and the symptomology of acute phase, TBI can be divided into mild, moderate and severe TBI. Mild TBI accounts for the vast majority of TBI (up to 80%) [1]. About 15% of patients still experience headache, dizziness, balance difficulties and other symptoms within 1–3 months after mTBI [2]. Indeed, over 22% of mTBI patients were still functionally impaired at 1year post injury [2]. Some studies have reported that Military personnel, athletes of full contact sports, and elderly people with reduced mobility are at higher risk of exposure to repetitive mild TBI (rmTBI),which can increase the incidence of chronic neurodegenerative disorders, such as Alzheimer's disease and chronic traumatic encephalopathy (CTE)

[3–5]. Persistent neuroinflammation occurs following rmTBI and promotes the accumulation of pathological proteins, which is a major mechanism of pathogenesis leading to chronic neurodegenerative diseases [6]. In recent years, more and more attention has been paid to the above-mentioned populations. Therefore, it is of great significance to explore new strategies to inhibit chronic neuroinflammation and improve the prognosis and cognitive function after rmTBI.

Microglia are the main mediators of the innate immune response in the central nervous system (CNS) and play a key role in neuroinflammation and secondary injury after TBI. Under physiological conditions, microglia are in an inactive "resting" state (M0) [7]. After TBI, microglia are activated, and two polarization states have been identified: M1 and M2 [8]. Significant differences in the roles of the two phenotypes of activated microglia have been documented: M1 microglia release pro-inflammatory cytokines and chemokines and exert pro-inflammatory and cytotoxic effects, and M2 microglia release anti-inflammatory cytokines and neurotrophic factors, and are important for suppressing neuroinflammation and promoting tissue remodelling [8–9]. As shown in our previous study, microglial activation occurs as a bimodal phenomenon with an initial peak of microglial with the M2 phenotype at 1 week and a secondary peak of microglia with the M1 phenotype at 4 weeks after rmTBI, which causes continuous, chronic immune and inflammatory reactions to promote cognitive impairment [6]. Therefore, the inhibition of the M1 phenotype and induction of the M2 phenotype are more feasible methods to inhibit neuroinflammation rather than simply inhibiting microglial activation. In summary, strategies regulating the polarization of microglia from the M1 phenotype to the M2 phenotype to reduce secondary brain injury are an important target for improving the prognosis and cognitive function after TBI.

Toll-like receptors (TLRs) are a family of highly conserved natural pattern recognition receptors (PRRs) that are considered a "bridge" between acquired immunity and innate immunity and play an important role in the development of neurodegenerative diseases. As we all know, TLR4 plays a key role in inflammation and mitochondrial DNA damage in trauma, shock and sepsis [10]. With the deepening of research, it is found that TLR4 is expressed at high levels in microglia and plays a vital role in regulating microglial activation and inflammatory responses after TBI [11]. Inhibition of the TLR4/NF-κB signalling pathway inactivates the NLRP3 inflammasome and increases the number of microglia with the M2 phenotype by shifting from an M1 phenotype, thereby improving neurological deficits in mice with ischaemic stroke [12]. Therefore, TLR4 is a key target for regulating immune and inflammatory reactions in the central nervous system. Resatorvid (TAK242), a small-molecule inhibitor of Toll-like receptor (TLR) 4 signalling, binds selectively to TLR4 [13]. Studies have confirmed that TAK242 penetrates the blood-brain barrier, inhibits microglial activation and exerts a neuroprotective effect on central nervous system diseases [14–15]. However, the effect of TAK242 on long-term cognitive impairment after TBI is less frequently reported.

Based on the research described above, we observed changes in neuroinflammation and pathological proteins in the chronic phase of rmTBI in mice, which were intraperitoneally injected with TAK242. The present study was designed to explore whether TAK242 is capable of improving prognosis and cognitive function after TBI by modulating the polarization of microglia from the M1 to the M2 phenotype.

Materials And Methods

Animals and rmTBI model

Adult male C57BL/6J mice (age: 10–12 weeks; weight, 20–25 g; Institute of Experimental Animals of the Chinese Academy of Medicine, Beijing, China) were used in this study. Animal care and experimental protocols were reviewed and approved by the Animal Research and Ethics Committee of Tianjin Medical University. The mice were housed at a temperature of 20 °C to 22 °C, humidity of 40% to 70% and a 12-h light/dark cycle. All mice ate and drank ad libitum prior to and following surgery.

A controlled cortical impact (CCI) method was used to establish the rmTBI model in this study [16]. The mice were anaesthetized with 4.6% isoflurane and then positioned in a stereotaxic frame using ear bars. A 3.0 mm diameter craniotomy was performed over the right parietal cortex between the right coronary suture and the herringbone suture and 2 mm lateral to the midline suture. Mild TBI was induced using a CCI device, and injury parameters were set to a controlled velocity of 3 m/s, depth of 1 mm and dwell time of 200 ms. Mice in the sham-operated group were anaesthetized, and a craniotomy was performed without CCI. Mice in the rmTBI group underwent four head impacts with a 24-hour interval between each impact [17]. After trauma or sham surgery, the mice were housed in separate cages until their consciousness was restored.

Experimental groups and treatment

Mice were randomly assigned to three groups (n=48 per group): the sham group,

rmTBI+PBS, and rmTBI+TAK242 groups. TAK242 was dissolved in DMSO and diluted with PBS to a final concentration of 0.4 mg/ml. The rmTBI+TAK242 groups received intraperitoneal injections of TAK242 (3 mg/kg) at 6 h, 1 day, 3 days, 7 days, 14 days, and 21 days post-last impact. The rmTBI+PBS groups received injections with equal volumes of DMSO/PBS. All time points used for detection started from the fourth strike. Mice were euthanized at 3 and 28 days following rmTBI to evaluate the levels of TLR4 and downstream signalling proteins, the polarized phenotype of microglia, inflammatory factors, and pathological proteins. Mice were evaluated for neurological function with the mNSS on days 1, 3, 7, 14, and 21 after rmTBI, and the cognitive function of mice was evaluated using the Morris water maze (MWM) at 28 days after rmTBI (Fig. 1).

Modified neurological severity score (mNSS) test

Each group of mice was evaluated for neurological function using the mNSS, including sensory tests, motor tests, reflexes, and balance tests. Mice were assessed with the mNSS prior to injury and on days 1, 3, 7, 14, and 21 after rmTBI to explore the cumulative effects of repeated injury on neurological function. A higher score indicates a more severe neurological deficit (normal score, 0; maximum score, 18).

Morris water maze (MWM)

The MWM test was conducted in mice to evaluate spatial learning and reference memory, as described previously [18]. The opaque water-filled pool (diameter, 122 cm; height, 55 cm) was divided into four equal quadrants and maintained at a temperature of 22 ± 2 °C. A target platform was placed in the centre of the northeast quadrant, submerged 2 cm below the water surface. The place navigation test started 28 days after rmTBI and was conducted for 5 consecutive days. The mice were placed in the pool at one of four fixed starting points and allowed to swim freely for 90 s or until they reached and stayed on the target platform. Each mouse was trained four times per day at 20-minute intervals. The time to reach the hidden platform (escape latency) was recorded and analysed by a video tracking system, and the values of 4 trials were averaged. A spatial probe test was performed 24 h after the last acquired training session. The platform was removed from the maze, and the mice were allowed to swim freely for 90 s. The swimming path and speed of the mouse, the number of target quadrants crossed, and the percentage of dwell time in the target quadrant were recorded and measured.

Tissue preparation

For immunofluorescence staining, the mice were sacrificed by transcardial perfusion with cold PBS at 3 and 28 days after rmTBI. The brain tissue was harvested on ice and then postfixed with 4% paraformaldehyde for 24 hours, followed by an incubation with 30% sucrose for 48 hours. After fixation, tissues were embedded in the optimum cutting temperature medium (Sakura, Torrance, CA, USA) on dry ice. Brain tissue at the injury area was sliced into 8 μm coronal sections using a -20℃ frozen microtome.

For western blotting and ELISA, the mice were sacrificed using the same method described above. The injured cerebral cortex with a 5-mm diameter and injured hippocampus were isolated immediately and combined. The separated brain tissue was stored in liquid nitrogen after removing microvessels for protein extraction.

Immunofluorescence staining

Immunofluorescence staining was performed to observe the expression of TLR4 and the polarization of microglia. After air drying, the sections were incubated with PBS containing 3% BSA for 30 min at 37 °C to block nonspecific staining. Then, sections were incubated with the primary antibody (anti-TLR4 antibody, 1:500, GeneTex, USA) overnight at 4 °C, followed by an incubation for 1 h at 37 °C with an appropriate secondary antibody. The nuclei were counterstained with DAPI. For the quantitative analysis, digital images of sections were captured under an immunofluorescence microscope (ZEISS, Germany) and quantified by a researcher who was blinded to the experiment. Five separate slides were selected from each brain sample. Digital images of positively labelled cells in three randomly selected 400× fields in each slide from the lesion boundary were captured. The lesion boundary was defined as the region surrounding the centre of the injury (0.5–1.5 mm to injured core), as previously reported. The mean number of positively labelled cells per visual field of each slide was counted for statistical analyses.

Double-label immunofluorescence staining for TLR4-Iba-1 and CD16/32-CD206 was performed to further observe the expression of TLR4 on microglia and the M1/M2 polarization of microglia. After blocking

with BSA, the sections were incubated overnight at 4 °C with anti-TLR4 antibody (1:500, GeneTex, USA) and anti-Iba-1 antibody (1:500, Abcam, MA, USA) or anti-CD16/32 antibody (1:100, Abcam, MA, USA) and anti-CD206 antibody (1:100, Abcam, MA, USA). The remaining steps were the same as described above.

Western blotting

Western blots were performed using a previously reported method [19]. The cerebral cortex and hippocampus on the injured side were separated and stored in liquid nitrogen until use. Total proteins were extracted, and then the protein concentration was measured using a BCA protein assay kit (Solarbio Science, Beijing, China). Protein samples were separated by SDS-PAGE and subsequently transferred onto PVDF membranes (Millipore, Bedford, MA). The blots were blocked with 5% non-fat milk for 2 h at room temperature, then immersed overnight at 4 ℃ in a solution containing the primary antibodies: anti-TLR4 (1:500, GeneTex, USA), anti-APP (1:500, CST, USA), anti-p-Tau(1:500, CST, USA), anti-MyD88 (1:1000, CST, USA), anti-NF-κB p65 (1:1000, CST, USA), anti-CD16/32 (1:1000, Abcam, MA, USA), and anti-CD206(1:1000, Abcam, MA, USA). Afterwards, membranes were washed with TBST and incubated for 1 h at room temperature with HRP goat anti-mouse and HRP goat anti-rabbit secondary antibodies (all diluted 1:5000, ZhongShan, Beijing, China). The blots were treated with enhanced chemiluminescence (ECL) reagents and visualized with a ChemiDoc^{TM} XRS + Image System (Bio-Rad, CA, USA). The intensity of each band was analysed using ImageJ software.

ELISA

The frozen brain tissue was weighed, homogenized in RIPA buffer containing protease inhibitors, and subsequently centrifuged for 20 minutes at 13,000 rpm at 4 ℃. The protein content of the supernatant was detected using the BCA Protein Assay Kit (Solarbio Science, Beijing, China). The protein levels of inflammatory mediators in all samples, including TNF-α, IL-10, IL-4 and IL-1β, were measured using ELISA kits (SBJ, Nanjing, China) according to the manufacturer's protocols. The optical density (OD) of each well was measured at 450 nm with a microplate reader (Thermo Forma, USA). After generating a standard curve, the OD value of the protein sample was substituted into the standard curve, and the actual concentration of the protein was quantified.

Statistical analyses

All data were obtained from at least three independent experiments. The data are presented as the means ± SD and were analysed using SPSS 17.0 software. For comparisons among multiple groups, one-way ANOVAs followed by Fisher's LSD post hoc test, were used to analyse the data. The results from the place navigation test in the MWM were compared using repeated measures ANOVAs. Differences between the two groups were analysed using unpaired t-tests. Statistical significance was set to $p< 0.05$.

Results

TAK242 inhibited the expression of TLR4 after rmTBI

We performed immunofluorescence staining for TLR4 to confirm that an intraperitoneal injection of TAK242 inhibited the expression of TLR4 after rmTBI. Microphotographs of TLR4 expression in each group are shown in Fig. 2A. The quantitative comparison revealed a significant increase in the expression of TLR4 around the lesion in the rmTBI + PBS group compared to the sham group at 3 and 28 days after rmTBI ($^{###}p < 0.001$; Fig. 2B). However, a significant reduction in TLR4 expression was observed in the rmTBI + TAK242 group compared to the sham group ($^{***}p < 0.001$; Fig. 2B). Based on these data, TAK242 effectively inhibited the expression of TLR4 in the acute and chronic phases of rmTBI.

TAK242 treatment reduced neurological deficits and improved cognitive function after rmTBI

The neurological deficit of rmTBI mice was evaluated using the mNSS comprising five aspects: motor function, sensory function, reflexes, epilepsy and myoclonus. Compared with the sham group, the mNSS score of mice in the rmTBI + PBS group increased significantly ($^{##}p < 0.01$; Fig. 3A). Moreover, the mNSS score of mice in the rmTBI + PBS group peaked at 1 day after rmTBI and then gradually decreased. The mNSS score of mice in the rmTBI + PBS group showed that neurological function recovered gradually with the prolongation of brain injury (Fig. 3A). The mNSS score of the rmTBI + TAK242 group was lower than the rmTBI + PBS group on days 1 and 3 after rmTBI, but the difference was not statistically significant ($p > 0.05$; Fig. 3A). Moreover, the mNSS score was obviously decreased in the rmTBI + TAK242 group at 7, 14, and 21 days after rmTBI compared with the rmTBI + PBS group ($^{*}p < 0.05$; Fig. 3A). TAK242 might promote the recovery of neurological deficits in mice after rmTBI.

Beginning at 28 days after rmTBI, the MWM was used to evaluate the spatial learning and memory ability of mice for 6 consecutive days. The escape latency, indicating the ability of mice to find a hidden platform, gradually decreased from 28 to 32 d after rmTBI, indicating that spatial memory was established. The mice in the rmTBI + PBS group showed a more obvious learning deficit than the sham group on days 3, 4 and 5 of training ($^{#}p < 0.05$ for day 3; $^{##}p < 0.01$ for days 4 and 5; Fig. 3B-C). However, compared with the rmTBI + PBS group, the escape latency was significantly shorter in the rmTBI + TAK242 group beginning on day 4 ($^{*}p < 0.05$ for days 4 and 5; Fig. 3B-C). In the spatial probe test, the number of times the animal passed over the previous location of the target platform and the percentage of time spent in the target quadrant in the rmTBI + PBS group were significantly lower than in the sham group ($^{#}p < 0.05$; Fig. 3B, D, and E). These two parameters were significantly increased in the rmTBI + TAK242 group compared to the rmTBI + PBS group ($^{*}p < 0.05$; Fig. 3B, D, and E). Thus, TAK242 improved memory and learning deficits in the chronic phase of rmTBI.

TAK242 treatment suppressed the expression of pathological proteins after rmTBI

The deposition of amyloid-β peptide in the cerebral cortex is a key factor contributing to the pathogenesis of neurodegeneration. APP is the precursor of β-amyloid protein, and its expression increases after TBI [20]. Under pathological conditions, the Tau protein is hyperphosphorylated to form neurofibrillary tangles, which leads to an abnormal cytoskeletal structure in neurons and finally results in cognitive impairment [21]. We detected the levels of the pathological proteins APP and p-Tau in the hippocampus

and cerebral cortex using Western blot analysis (Fig. 4A). At 3 and 28 d postinjury, the expression of the APP and p-Tau proteins was significantly increased in the rmTBI + PBS group compared with the sham group ($^{###}p < 0.001$; Fig. 4B-C). Furthermore, the administration of TAK-242 significantly reduced the expression of the APP and p-Tau proteins compared to the rmTBI + PBS group ($^{***}p < 0.001$ and $^{**}p < 0.01$; Fig. 4B-C). Taken together, a series of pathological changes associated with secondary brain injury after rmTBI promote the expression of APP and the hyperphosphorylation of the Tau protein, which leads to a decline in cognitive function. Moreover, the TAK242 treatment reduced the expression of pathological proteins and improved cognitive function.

TAK242 treatment regulated microglial M1/M2 polarization and the release of inflammatory cytokines after rmTBI

We measured the ratio of M1/M2 microglia in this study to determine whether TAK242 regulates the polarization state of microglia in the acute and chronic phases after rmTBI. CD16 and CD206 are specific membrane proteins and were visualized as biomarkers of M1 and M2 microglia, respectively [22], as determined using western blot analysis (Fig. 5A). Based on the results of the quantitative analysis, a significantly higher ratio of CD16/CD206 was detected in the rmTBI + PBS group than in the sham group ($^{###}P < 0.001$; Fig. 5B), and this ratio was decreased significantly upon treatment with TAK242 at 3 d and 28 d after rmTBI ($^{***}p < 0.001$; Fig. 5B). This result further confirms that microglia are polarized into two different phenotypes, M1 and M2, during the acute and chronic phases of rmTBI and is consistent with previous studies[6]. Furthermore, microglia were continuously polarized towards the M1 phenotype in the chronic phase of rmTBI, while TAK242 promoted microglial polarization from the M1 to the M2 phenotype by reducing the ratio of M1/M2 microglia after rmTBI.

A persistent neuroinflammatory response occurs after TBI that is characterized by a rapid increase in cytokine and chemokine levels [5]. We measured the levels of pro-inflammatory cytokines (TNF-α and IL-1β) and anti-inflammatory cytokines (IL-4 and IL-10) using ELISAs to evaluate the effects of TAK242 on inflammatory cytokines after rmTBI. Compared with the sham group, the levels of TNF-α and IL-1β were increased significantly at 3 and 28 days after rmTBI ($^{###}P < 0.001$, Fig. 5C-D), while the levels of IL-4 and IL-10 were also increased significantly at 3 days after rmTBI, but subsequently decreased significantly at 28 days after rmTBI in the rmTBI + PBS group ($^{##}P < 0.001$; Fig. 5E-F). In contrast, TAK242 substantially reduced the levels of TNF-α and IL-1β and increased the levels of IL-4 and IL-10 compared with the rmTBI + PBS group at 3 and 28 days after rmTBI ($^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$; Fig. 5C, D, E, and F). Thus, TAK242 exerted anti-inflammatory effects after rmTBI.

In summary, TAK242 promotes the polarization of microglia from the M1 to M2 phenotype, thereby promoting the release of anti-inflammatory factors and inhibiting the release of pro-inflammatory factors.

TAK-242 treatment downregulates the expression of signalling molecules downstream of TLR4 after rmTBI.

Immunofluorescence staining was performed to measure the colocalization of Iba-1 and TLR4 and to further investigate the molecular signalling mechanism by which TAK242 promotes microglial polarization to the M2 phenotype and inhibits neuroinflammation (Fig. 6A), and western blotting was performed to detect the levels of TLR4 and its downstream signalling proteins MyD88 and NF-κB (Fig. 6C). TLR4 plays important roles in innate defence mechanisms and efficient clearance of damaged tissues [23]. Furthermore, TLR4 has consistently been shown to be involved in the activation of neuroglia [23–24]. Iba-1 is considered a biomarker of microglia. As shown in Fig. 6A-B, a large amount of TLR4 was localized to microglia and its expression was obviously increased in the rmTBI + PBS group ($^{###}p < 0.001$ compared with the sham group), whereas TLR4 expression was markedly decreased in the rmTBI + TAK242 group compared with the rmTBI + PBS group ($^{***}p < 0.001$ and $^{*}p < 0.05$) at 3 and 28 days after rmTBI. As shown in Fig. 6C-D, the expression of TLR4, MyD88 and NF-κB p65 was significantly elevated in the rmTBI + PBS group ($^{###}p < 0.001$ compared with the sham group), and these changes were obviously inhibited by TAK242 ($^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$ compared with the rmTBI + PBS group). Overall, our study suggested that TAK242 inhibits the TLR4/MyD88/NF-κB signalling pathway.

Discussion

Research status of rmTBI and TAK242

TBI has high incidence, disability, and fatality rates and is generally prevalent in people of all ages [25]. Increasing evidence suggests that rmTBI is more likely to cause long-term cognitive impairment [17, 26]. Cognitive dysfunction in the chronic phase after rmTBI is a hot topic in the field of brain trauma. Therefore, studies exploring the pathological mechanism of and intervention measures for long-term cognitive impairment in individuals with rmTBI are clinically important. Immune/inflammatory responses play a crucial role in the mechanism of secondary injury after TBI [27]. Microglia, as the main mediator of the innate immune response in the central nervous system, are the first line of defence against damage to the central nervous system and play a key role in the neuroinflammatory mechanism of secondary injury after TBI [28]. The deposition of Aβ and hyperphosphorylated Tau (p-Tau) proteins is a key pathological feature of Alzheimer's disease (AD) that is necessary for the neuropathological diagnosis of AD [29]. The microglia-induced inflammatory response interacts with the clearance of Aβ and internalization and degradation of Tau [30–31]. High expression of TLR4 in microglia regulates innate immune/inflammatory responses [10], and thus strategies targeting TLR4 are considered to regulate immune/inflammatory responses and effectively improve cognitive function after rmTBI.

TAK242, a specific inhibitor of TLR4, selectively binds to Cys747 in the intracellular domain of TLR4 and disrupts the interaction of TLR4 with adaptor molecules [12]. TAK242 has been shown to pass through the BBB, inhibit neuroinflammation, and exert neuroprotective effects on cerebral ischaemia-reperfusion injury [14]. In addition, TAK242 promotes neurological recovery by attenuating cerebral oedema in individuals with subarachnoid haemorrhage [32]. Importantly, TAK242 has been used in clinical studies of sepsis and was proven to be safe for humans [33]. TAK242 represents a novel therapeutic strategy for the

treatment of nervous system disease. However, researchers have not clearly determined whether TAK242 improves the prognosis and cognitive dysfunction after rmTBI.

Focus on the effect and mechanism of TAK242 on long-term cognitive impairment after rmTBI

In the current study, we intraperitoneally injected TAK242 into rmTBI mice to explore whether the prognosis and cognitive function were improved. Through pathological observations and behavioural testing, we found that the TAK242 treatment significantly reduced the expression levels of APP and p-Tau, promoted neurological recovery, and improved learning and memory after rmTBI. Based on previous studies, we hypothesized that the molecular mechanism by which TAK242 improves cognitive function is related to the inflammatory response induced by microglial activation.

Based on accumulating evidence, microglia are a key component of chronic neuroinflammation and degenerative processes after rmTBI [25, 34]. Neuroinflammation is considered to exert both detrimental and beneficial effects, which are related to microglial polarization towards the M1 or M2 phenotype. The release of pro-inflammatory mediators by activated microglia further promotes microglial polarization towards the M1 phenotype, resulting in a progressive, chronic cycle of neuroinflammation, which aggravates neuropathological damage and thereby drives degeneration [35, 36]. Additionally, significant benefits have been achieved when M2 microglia are activated to suppress the inflammatory response [35, 36]. In the present study, the numbers of both pro-inflammatory M1 and anti-inflammatory M2 microglial cells were increased, accompanied by the upregulation of inflammatory mediators, including TNF-α, IL-1β, IL-4, and IL-10, 3 days after rmTBI, indicating that the M2 phenotype is transiently activated to protect against inflammatory damage in the acute stage after rmTBI. However, microglia with a predominant M1 phenotype were observed 28 days after rmTBI, along with an increase in the levels of the pro-inflammatory mediators TNF-α and IL-1β and a reduction in the levels of the anti-inflammatory mediators IL-4 and IL-10, suggesting that persistent inflammation occurs in the chronic stage after rmTBI and that the changes in inflammatory factors are consistent with microglial M1/M2 polarization. These results are consistent with previous studies. Furthermore, our results confirmed that TAK242 significantly reduced the ratio of M1/M2 microglia in the acute and chronic stages after rmTBI, indicating that TAK242 promoted microglial polarization towards the M2 phenotype and inhibited M1 polarization. TAK242 also markedly stimulated the release of IL-4 and IL-10 and decreased the release of TNF-α and IL-1β. In summary, we speculated that TAK242 promoted tissue repair, reduced the accumulation of pathological proteins, and ultimately improved long-term cognitive function after rmTBI probably by inducing the pro-inflammatory M1 phenotypic switch to the anti-inflammatory M2 phenotype and attenuating the inflammatory response.

TLR4, a key PRR expressed on the surface of microglia, participates in the pathological process of secondary injury after TBI and plays an important role in the initiation and regulation of immunity/inflammation [37–38]. Immunofluorescence staining was performed to measure the localization of Iba-1 and TLR4 and to ascertain whether the TAK242 treatment microglial polarization through changes in TLR4 expression. We observed a significant increase in the expression of TLR4 on

microglia in the acute and chronic stages after rmTBI. However, the administration of TAK242 significantly reduced the expression of TLR4 on microglia. Based on these findings, we speculated that TLR4 might play an essential role in the regulation of microglial polarization and further rescue cognitive deficits after TAK242 treatment in rmTBI mice.

TLR4 signalling pathways include MyD88-dependent and MyD88-independent pathways [39–40]. MyD88 is the central adaptor protein involved in the inflammatory response [22]. TLR4 and MyD88 signalling pathways activate downstream IKK, resulting in the nuclear translocation of NF-κB, which triggers and regulates the expression of pro-inflammatory cytokines [39–40]. NF-κB is expressed in almost all cells and plays an important role in the neuroinflammatory response caused by M1-type microglia [41]. Ye et al reported that TLR4/MyD88/NF-κB signalling pathways are activated to suppress neurogenesis after TBI [42]. The data obtained from the present study are consistent with previous studies, confirming that the expression of TLR4, MyD88 and NF-κB is upregulated to stimulate the release of pro-inflammatory factors and induce neurodegeneration after rmTBI. Additionally, the TAK242 treatment suppressed the expression of TLR4, MyD88, and NF-κB P65, proteins involved in TLR4 signalling pathways. Collectively, in the canonical pathways, activation of TLR4/MyD88/NF-κB signalling may be a fundamental step in the M1-type microglia-induced inflammatory response. Therefore, we speculated that inhibition of the TLR4/MyD88/NF-κB signalling pathway might be involved in TAK242-mediated regulation of microglial polarization after rmTBI.

In subsequent studies, we will further explore the central nervous system concentration of TAK242 and its different effects on the long-term cognitive function of a mouse model of rmTBI through repeated administration and single administration. Moreover, we will identify adverse effects after repetitive drug delivery, which will provide a theoretical basis for the clinical application of TAK242.

Conclusions

Our results provide new insights into the role of TAK242 in improving cognitive impairment after rmTBI. TAK242 promotes motor function recovery and effectively improves learning and memory, probably by regulating microglial polarization from the M1 to M2 phenotype and inhibiting the inflammatory response after rmTBI (Fig. 7). Furthermore, we hypothesized that these effects might be associated with inhibition of the TLR4/MyD88/NF-κB signalling pathway. In summary, inhibiting TLR4 to regulate microglia polarization may be a key target to improve cognitive function and the development of neurodegenerative diseases after rmTBI, which provide a new strategy for the treatment of rmTBI.

Abbreviations

TBI: Traumatic brain injury; rmTBI: repetitive mild TBI; CNS: central nervous system; TLR4: Toll-like receptor 4; TLRs: Toll-like receptors; PRRs: pattern recognition receptors; CCI: controlled cortical impac; MWM: Morris water maze; mNSS: Modified neurological severity score; AD: Alzheimer’s disease; p-Tau: hyperphosphorylated Tau;

CTE: chronic traumatic encephalopathy.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors have no conflicts of interest to declare.

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Authors' contributions

SSZ, SH, FW, YYZ,DW, ZLH, DL, JZ and YFW performed the experiments. FC was responsible for technical support. XDK were in charge of methodology. SSZ and SH explained the results and created the figures.FW performed data statistical analysis. SSZ completed the manuscript.XTG and PL provided experimental design and revised the manuscript.

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Declarations

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Authors' contributions

SSZ, SH, FW, YYZ,DW, ZLH, DL, JZ and YFW performed the experiments. FC was responsible for technical support. XDK were in charge of methodology. SSZ and SH explained the results and created the figures.FW performed data statistical analysis. SSZ completed the manuscript.XTG and PL provided experimental design and revised the manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

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Competing interests

The authors have no conflicts of interest to declare.

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Figures

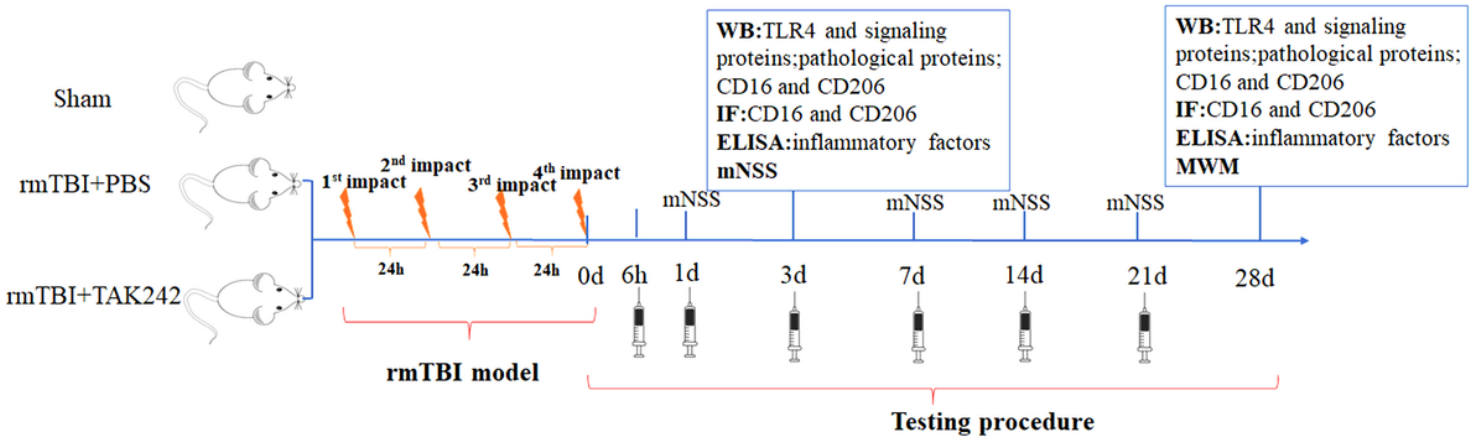

Figure 1

Schematic diagram of biochemical analyses and neurobehavioural tests of rmTBI mice at different time points.

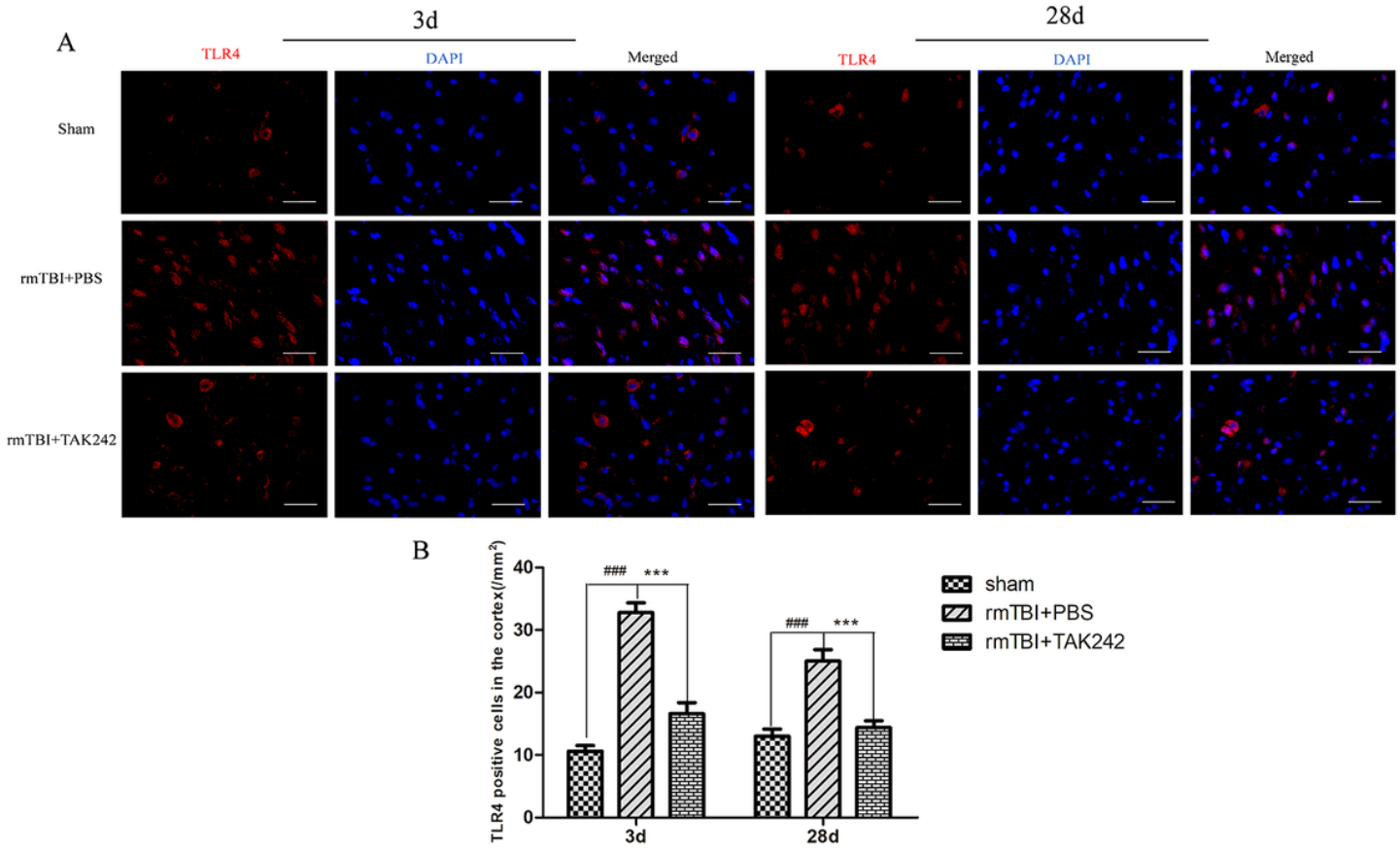

Figure 2

Detection of TLR4 expression using immunofluorescence staining. (A) TLR4 immunofluorescence staining was performed at 3 and 28 days after rmTBI (red, TLR4; blue, nuclei; scale bars: 50 μm). (B) The number of TLR4-positive cells in the injured cerebral cortex was quantitatively compared among the sham group, the rmTBI+ PBS group and the rmTBI+TAK242 group. The expression of TLR4 was significantly increased after rmTBI, whereas TLR4 expression was remarkably reduced after TAK242

treatment at 3 and 28 days postinjury (n=6; ###p < 0.001: the rmTBI+ PBS group compared with the sham group, ***p < 0.001: the rmTBI+TAK242 group compared with the rmTBI + PBS group).

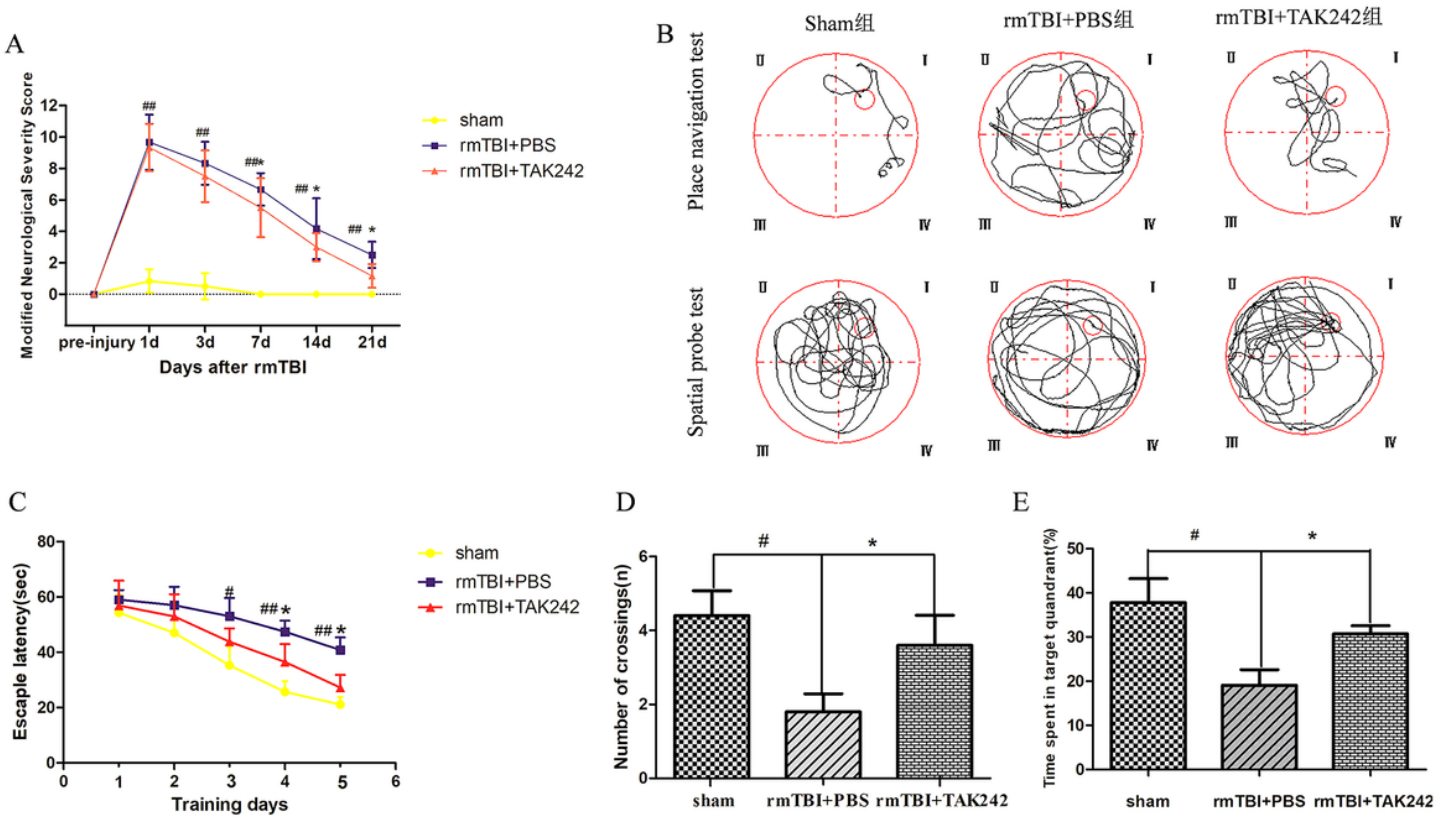

Figure 3

Evaluation of sensorimotor function using the mNSS and learning and memory ability using the MWM. (A) Compared with the sham group, the mNSS score of mice in the rmTBI+PBS group increased significantly(##p < 0.01 compared with the sham group),peaked at 1 day after rmTBI, and then gradually decreased. The mNSS score of the rmTBI+TAK242 group showed no statistically significant decrease at 1 and 3 days after rmTBI but was obviously decreased at 7, 14, and 21 days after rmTBI (p>0.05 and *p<0.05, respectively, compared with the rmTBI+PBS group). (B) Representative training and spatial probe traces of mice in the sham group, the rmTBI+PBS group and the rmTBI+TAK242 group on the fifth day. (C) The escape latency of the rmTBI+PBS group was delayed significantly beginning on day 3 of training (##p < 0.01 and #p < 0.05 compared with the sham group). The TAK242 treatment obviously shortened the time to find the platform beginning on day 4 of training (*p < 0.05 compared with the rmTBI+PBS group). (D) and (E) The number of target platform crossings and the percentage of time spent in the target quadrant were significantly decreased in the rmTBI+PBS group (#p < 0.05 compared with the sham group) and were significantly increased in the rmTBI+TAK242 group (*p < 0.05 compared with the rmTBI + PBS group).

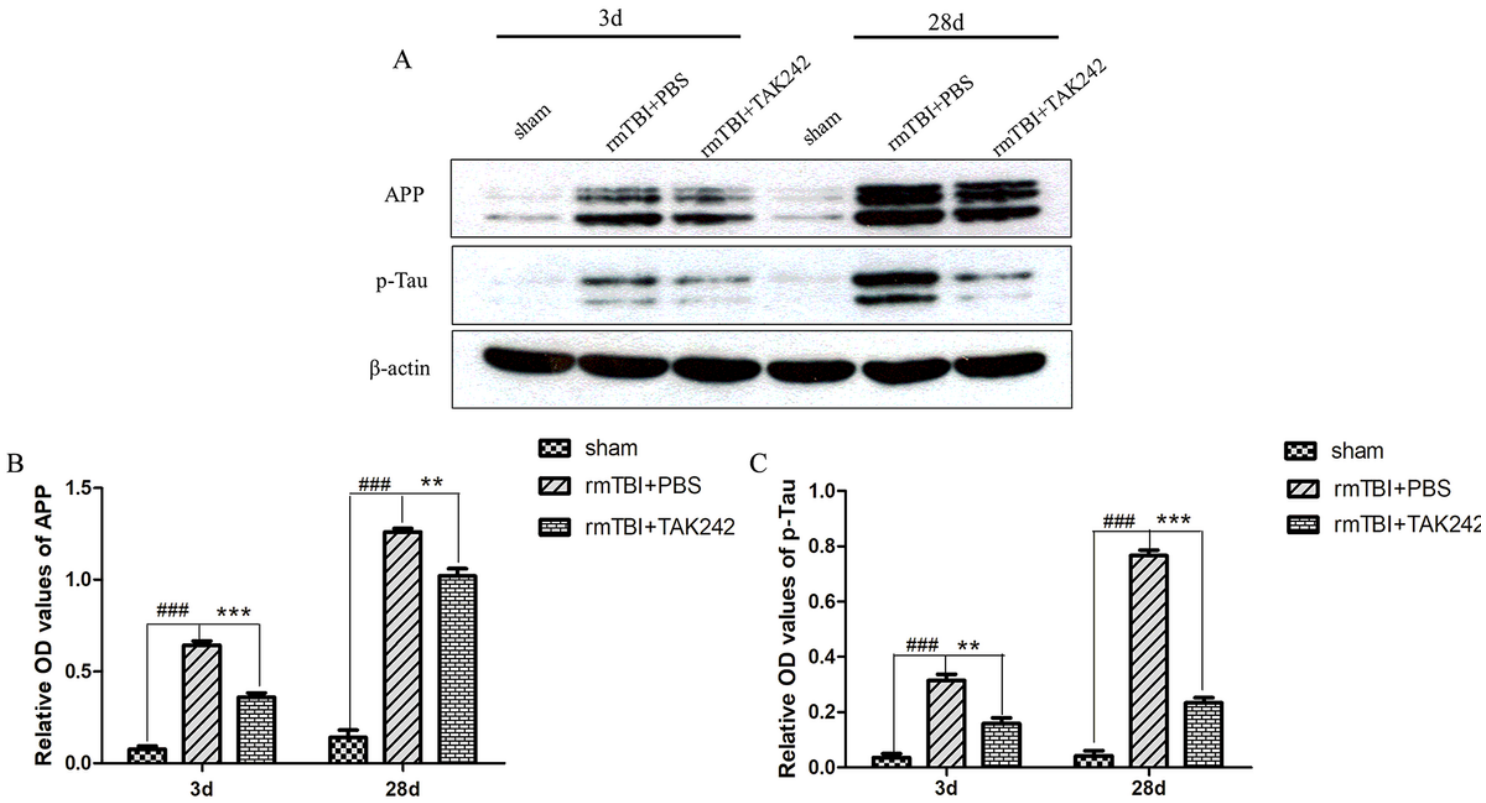

Figure 4

The expression levels of APP and p-Tau in the brain. (A) western blot analysis showing the levels of APP and p-Tau at 3 and 28 days after rmTBI. (B) The levels of APP and p-Tau were increased remarkably in the rmTBI+PBS group compared with the sham group at 3 and 28 days after rmTBI, but decreased remarkably after treatment with TAK242 compared with the rmTBI+PBS group (n = 6; ###p < 0.001: the rmTBI + PBS group compared with the sham group, ***p<0.001 and **p<0.01: the rmTBI+TAK242 group compared with the rmTBI + PBS group).

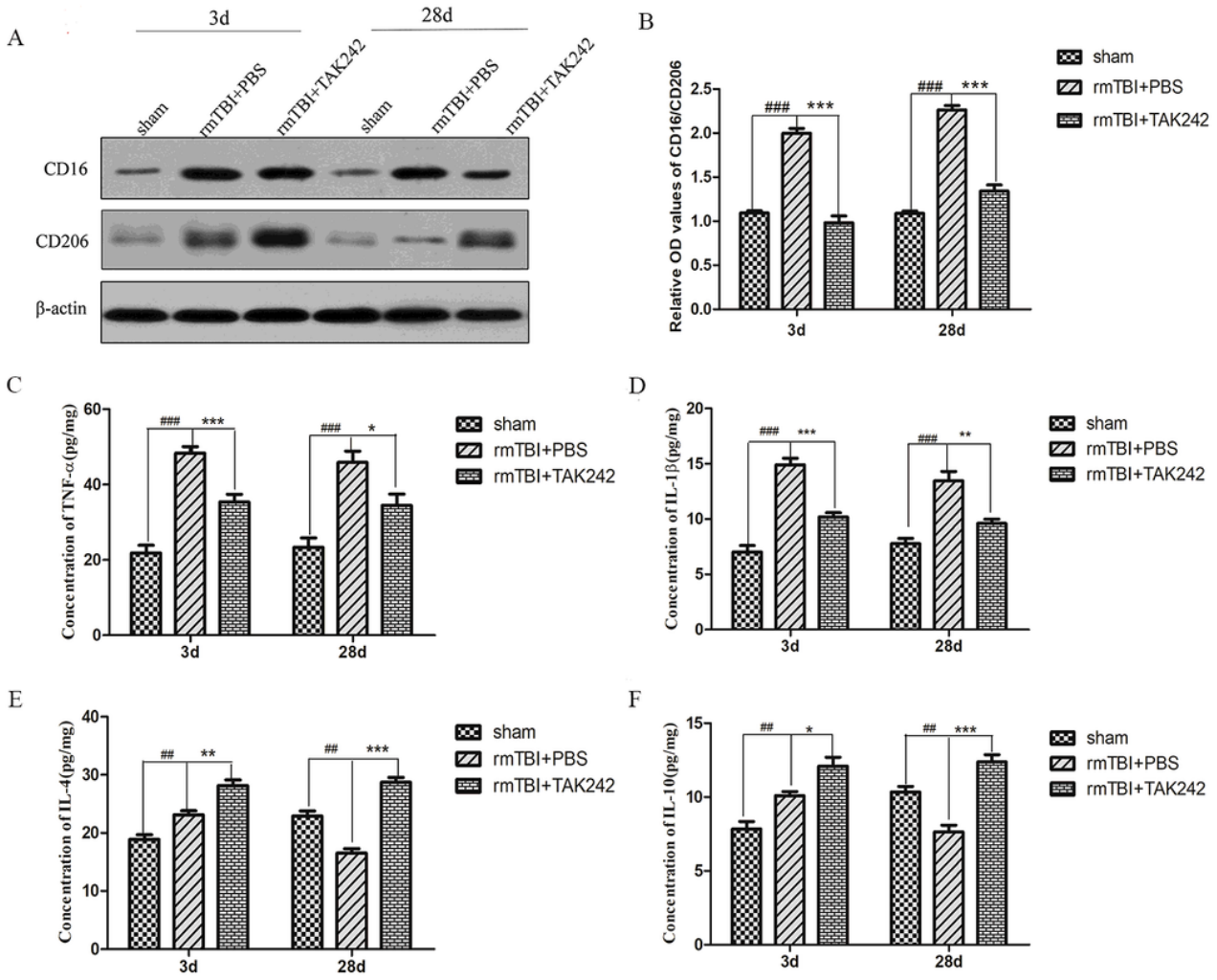

Figure 5

Observation of microglial M1/M2 polarization and measurements of the levels of inflammatory cytokines. (A) western blot showing the levels of CD16 (M1 marker) and CD206 (M2 marker) as indicators of the M1/M2 polarization of microglia at 3 and 28 days after rmTBI. (B) The ratio of CD16/CD206 was significantly higher in the rmTBI+PBS group than in the sham group (n=6, ###P<0.001: the rmTBI + PBS group compared with the sham group), and this ratio was decreased significantly upon treatment with TAK242 at 3 d and 28 d after rmTBI (***p<0.001: the rmTBI+TAK242 group compared with the rmTBI + PBS group). (C)-(F) We measured the levels of inflammatory cytokines using ELISAs. (C) and (D) The concentrations of TNF-α and IL-1β in each group. At 3 d and 28 d postinjury, the levels of the pro-inflammatory cytokines TNF-α and IL-1β were significantly increased in the rmTBI+PBS group (###P<0.001 compared with the sham group), but were obviously reduced after the TAK242 treatment (***p<0.001, **p<0.01, and *p<0.05 compared with the rmTBI + PBS group). (E) and (F) The concentrations of IL-4 and IL-10 in each group. The d levels of the anti-inflammatory cytokines (IL-4 and

IL-10) were increased significantly at 3 days after rmTBI and subsequently decreased significantly at 28 days after rmTBI in the rmTBI+PBS group (##P<0.01 compared with the sham group), but were obviously increased in the rmTBI+TAK242 group (***p<0.001, **p<0.01, and *p<0.05 compared with the rmTBI + PBS group).

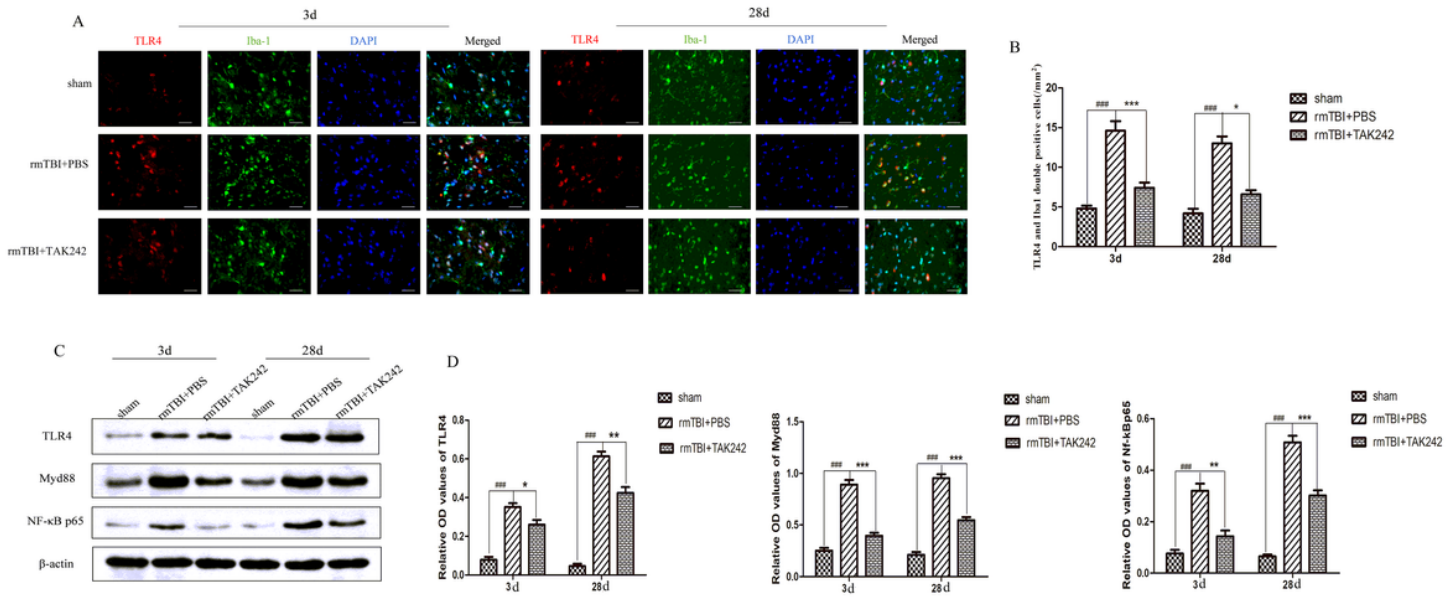

Figure 6

The expression of TLR4 signalling pathway-related molecules. (A) Immunofluorescence staining was performed to observe TLR4-positive cells colocalized with Iba-1 (red, TLR4; green, Iba-1; blue, nuclei; scale bars: 50 μm). (B) Quantification of cells positive for both TLR4 and Iba-1. The statistical analysis showed that TLR4 expression in microglia was significantly upregulated after rmTBI, but TAK242 administration reduced the expression of TLR4 in microglia at 3 and 28 days after rmTBI (###P<0.001: the rmTBI + PBS group compared with the sham group, ***p<0.001 and *p<0.05: the rmTBI+TAK242 group compared with the rmTBI + PBS group). (C) TLR4, MyD88 and NF-κB p65 were detected using western blotting. (D) The TLR4 signalling pathway was activated following rmTBI, whereas TAK242 reduced the expression of TLR4 and the downstream signalling molecules MyD88 and NF-κB at 3 and 28 days after rmTBI (###P<0.001: the rmTBI + PBS group compared with the sham group, ***p<0.001, **p<0.01, and *p<0.05: the rmTBI+TAK242 group compared with the rmTBI + PBS group).

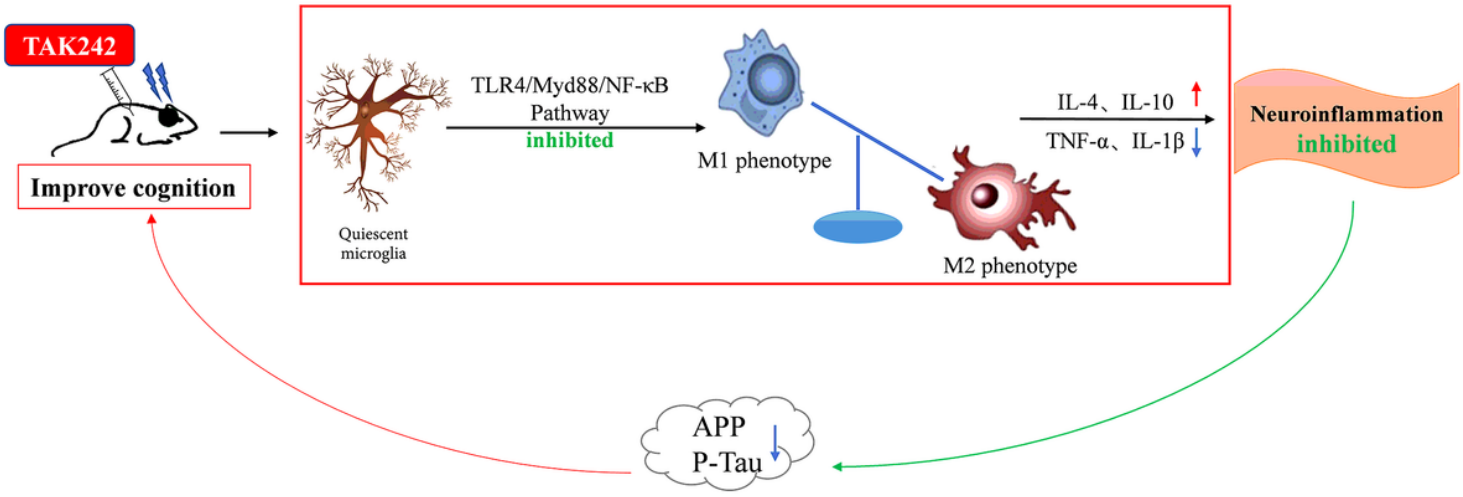

Figure 7

Schematic representation of the mechanism by which TAK242 improves cognitive function after rmTBI. TAK242 promotes microglial polarization from the M1 to M2 phenotype, attenuates the inflammatory response, and reduces the accumulation of pathological proteins, ultimately improving cognitive function after rmTBI.