Research Report

Expression and cell distribution of myeloid differentiation primary response protein 88 in the cerebral cortex following experimental subarachnoid hemorrhage in rats: A pilot study

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A B S T R A C T

Subarachnoid hemorrhage (SAH) which is mostly caused by aneurysm rupture causes a lot of death every year. Convincing evidence can be made that inflammation contributes to the poor outcome caused by SAH. Toll like receptors (TLRs), nuclear factor-kappaB (NF-κB), Interleukin 1β (IL-1β) and tumor necrosis factor-α (TNF-α) are involved in the damaging inflammation process after SAH. Myeloid differentiation primary response protein 88 (MyD88) is essential to deliver TLRs signals down to NF-κB and pro-inflammatory factors. The study aims to detect the expression level of MyD88 and know more about the role of MyD88 after SAH. Sprague Dawley (SD) rats were randomly divided into sham group and SAH groups at 2 h, 6 h, 12 h and on day 1, day 2, day 3, day 5 and day 7. SAH groups suffered experimental subarachnoid hemorrhage by injection of 0.3 ml autoblood into the pre-chiasmatic cistern. MyD88 expression is measured by western blot analysis, real-time polymerase chain reaction (PCR), immunohistochemistry and immunofluorescence. The levels of TNF-α and IL-1β were measured by real-time PCR. Our results demonstrated MyD88 expression was increased after SAH, and peaked on day 1 and day 5, which showed a parallel time course to the up-regulation of IL-1β, there was a highly positive relationship between them. Immunohistochemistry and immunofluorescence results indicated up-regulated MyD88 was mainly located in neurons while over expressed MyD88 could also be found in astrocytes and microglia. These results might have important implications during...
1. Introduction

Subarachnoid hemorrhage (SAH), especially aneurysm subarachnoid hemorrhage, is a life-threatening disease of central nervous system (CNS). The incidence of SAH is about 22.5 cases per 100,000 population according to a World Health Organization Study (Schuette and Barrow, 2012). Although its relatively low incidence, its early age of onset and poor outcome results in a lot of life years lost. Both clinical and experimental evidence supports that inflammatory factors are involved both in the early brain injury and delayed cerebral vasospasm after SAH (Cahill et al., 2006; Fassbender et al., 2001; Gaetani et al., 1998; Hanafy et al., 2010a; Hendryk et al., 2004; Mathiesen et al., 1997; Pradilla et al., 2010; Sehba et al., 2011). Toll like receptors (TLRs), nuclear factor-kappaB (NF-κB), Interleukin-1 receptor (IL-1R) and tumor necrosis factor-α (TNF-α) have been proved to participate in the damaging inflammation process after SAH (Greenhalgh et al., 2012; Zhou et al., 2007). Moreover, clinical studies have shown that increased level of pro-inflammatory factors in cerebrospinal fluid (CSF) of SAH patients is associated with poor outcome (Fassbender et al., 2001; Gaetani et al., 1998; Hanafy et al., 2010b; Hendryk et al., 2004). Elevated inflammatory factors contribute to the breakage of blood brain barrier (BBB), brain edema, neuroglia cells apoptosis and death (Greenhalgh et al., 2012; Larysz-Brysz et al., 2012; Sozen et al., 2009). Administration of the antagonists of the pro-inflammatory factors confers neuroprotective effect in library experimental studies (Greenhalgh et al., 2012; Jiang et al., 2012; Sozen et al., 2009).

Myeloid differentiation primary response protein 88 (MyD88) is now known to function as an adapter protein for inflammatory signal pathways including TLRs and IL-1 family signal transduction. Several studies examining TLRs in the CNS indicate that these receptors may take an important part in CNS autoimmunity, neurodegeneration, subarachnoid hemorrhage, traumatic brain injury especially in the ischemia/reperfusion injury (Kielian et al., 2007; Mishra et al., 2009; Zhou et al., 2007). Moreover, the pro-inflammatory cytokine such as IL-1 family has been reported to induce breakdown of the blood brain barrier (BBB), leading to cerebral edema and secondary neuronal damage following SAH (Greenhalgh et al., 2012; Larysz-Brysz et al., 2012). Thus, we suppose that the cytoplasmic signal transducer MyD88 may take a great part in inflammation and damage development after SAH. However, there were few studies exploring the expression of MyD88 in the brain tissue after SAH. Therefore, this study aimed to identify the time course of MyD88 expression in brain tissue and to clarify the potential role of MyD88 in brain injury following SAH.

2. Results

2.1. The level of MyD88 protein expression in brain cortex following SAH

Low level of MyD88 was identified in the sham group while the level of MyD88 protein increased significantly by 12 h after experimental SAH, peaked on day 1, day 5 and remained ascended till day 7 post-SAH. There was a statistically significant difference between the sham group and 12 h, day 1, day 2, day 3, day 5, day 7 group (p<0.05) (Figs. 1 and 2). The above result showed that SAH could cause significant activation of MyD88 in the brain tissue in the early part of 12 h post-injury, and MyD88 is now known to function as an adapter protein for inflammatory signal pathways including TLRs and IL-1 family signal transduction.

Fig. 1 – Schematic representation of the areas taken for assay. Left one: sham group rat brain and right one: SAH rat brain harvested 2 h after SAH was induced.
keep it at a high level for 7 days. Moreover, the level of MyD88 protein featured a biphasic expression following SAH.

2.2. MyD88 mRNA level in the brain cortex after SAH

The expression of MyD88 mRNA was identified by quantitative real-time PCR. This study showed that the MyD88 mRNA expression was at a low level in the sham group, while the MyD88 mRNA was significantly increased in the SAH group in a time-dependent manner which was similar to the western blot result. There was a significant difference between the sham group and day 1 (p<0.05), day 5 group (p<0.01) (Fig. 3).

Fig. 2 – Top: representative autoradiogram of MyD88 expression in the cortex of the brain after SAH. It shows that the expression of MyD88 protein was increased in the SAH groups and peaked approximately on day 1 and day 5. Lane 1, sham; lanes 2, 3, 4, 5, 6, 7, 8 and 9 represent 2 h, 6 h, 12 h, day 1, day 2, day 3, day 5 and day 7 groups, respectively. Bottom: quantitative analysis of the western blot results for MyD88. It shows that MyD88 levels in SAH groups are significantly higher than in sham group. Obvious difference can also be detected between day 3 group and day 1, and 5 groups. Bars represent the mean±SE (n=6, each group). *P<0.01 compared with sham group. **P<0.05 compared with day 1 group and day 5 group.

Fig. 3 – Representative real-time PCR for the expression of MyD88 mRNA in the brain cortex. The level of MyD88 mRNA expression was increased after SAH. Lane 1, sham group; lanes 2, 3, 4, 5, 6, 7, 8 and 9 represent the 2 h, 6 h, 12 h, day 1, day 2, day 3, day 5 and day 7 groups, respectively. Quantitative analysis of the level of MyD88 mRNA. It shows that MyD88 mRNA levels in day 1 and day 5 groups are significantly higher than in sham group. Bars represent the mean±SE (n=6, each group). *P<0.05, **P<0.01 compared with sham group.

Fig. 4 – Representative real-time PCR for the expression of IL-1β mRNA in the brain cortex. The level of IL-1β expression was increased after SAH. Lane 1, sham group; lanes 2, 3, 4, 5, 6, 7, 8 and 9 represent the 2 h, 6 h, 12 h, day 1, day 2, day 3, day 5 and day 7 groups, respectively. Quantitative analysis of the level of IL-1β mRNA. It shows that IL-1β mRNA levels in day 1, day 3, and day 5 groups are significantly higher than in sham group. Bars represent the mean±SE (n=6, each group). *P<0.05, **P<0.01 compared with sham group.

Fig. 5 – Representative real-time PCR for the expression of TNF-α mRNA in the brain cortex. Lane 1, sham group; lanes 2, 3, 4, 5, 6, 7, 8 and 9 represent the 2 h, 6 h, 12 h, day 1, day 2, day 3, day 5 and day 7 groups, respectively. Quantitative analysis of the level of TNF-α mRNA. It shows that TNF-α mRNA levels in 2 h, 6 h, and 12 h groups are significantly higher than in sham group. Bars represent the mean±SE (n=6, each group). *P<0.01 compared with sham group.
2.3. Expression and distribution of MyD88 in brain cortex after SAH

The expression and distribution of MyD88 was identified by immunohistochemical and immunofluorescence staining. MyD88 was seldom detected in the sham group while MyD88 was highly expressed in the brain cortex in the day 1 (33.67±4.36%) and day 5 groups (29±2.35%) (Fig. 6). Semi-quantitative analysis showed that there was a significant difference between the sham group and the day 1, day 5 group (p<0.01) (Fig. 6). To identify in which kind of brain cells MyD88 mainly expressed in early phase day 1 and the late phase day 5 group, double immunofluorescent staining was performed for MyD88 and neuron-specific nuclear protein (NeuN), ionized calcium binding adapter molecule 1 (Iba1) or glial fibrillary acidic protein (GFAP). As shown in Fig. 7, MyD88 was weakly expressed in sham group. Compared with sham group, over-expressed MyD88 was mainly detected in NeuN-positive cells in the cortex near subarachnoid clot following SAH. In details, approximately 87% of cells staining positively for MyD88 also stained positively for NeuN in day 1 group while about 71% of cells staining positively for MyD88 were positive for NeuN in the late phase day 5 group. Furthermore, in comparison with the sham group, SAH induced elevated MyD88 expression in cells positive for GFAP and Iba-1 (Figs. 8 and 9). More GFAP or Iba-1 positive cells were also detected positive for MyD88 in day 5 group compared with day 1 group. These results suggest that MyD88 was mainly expressed in neurons in the injured cortex following SAH. Meanwhile more GFAP and Iba-1 positive cells were activated in the late phase following SAH (Figs. 8 and 9).

2.4. TNF-α and IL-1β expression increased in brain cortex following SAH

Our data demonstrated that the mRNA expressions of TNF-α and IL-1β were low in the sham rat brain (Figs. 4 and 5). Compared with that of the sham group, TNF-α mRNA level in

![Fig. 6 - MyD88 expression detected by immunohistochemistry in brain cortex. (A) MyD88 expression in the sham group: MyD88 expression could be observed seldom in the sham group; (B) and (C) MyD88 expression in the day 1 and day 5 groups post-SAH, respectively. MyD88 expression obviously increased in the SAH group at day 1 and day 5 after SAH. D, E and F Enlarged images of A, B and C, respectively; Positive cells were defined as presenting buffy grains in cytoplasm as shown by arrows. Scalar bars in A, B, C present 50 μm, while 20 μm in D, E, F. (G) Quantification of density of cells positive for MyD88. Values were obtained from averaging three section per animal. SAH induced up-regulation of MyD88 expression in cortex near subarachnoid space. Bars represent the mean±SE (n=6). #p<0.01 compared with sham group.](image-url)
the injured brain tissue was significantly increased as early as 2 h after SAH while IL-1β mRNA level with a maximum on day 1 and day 5 post-injury in this study. There is obviously positive relationship between IL-1β and MyD88 in mRNA level ($r=0.728$, $P<0.01$). Positive relationship between MyD88 protein and IL-1β mRNA was also found in this study ($r=0.286$, $P<0.05$).

3. Discussion

In this study, we first reported the up-regulated protein and gene expression of MyD88 in the brain cortex near the subarachnoid clot following experimental SAH. Immunohistochemical and immunofluorescence staining demonstrated that the MyD88-positive cells were obviously increased in the
brain cortex near the subarachnoid clot. Most of the MyD88-positive cells were the neurons while the over-expressed MyD88 could also be found in astrocytes and microglia. Moreover, the downstream IL-1β and TNF-α were also dramatically improved in the mRNA level (Figs. 4 and 5). A positive relationship between MyD88 and IL-1β could be found through statistical analysis. This positive relationship demonstrated that MyD88 might play an essential role in triggering inflammation following SAH, which may contribute to the poor outcome of SAH.

### 3.1. Increased expression of MyD88 in both early stage and late stage after SAH

Convincing evidence indicates that inflammation process plays an essential role in the pathophysiological process of brain injury after SAH (Cahill et al., 2006; Pradilla et al., 2010; Sehba et al., 2011). An increased level of pro-inflammatory factors in cerebrospinal fluid (CSF) of SAH patients is associated with poor outcome (Fassbender et al., 2001; Gaetani et al., 1998; Hanafy et al., 2010a; Hendryk et al., 2004). Administration of the antagonists of the pro-inflammatory factors confers neuroprotective effect in library experimental studies (Greenhalgh et al., 2012; Jiang et al., 2012; Sozen et al., 2009).

**TLRs/MyD88/NF-κB pathway is an important pathway in inflammatory response.** Previous studies have suggested the elevated levels of TLR4 in brain tissue in the early stage (Ma et al., 2009; Vecchione et al., 2009) and the late stage (Ma et al., 2009; Zhou et al., 2007) following SAH. Up-regulated level of TLR4 (Zhou et al., 2007 and IL-1p, TNF-α (Greenhalgh et al., 2012) after SAH induced the brain inflammation, endothelium dysfunction, blood-brain barrier disruption, brain edema, neuroglia cells apoptosis and death, causing brain damage. However, the upstream and downstream pathways were not fully investigated.

MyD88 was an important downstream adapter of TLR family and IL-1 family (Muzio et al., 1997; Wescue et al., 1997). Association of MyD88 with TLRs and IL-1 receptor can induce the activation of NF-κB, which can enlarge the inflammatory response by regulating transcription of inflammatory factors, such as IL-1β and TNF-α, the former could in turn trigger the NF-κB activity through association with IL-1 receptor and MyD88 (Dunne and O’Neill, 2003). IL-1β also increases release of endothelin-1 and TNF-α as well as reactive oxygen species after SAH (Larysz-Brysz et al., 2012). In the current research, our data demonstrated that MyD88 level in brain cortex was significantly up-regulated after SAH and peaked on the day 1 group and day 5 group which featured a biphasic expression. Biphasic expression of TLR4 was reported in previous studies (Ma et al., 2009; Zhou et al., 2007). Peak time of TLR4 level in their researches is similar to the time-course of MyD88 protein in our study. These results suggested the strong relationship between TLR4 and its downstream protein MyD88. Moreover, MyD88 deficiency significantly reduced macrophage and T cells recruitment after injury. The pro-inflammation cytokine expressions such as TNF-α, IL-1β were significantly decreased in MyD88 deficient mice (Babcock et al., 2008). Furthermore, the first peak time of MyD88 was also a peak time of inflammatory factors, cell death, brain edema and high mortality in SAH models (Jeon et al., 2010; Thal et al., 2009). Above publications and our data in this study indicate that MyD88 might play a key role in the inflammatory response following SAH. However, further researches using MyD88 special inhibitor in SAH models (Loiarro et al., 2007)

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**Fig. 9** – Representative photomicrographs showed brain cortex double immunofluorescent staining for MyD88 (red) and Iba-1, a microglia marker (green) in brain cortex in the sham (A–E), day 1 (F–J) and day 5 (K–O) post-SAH groups. Nucleus was counterstained with DAPI (blue) in the same view in each section. D, I, N present merged images of MyD88 (red) and DAPI (blue); E, J, O present merged images of MyD88 (red) and Iba-1 (green). Compared with the sham group (D), the SAH groups (I, N) showed up-regulated MyD88 in cytoplasm. Overlapped images showed that the number of cells positive both for MyD88 and Iba-1 increased in SAH groups (f, o) compared with sham group (e). The number increased more in day 5 group compared with day 1 group. Arrows indicated the co-localization of MyD88 and Iba-1. Scale bar: 20 μm.
are still required to confirm the key role of MyD88 in the brain injury following SAH.

Inflammation in brain composes a biphasic response with an acute and a chronic period after SAH (Pradilla et al., 2010; Serhan, 2007). Acute inflammation results from the combination of local cellular response (including endothelial cells and neuroglia cells) and neutrophils, macrophages, monocytes, which migrate to the inflamed site and phagocyte the particles that stimulated the inflammatory response. In the late chronic stage, the trapped intrathecal inflammatory cells from extravasated blood or migrating to subarachnoid space from other places eventually undergo apoptosis within days and release free radicals, endothelin-1 and pro-inflammatory cytokines (Pradilla et al., 2010). The chronic inflammatory response in the subarachnoid space enhanced the inflammatory response again in the cortex near the clot. In our study, we observed the second peak of MyD88 protein and mRNA level in the late stage of SAH with up-regulated IL-1β mRNA level. The chronic inflammation in the subarachnoid space might be the responsible reason. At the meantime, cerebral ischemia caused by delayed vasospasm could be observed both in clinical SAH cases and experimental SAH models (Chen et al., 2013; Pradilla et al., 2010). Delayed cerebral vasospasm usually happens in the late stage of SAH. Serious cerebral vasospasm was detected in the day 3, day 5 group in our prechiasmatic injection SAH model according to previous study (Chen et al., 2013). Cerebral vasospasm causing delayed cerebral ischemia and infarction might be another responsible reason for the inflammatory response in the late stage of SAH (Joo et al., 2013; Tam et al., 2010).

3.2. Neural cellular localization of MyD88 and increased level of inflammatory factors after SAH

TLRs are present in the brain, where their expression and inflammatory response are believed to be limited to glial cells (microglia and astrocytes) (Jack et al., 2005; Olson and Miller, 2004). However, recent findings suggest neuron cells could also express at least some TLRs responsive to stimulation (Lafon et al., 2006; Tang et al., 2007). Specifically, Sung-Chun Tang’s research indicates that inflammatory response after stroke is not strict only to immune cells but also to neuron cells (Tang et al., 2007). The levels of TLR2 and TLR4 are increased in neurons in response to IFN-γ stimulation and energy deprivation associated with elevated MyD88 level. Neurons from both TLR2 knockout and TLR4 mutant mice are protected against energy deprivation-induced cell death. These results indicate that TLRs/MyD88 pathways in neurons play a pivotal role in stroke which might lead to cell damage. Moreover, Lara and colleagues’ research indicate that neurons are more efficient in uptake of extravasation of blood than astrocytes in intracerebral hemorrhage (Lara et al., 2009). It seems that neurons are actively involved in brain damaging process following SAH. In our research, we detected that up-regulated MyD88 protein was mainly located in neurons in the cortex near the clots (Fig. 7). Moreover, in our previous study (Li et al., 2012a), cultured neurons could up-regulate MyD88/NF-κB and the downstream inflammatory factors such as IL-1β, TNF-α by the stimulation of recombinant high-mobility group box 1, a component released from death cells and detected to increase after SAH (Murakami et al., 2011). Thus, our data in this study and the above publications suggested that activated neurons following SAH might contribute to the production of pro-inflammatory factors.

TNF-α is a major initiator of inflammation and increased early after SAH. TNF-α can enhance vascular permeability and play an important role in the recruitment of inflammatory and immune cells to the injured site. TNF-α could also induce both apoptosis and necrosis via intracellular signaling (Verbrugge et al., 2010). IL-1β is another pro-inflammatory cytokine that signals through the IL-1 receptor complex to generate multiple cellular response. Neurotoxicity of TNF-α and IL-1β was critically tested by experimental administration of anti-TNF-α antibody and IL-1 receptor antagonists (Greenhalgh et al., 2012; Jiang et al., 2012). Decreased levels of these pro-inflammatory factors have been shown to delay pro-inflammatory cytokine, reduce BBB destruction, lessen apoptotic or dead cells and improve neurological recovery after SAH. MyD88 is a key protein that participates in the IL-1 signaling transduction. Accumulating evidence has demonstrated that the positive feedback of the IL-1 induces MyD88 through IL-1 receptors. Consistent with other studies, we showed that pro-inflammatory cytokine TNF-α and IL-1β markedly increased after SAH. Positive relation could be found between IL-1β and MyD88. It may be related to the amplification and maintenance of the inflammation after SAH.

These above evidence indicated that the neurons took great part in the inflammatory response and cell damage process following SAH and neuronal MyD88 might play an important role in brain injury and functional deficits following SAH.

3.3. Assessing therapeutic potential of MyD88 in the brain injury after SAH

As described above, the MyD88 is the common and essential adapter protein to TLRs, IL-1 signal transduction; therefore inhibition the MyD88 would perhaps more markedly attenuate inflammation response and brain damage than blocking single signal transduction pathway of TLRs and IL-1. However, the therapeutic potential of MyD88 modulation to regulate the TLRs and IL-1 dependent biological effects in the injured brain following SAH has not been previously investigated. Further design of the study was required to aim at assessing the therapeutic potential of MyD88 in the brain injury after SAH.

3.4. Summary

To the best of our knowledge, this is the first study to show the expression of MyD88 in the brain after SAH. We found that SAH caused an obvious up-regulation of MyD88 in brain. Both MyD88 protein and mRNA expressions were significantly increased after SAH. It could be postulated that MyD88 might play an important role in the secondary brain injury after SAH. However, the full role of MyD88 and its therapeutic potential in secondary brain injury need further research.
4. **Experimental procedures**

4.1. **Animal preparation**

Male Sprague-Dawley rats (280–320 g) were purchased from Animal Center of Jinling Hospital. The rats were raised on a 12-h dark-light cycle circumstance with free access to food and water. All procedures were approved by the Animal Care and Use Committee of Nanjing University and accorded to Guide for the Care and Use of Laboratory Animals by National Institutes of Health.

4.2. **Animal model of SAH**

The prechiasmatic injection model was used to introduce SAH (Jeon et al., 2010). Specifically, after intraperitoneal anesthesia with pentobarbital sodium (50 mg/kg) (Sigma, St. Louis, MO, USA). Then they were positioned prone in a stereotactic frame. After careful disinfection, a midline scalp incision was made and a 1 mm hole was drilled 7.5 mm anterior to the bregma in the midline, at an angle of 30E caudally. Then they were positioned in supine position. After carefully disinfecting again, we use insulin injection needle (BD Science) to get 300 μl of their blood from femoral artery. The needle (BD Science) was advanced 11 mm into the prechiasmatic cistern through this burr hole, and the 300 μl blood was injected into the prechiasmatic cistern over 20 s. Intracranial pressure was monitored for 45 min and 60 min after SAH. After completing these procedures, 1 ml of 0.9% NaCl solution was injected subcutaneously to prevent dehydration and the rats were returned to their cages and raised at 23 ± 1°C.

Rats with SAH were randomly divided into eight subgroups and killed by ventricle perfusion at 2 h, 6 h, 12 h, and on day 1, day 2, day 3, day 5, day 7 post-SAH, (n=6/subgroup). Another 12 rats with SAH were selected randomly for immunohistochemical and immunofluorescence study of day 1 and day 5 groups (n=6). Sham animals experienced the same surgery process except for injection with anything into the prechiasmatic cistern. In our pilot study, we found that there was no statistical difference of all detected variables among sham groups at each time point (data not shown). Therefore, animals in sham group were sacrificed at 24 h after sham operation.

4.3. **Perfusion-fixation and tissue preparation**

They were anesthetized as above, and perfused through the left cardiac ventricle with 0.9% NaCl solutions until the effluents from the right atrium was clear. Animals which had obvious clots in the prechiasmatic cistern were selected for further analysis. After blood clots on the brain tissue were cleared carefully, the temporal lobe tissue (see Fig. 1) near the hematoma was harvested on ice and stored in −80°C for western blot and real-time-PCR analysis. For immunohistochemistry and immunofluorescence study, the rats were perfused with 0.9% NaCl solutions followed by 4% buffered paraformaldehyde; the brain was immersed in 4% buffered paraformaldehyde overnight and then embedded in paraffin for immunohistochemistry study while frozen in OCT for immunofluorescence study.

4.4. **Western blot analysis**

For western blot analysis, proper size of tissues were completely homogenized and centrifuged at 14,000 × g for 15 min at 4°C. The supernatant was collected. After adding SDS sample buffer, the samples were then boiled for 5 min at 100°C. Samples (70 μg/lane) were subjected to electrophoresis on 10% SDS-polyacrylamide gels for 30 min at 80 V followed by 100 min at 110 V and then transferred onto PVDF for 2 h at 200 mA. The membrane was blocked with 5% defatted milk for 2 h at room temperature, then incubated with primary anti-MyD88 antibody (diluted 1:200 in 5% defatted milk, Santa Cruz Biotechnology, Santa Cruz, CA) and β-actin (diluted 1:3000 in 5% defatted milk, Bioworld, USA) at 4°C with gentle shaking, overnight. After that the membrane was washed for 10 min each for four times in TBS+TWEEN 20 (TBST), followed by incubation in the appropriate HRP-conjugated secondary antibody (diluted 1:5000 in TBST) for 2 h at room temperature. The blotted protein bands were visualized by enhanced chemiluminescence (ECL, Thermo Scientific, USA) and were exposed to X-ray film. Relative changes in protein expression were estimated from the mean pixel density using UN-SCAN-IT, normalized to β-actin, and calculated as target protein expression/β-actin expression ratios.

4.5. **RNA isolation and quantitative real-time PCR**

Rat brain tissues were isolated using TRIzol Reagent (TAKARA Biotechnology) as manufacturer’s instructions. The concentration of the RNA was determined by spectrophotometric analysis (OD260/280). The quantity of RNA was measured using OD260. The isolated RNA was stored at −80°C until being analyzed. RNA was reverse-transcribed to cDNA using Reverse Transcriptase Reagent (TAKARA Biotechnology) and oligodT primers. Quantitative real-time PCR analysis was performed using the Agilent Technologies Stratagene Mx3000P real-time PCR system (Genetechines Technology, Inc.), applying real-time SYBR Green PCR technology. The reaction mixtures contained 1 μl cDNA, 12.5 μl SYBR Green (TAKARA Biotechnology), 1 μl of each forward and reverse primer (10 μM) and nuclease-free water to a final volume of 25 μl. The primers were synthesized by Life Technologies (Invitrogen, Shanghai, China) and the sequences used were from a database at NCBI for rat MyD88, TNF-α, IL-1β and β-actin. MyD88 forward and reverse primers were 5′-TTCTCCAAACGCTGTCCTGTC-3′ and 5′-AACTGAGATGTGTGCGCCTAGG-3′; TNF-α forward and reverse primers were 5′-TGCCATTAGTTCAGCTCCCTCTC-3′ and 5′-GAGGCCATTTGGGAACTTCTC-3′; IL-1β forward and reverse primers were 5′-TGCACCCCTTCTTCTTCTC-3′ and 5′-TTGCTCAGTATTCTTCTGCTC-3′; β-actin forward and reverse primers were 5′-AGGGAAAATGGTCTGACG-3′ and 5′-CGCTCATTGCGGATAGTG-3′. After 95°C for 30 s, 40 PCR cycles were performed, each consisting of a denaturation step (95°C, 5 s) and an annealing step (60°C, 30 s). Total RNA concentrations from each sample were normalized by quantity of β-actin.
messenger RNA (mRNA), and the expression levels of target genes were evaluated by using the 2^−ΔΔCq method. All samples were analyzed in triplicate.

4.6. Immunohistochemical staining

The tissue was fixed with the 4% paraformaldehyde and embedded in paraffin. Immunohistochemical staining was performed as our previous study (Li et al., 2011). Detaileadly, the tissue sections (4 μm) were used for immunohistochemical staining; the sections were deparaffinized as usual and incubated with 3% H2O2 in phosphate-buffered saline (PBS) for 10 min. Sections were blocked with 5% normal fetal bovine serum in PBS for 2 h followed by incubation with anti-MyD88 antibody (diluted 1:100, Santa Cruz, USA). After washing carefully for half an hour, each of the sections was incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (diluted 1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 60 min at room temperature. After washing for half an hour again, diaminobenzidine (DAB) was used as a chromogen and counterstaining was done with hematoxylin. The negative control was also performed without adding MyD88 antibody, and the other steps were the same between the experiment sections and negative control. MyD88-positive cells were defined as presenting buffy grains in cytoplasm of neuroglia cells and neuron as shown by arrows in Fig. 6. Analysis of the immunohistochemical staining was performed according to our previous studies (Li et al., 2011, 2013). In detail, three coronary sections of temporal lobe tissue in each rat brain sample, with a minimum of 150 μm from the adjoining section, were used for cell counting in each sample. The number of MyD88-positive cells was presented as the percentage of total cells in each visual field. Six randomly non-overlapping high power areas (×400) per section were selected and observed. Then mean percentage of MyD88-positive cells in the six views was regarded as the data for each section. The final average percentage of the three sections was regarded as the data for the sample. Six samples in each group were employed for the statistical analysis. The percentage of MyD88-positive cells was identified, calculated, and analyzed under a light microscope by an investigator blinded to the grouping.

4.7. Immunofluorescence staining

Immunofluorescence staining was performed according to our previous study in our laboratory (Li et al., 2012a). Brain tissue was fixed with 4% paraformaldehyde overnight and dipped in 20% saccharose PBS for 2 days and then in 30% saccharose PBS for another 2 days to remove water in the tissue. Sections 6 μm in thickness were sliced and blocked with 5% normal fetal bovine serum in PBS containing 0.1% Triton X-100 for 2 h at room temperature prior to incubation with anti-neuron-specific nuclear protein (NeuN) antibody (Millipore, USA, 1:200) and anti-MyD88 antibody (Santa Cruz, USA, 1:100) or anti-ionized calcium binding adapter molecule 1 (Iba1) antibody (abcam, 1:200) and anti-MyD88 antibody (Santa Cruz, USA, 1:100) or anti-glial fibrillary acidic protein (GFAP) antibody (Millipore, USA, 1:200) and anti-MyD88 antibody (Santa Cruz, USA, 1:100) overnight at 4 °C. After sections were washed three times with PBS for 45 min, they were incubated with proper secondary antibodies (Alexa Fluor 488 and Alexa Fluor 594,1:200) for 1 h at room temperature. The slides were washed with PBS again three times for 45 min prior to be counterstained by DAPI for 2 min. After three washes again, the slides were covered by microscopic glass with Anti-fade Mounting Medium for further study. Negative controls were prepared by omitting the primary antibodies. Fluorescence microscopy imaging was performed using ZEISS HB050 inverted microscope system and handled by Image-Pro Plus 6.0 software (Media Cybernetics, USA) and Adobe Photoshop CS5 (Adobe Systems, San Jose, USA). MyD88 in cytoplasm was defined as MyD88-positive cells according to our previous study (Li et al., 2013).

4.8. Statistical analysis

All data were presented as mean ± SE. SPSS 17.0 (SPSS Inc., Chicago) was used for statistical analysis of the data. The measurements were subjected to ANOVA followed by Tukey’s post-hoc test. The relationship between MyD88 protein and IL-1p mRNA expression was analyzed using the linear regression model. A value of P < 0.05 was considered statistically significant.

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