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Original Article

Expression of cysteinyl leukotriene receptor in brain tissues of rats with Streptococcus pneumoniae meningitis

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Abstract: *Streptococcus pneumoniae* meningitis is an infection of the central nervous system associated with high mortality rates and serious neurologic sequelae in children. The principal reason for the severity of *S. pneumoniae* meningitis is widespread ignorance of the pathogenesis of the disease. This study aimed at exploring whether cysteinyl leukotriene receptor (CysLTR) participates in the inflammatory response and elucidates the pathologic process of *S. pneumoniae* meningitis. Bacterial meningitis disease models were constructed by intracisternal inoculation of rats with serotype III *Streptococcus pneumoniae* while control models were inoculated with the same volume of normal saline. Rats were sacrificed at different time points (1 d, 2 d, and 5 d) following the administration of *Streptococcus pneumoniae.* Results from the body-weight, Loeffler neurologic deficit score, and cerebrospinal fluid culture confirmed that a successful pneumococcal meningitis rat model was established. Pathologic changes in brain tissues mainly consisted of inflammation in the meninges and subarachnoid space and significant neuronal injury in the cerebral cortex and hippocampus ($P < 0.05$). Immunohistochemical analysis revealed that microglial activation and astrocyte proliferation were associated with the development of bacterial meningitis. The expression levels of CysLTR and inflammatory factor tumor necrosis factor-α (TNF-α) were examined by quantitative real-time polymerase chain reaction (qRT-PCR) and western blot analysis. The results of this study indicate that CysLTR expression was markedly elevated in the 5 d infection group ($P < 0.05$), which was consistent with time-dependent release of TNF-α. The findings of this study indicate that CysLTR participates in the pneumococcal meningitis infection process by mediating neuronal injury and glial cell proliferation. Cysteinyl leukotriene receptors could, therefore, be novel targets to mitigate the progression of pneumococcal meningitis.

Keywords: Cysteinyl leukotriene receptor, *Streptococcus pneumoniae,* meningitis

Introduction

Bacterial meningitis (BM) is a severe infection of the central nervous system associated with high mortality rates in pediatrics [1]. In China, the annual BM incidence from the years, 2006 to 2009 ranged between 6.95 to 22.30 cases in children < 5 years old and between 1.84 to 2.93 cases per 100,000 population in the entire population [2]. Among the major BM causative pathogens is *Streptococcus pneumoniae* (*S. pneumoniae*) which mostly inflicts children older than a few months [3]. Furthermore, *S. pneumoniae*-associated meningitis is the leading cause of serious neurologic sequelae, including epilepsy, hearing loss, seizures, learning and memory deficit [4-7]. In high-income economies, however, the improvement of antibiotics and widespread use of conjugate vaccines has resulted in a decrease in the incidence of BM [8]. However, the pneumococcal conjugate vaccine is not universally used in China [9] and antibiotic/multidrug resistance of *S. pneumoniae* is an emerging challenge in mainland China [10]. There is, therefore, no effective treatment for pneumococcal meningitis in parts of China.

Inflammation in the brain is regulated by large molecules such as cytokines and small molecular inflammatory mediators. Cysteinyl leukotrienes (CysLTs) such as LTE_4, LTD_4, and LTC_4 modulate inflammatory responses by the metabolism of arachidonic acid in the 5-lipoxygen-

ase pathway. Cysteinyl leukotrienes are predominantly synthesized by inflammatory cells such as microglia, astrocytes, and leukocytes [11-13]. Additionally, CysLTs and their receptors, mostly $CysLT_2R$ and $CysLT_1R$, have been found to play a role in the development of various inflammatory diseases such as those affecting the central nervous system [11, 14, 15]. We have previously demonstrated that $CysLT_1R$ blockers montelukast and pranlukast protect against acute and chronic injury induced by global or focal cerebral ischemia in rodents, and their neuroprotective effects may be indirectly through the regulation of microglia. Moreover, $CysLT_1R$ also has a clear inhibitory effect on the proliferation of astrocytes and the formation of glial scar. Intracerebroventricular administration of HAMI 3379, a $CysLT_2R$ antagonist has been reported to abrogate focal cerebral ischemia-induced acute injury in rats [16-20]. Interestingly, in the model of cryptococcal meningoencephalitis, CysLTs have been found to facilitate the passage of bacteria across the blood-brain barrier [21]. There is, however, no evidence to date whether either $CysLT_1$ or $CysLT_2$ receptors participate in the pneumococcal meningitis inflammatory reaction and pathologic process.

The present study aimed at using a clinically relevant strain of *S. pneumoniae* to evaluate a pneumococcal meningitis model in rats. We examined the transcriptional and protein expression of cysteinyl leukotriene receptors during disease progression of pneumococcal meningitis. In addition, the study examined inflammatory factor expression, neuronal injury, and proliferation of microglia and astrocytes at various time-intervals following *S. pneumoniae* injection. This study demonstrates the expression and role of cysteinyl leukotriene receptors in pneumococcal meningitis disease progression.

Materials and methods

Selection of animals

Male Sprague-Dawley rats (3 weeks old, weighing 50-60 g) were purchased from the Experimental Animal Center, Zhejiang Academy of Medical Sciences, (Hangzhou, China; Certificate no. SCXK (Zhe) 2014-0001). The rats were housed in an animal facility maintained at a temperature of 20-24°C and adjusted to a photoperiod of 12 h dark and 12 h light. The rodents were fed on standard water and food.

All protocols used in this study conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Utmost care was taken to use the least possible number of rats and minimize pain.

Bacterial strain

This study used a *Streptococcus pneumoniae* serotype III standard strain (strain number 49619). The bacterial strain was cultured on sheep blood agar plates infused into a broth and incubated overnight at 37°C under anaerobic conditions of 5% CO_2. Bacterial cells were harvested by 20 min centrifugation at 4000 rpm and washed twice with saline. The bacterial pellet was then resuspended in saline solution and the concentration was adjusted to 1×10^7 colony forming units (CFU)/mL using a nephelometer.

Induction of bacterial meningitis rat models

Bacterial meningitis models were constructed in accordance with a previous study [22]. Anesthesia induction prior to operation was achieved by intraperitoneal administration of 40 mg/kg of sodium pentobarbital. To collect cerebrospinal fluid (CSF), the head of a rat was first put in the brain stereotactic apparatus, an intracisternal puncture was performed, and a gas chromatography sampling needle was used to extract 20 μL of CSF. An equal volume (20 μL) of *Streptococcus pneumoniae* (1×10^7 CFU/mL) or saline were inoculated into the rats. At the end of the operation, rats were first put in a warm box until they reverted to a conscious state after which they were taken back to their home cages and weighed at appropriate periods. The meningitis model was confirmed by culturing CSF (5 μL) after 24 h of injection. Sixty-four rats were randomized into the saline control group (n = 16) and model group [1 d (n = 16), 2 d (n = 16) or 5 d (n = 16)]. Disease severity was assessed in accordance to the neurological scoring system [23]: 1, coma; 2, unable to stand upright or turn upright when supine; 3, unable to stand upright when supine within 30 s; 4, spontaneous activity decreased, not standing upright within 5 s; 5, normal. Then, *Streptococcus pneumoniae* and saline-inoculated rats were scored at pre-determined time-intervals (1 d, 2 d, and 5 d post-infection).

Histopathology and immunohistochemistry

After being anesthetized as previously described, the rats were first washed with a saline solution and transcardially perfused with 4% paraformaldehyde. The rats were then decapitated to obtain the brain tissue. This was followed by a 24 h tissue fixation in 4% paraformaldehyde and 5 d tissue bathing in 30% sucrose at 25°C. Thereafter, 10 μm-thick slices of the frozen coronal tissues were sectioned using a CM 1900 cryomicrotomy (Leica, Wetzlar, Germany). The serial sections were mounted on gelatin-coated glass slides and stained using the hematoxylin and eosin dyes, and cresyl violet staining [24]. The staining of brain specimens was carried out as described in the literature [20]. Stained specimens were examined and photographed using a BX-51 Olympus fluorescence microscope and number of neurons was calculated using ImageJ software. For each site, 9 non-overlapping neurons were randomly selected to obtain the average value.

Immunohistochemical assays were conducted by first blocking the specimen for 30 m with 0.3% hydrogen peroxide in methanol, followed by sequential hydration in distilled water. Finally, the specimens were blocked at room temperature (RT) with 5% goat serum for 2 h. This was followed by overnight incubation at 4°C in glial fibrillary acidic protein (GFAP) antibody, a biomarker of astrocytes, polyclonal anti-rabbit anti-ionized calcium-binding adaptor molecule-1 (Iba-1), a biomarker of macrophages/microglia, (1:200, Proteintech, USA). The specimens were rinsed and treated with biotinylated goat mouse IgG or anti-rabbit (1:200, Vectorlab, USA) for 2 h, and then with streptavidin horseradish peroxidase (1:200, Vectorlab) for 2 h. The tissue specimens were bathed in 3, 3-diaminobenzidine (DAB). Finally, specimens were dried, bathed in xylene, and mounted permanently. Using a microscope, GFAP and Iba-1-positive cells were counted.

Isolation and quantification of mRNA levels by RT-PCR

Total RNA was isolated from brain tissues using Trizol reagent (Invitrogen, USA) as previously detailed [25]. Complementary DNA (cDNA) was synthesized from the total RNA using 200 U M-MuLV reverse transcriptase and 20 U RNasin in accordance with manufacturer's instruction. Reverse transcriptase was deactivated by heating the mixture at 42°C for 60 m then at 70°C for 5 m.

Messenger RNA (mRNA) levels of Tumor Necrosis Factor-alpha (TNF-α), and cysteinyl leukotriene receptors CysLT2R and CysLT1R were quantified by Reverse Transcriptase PCR (RT-PCR) analysis. The cDNA was amplified by RT-PCR on ABI7500 system (Applied Biosystems, Life Technologies) using SYBR green master mix (Roche, Mijdrecht, the Netherlands) as follows: 95°C initial denaturation for 10 m, 40 cycles of denaturation at 95°C for 15 s, annealing, and extension at 60°C for 1 m Messenger RNA levels were normalized to that of GAPDH. Gene expression levels were analyzed using the comparative Ct ($2^{-\Delta\Delta Ct}$) method [26].

Western blotting assay

Proteins were isolated from brain tissues using a lysis buffer at 4°C. The lysate was centrifuged for 30 m at 12,000 g for 4°C. The concentration of the proteins was measured using the Bradford assay. About 80 μg of samples were resolved in 10% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) after which they were electrically transferred to polyvinylidene difluoride (PVDF) membranes. This was followed by 1 h blocking of the membrane at RT in 5% non-fat dry milk and incubation at 4°C with primary antibodies diluted as follows: rabbit polyclonal antibodies against $CysLT_1$ receptor (1:1000, Proteintech, Chicago, USA) and $CysLT_2$ receptor (1:1000, Santa Cruz, CA, USA), anti-β-actin (1:2000, Service Biotechnology, Wuhan, China). Following overnight incubation, repeated washes were done and the membranes were incubated at RT with HRP conjugated secondary antibody for 1 h. The immunoblots were then scanned using an EPSON scanner. The protein bands were developed and protein expression was normalized to β-actin.

Statistical analysis

GraphPad Software was employed to perform data analysis. Values are presented as mean ± standard error mean (SEM). Multiple groups were compared using a one-way analysis of variance (ANOVA), and mean separation was done using by Newman-Keuls post hoc. The Kruskal-Wallis test was used to determine the

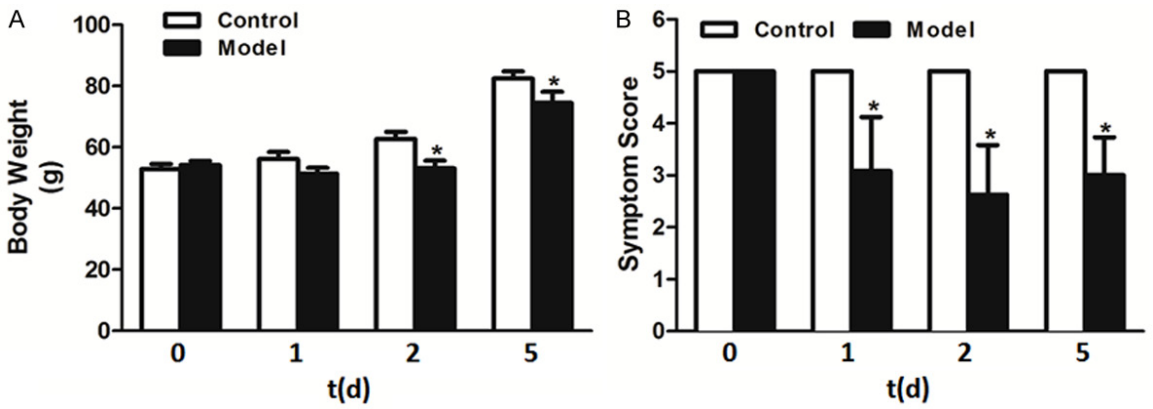

Figure 1. Clinical findings of rats in different groups. A. The weight of rats on days 1, 2 and 5 post-infection; B. Symptom scores of experimental rats. n = 12-16 rats; *P < 0.05 versus the saline control group.

neurological scores. A value of $P < 0.05$ was considered significant.

Results

Clinical observation and CSF examination of infected rats

The body weight of rats with bacterial meningitis decreased significantly on the second and fifth days ($P < 0.05$), while the weight of rats in the control group increased steadily (**Figure 1A**). Twenty-four hours after bacterial infection, all rats started to manifest different degrees of neurologic deficit symptoms such as poor spirit, reduced activity, dyskinesia, convulsions, coma, and even death. The rats in the control group either had normal neurological functions or died during the experimental period. After infection, the neurological score in 1 d, 2 d, and 5 d groups was markedly smaller relative to those of the control group ($P < 0.05$) (**Figure 1B**). In the model group, all rats were cultured the same strain of *Streptococcus pneumoniae* in CSF after 24 h of infection. There was, however, no bacterial growth in CSF of rats in the control group.

Histopathology of meningitis

Changes in the morphological state of tissues were assessed by conducting hematoxylin and eosin staining at 1 d, 2 d, and 5 d post-infection. The results indicated pools of infiltrated cells and inflammatory exudate in the subarachnoid space in all infected rats. Saline-inoculated control rats did not show any inflammatory cell response in the meninges (**Figure 2A**). Moreover, neuronal damage occurred in the cortex and hippocampus of the disease model rats as revealed by cresyl violet staining. The Nissl bodies of the cortex and hippocampus of disease model rats were obscure and the cell bodies were either deeply stained and shrunken or completely lost (**Figure 2B**). On the contrary, the arrangement of neurons in the hippocampus and cerebral cortex were orderly and regular, and the Nissl bodies were evenly distributed. The number of neurons was significantly reduced in the cortex and hippocampus at different time points relative to the control group ($P < 0.05$) (**Figure 2C**). The present study, therefore, demonstrates a complete-time course of significant neuronal injury in the hippocampus and cerebral cortex following administration of *Streptococcus pneumoniae*.

Immunohistochemistry

The possibility of the existence of a correlation between microglial activation and astrocyte proliferation with brain injury in bacterial meningitis was evaluated by Iba-1, microglia (**Figure 3**) and anti-Glial Fibrillary Acidic Protein (GFAP), astrocyte (**Figure 4**) immunostaining analysis. **Figure 3A** shows that the ramified Iba-1-positive microglial cells were arranged in a diffuse manner in the cerebral cortex in control rats. At 2 d and 5 d post-infection, the number of cells testing positive for Iba-1 i.e., activated (round/ameboid) macrophages/microglia and ramified microglia were very elevated in comparison with the control rats (P < 0.05, **Figure 3B**). Astrocytes became hypertrophic (**Figure**

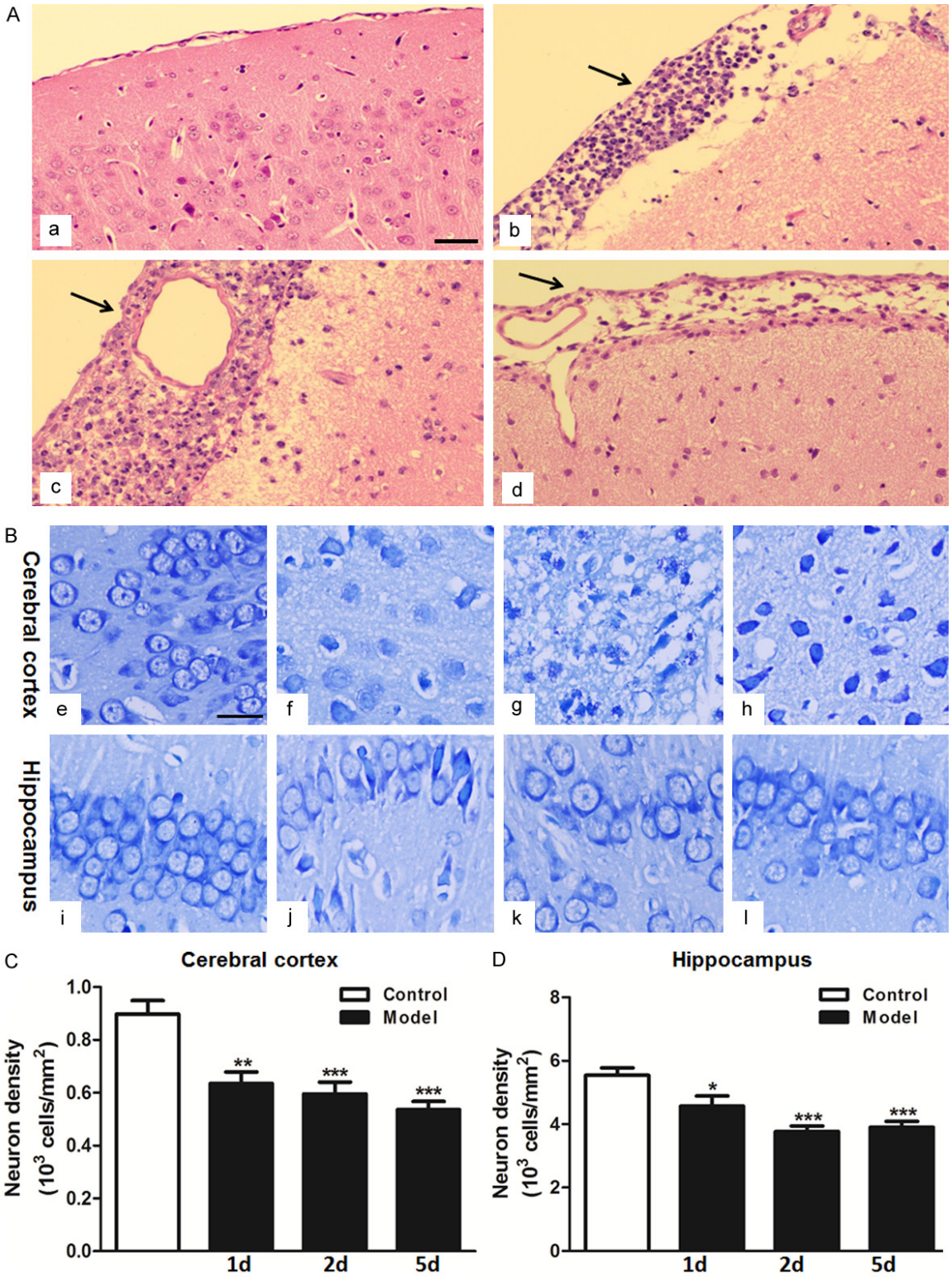

Figure 2. Intracisternal injection of Streptococcus pneumoniae induced changes in neuron density and histopathologic changes in the cerebral cortex and hippocampus at standardized times. A. Hematoxylin and eosin (H&E) staining showing the structure of meninges following *S. pneumoniae*-induced meningitis. B. Cresyl violet staining showing neuronal density was altered in the cerebral cortex and hippocampus following injection. C. Number of cresyl violet-stained neurons in the cerebral cortex. D. Number of cresyl violet-stained neurons in the hippocampus. Scale bar, 50 μm. (a, e and i) Control group, (b, f and j) 1 d group, (c, g and k) 2 d group, (d, h and l) 5 d group. n = 7-8 rats; **P < 0.01 and ***P < 0.001 relative to the control group.

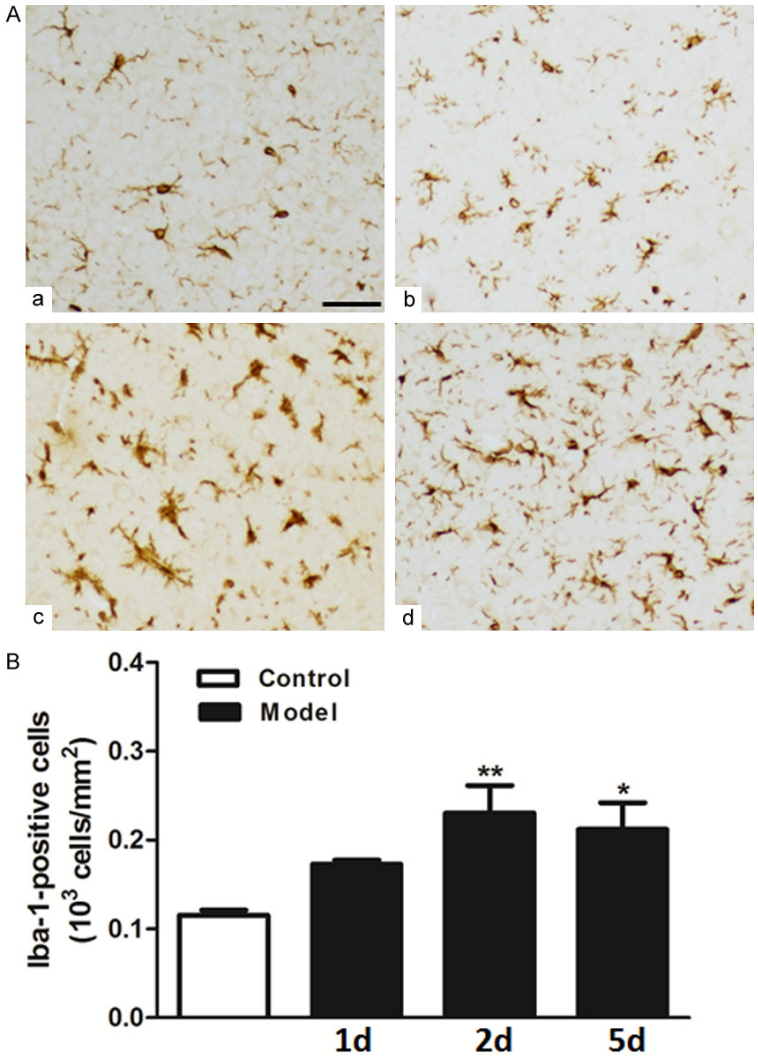

Figure 3. Impact of bacterial meningitis on microglial activation in rats. A. Images showing Iba1-immunopositive microglia in the cerebral cortex at 1, 2 and 5 days post-infection (scale bar, 50 μm). B. Quantification of results. (a) Control group, (b) 1 d group, (c) 2 d group, (d) 5 d group. n = 7-8 rats; *P < 0.05 and **P < 0.01 versus the control group.

4A) and the density of GFAP-positive astrocytes was gradually increased in all groups of rats that received *S. pneumoniae* (P < 0.01, **Figure 4B**). Overall, these results indicate that microglial activation and astrocyte proliferation are closely related to the development and progression of bacterial meningitis.

Expression of cysteinyl leukotriene receptor and cytokine secretion following S. pneumoniae infection

The transcript levels of cysteinyl leukotriene receptors $CysLT_1R$ and $CysLT_2R$ and inflammatory factor Tumor Necrosis Factor-alpha (TNF-α) were evaluated through quantitative RT-PCR at 1, 2 and 5 days. The mRNA expression levels of $CysLT_1R$ in the brain began to increase at day one and by day five, there was a significant difference in the expression levels between the test and control group. The mRNA level of $CysLT_2R$ in the brain at 5 d post-infection with *S. pneumoniae* was higher than that of the control group and 1 d group (P < 0.01). Furthermore, this study revealed that the level of TNF-α was markedly elevated in a time-dependent manner in all the infected rats in comparison with those in the control group (P < 0.05, **Figure 5**).

Western blot analysis of the $CysLT_1R$ and $CysLT_2R$ protein expression levels revealed a change similar to that obtained in its mRNA (**Figure 6**). Taken together, these data indicate a complete time course of $CysLT_1R$ and $CysLT_2R$ expression following administration of *S. pneumoniae*.

Discussion

Since there is still a dearth of efficient preventive and adjuvant therapeutic drugs other than antibiotics, the consequences of *S. pneumoniae* meningitis are so devastating that the construction of an animal model is the most ideal way of examining the mechanism and treatment of bacterial meningitis. A bacterial meningitis animal model can be inoculated with bacteria in various ways, such as through the nose, vein and abdominal cavity. Host and bacterial factors, however, influence bacteremia and its invasion of the central nervous system. These methods do not therefore reliably induce meningitis. On the other hand, direct inoculation of bacteria into cerebrospinal fluid can produce intracranial infection within the expected duration. This study constructed a pneumococcal meningitis model by injecting *S. pneumoniae* into the posterior cistern of rats. The success and efficiency of the model were confirmed by the nervous

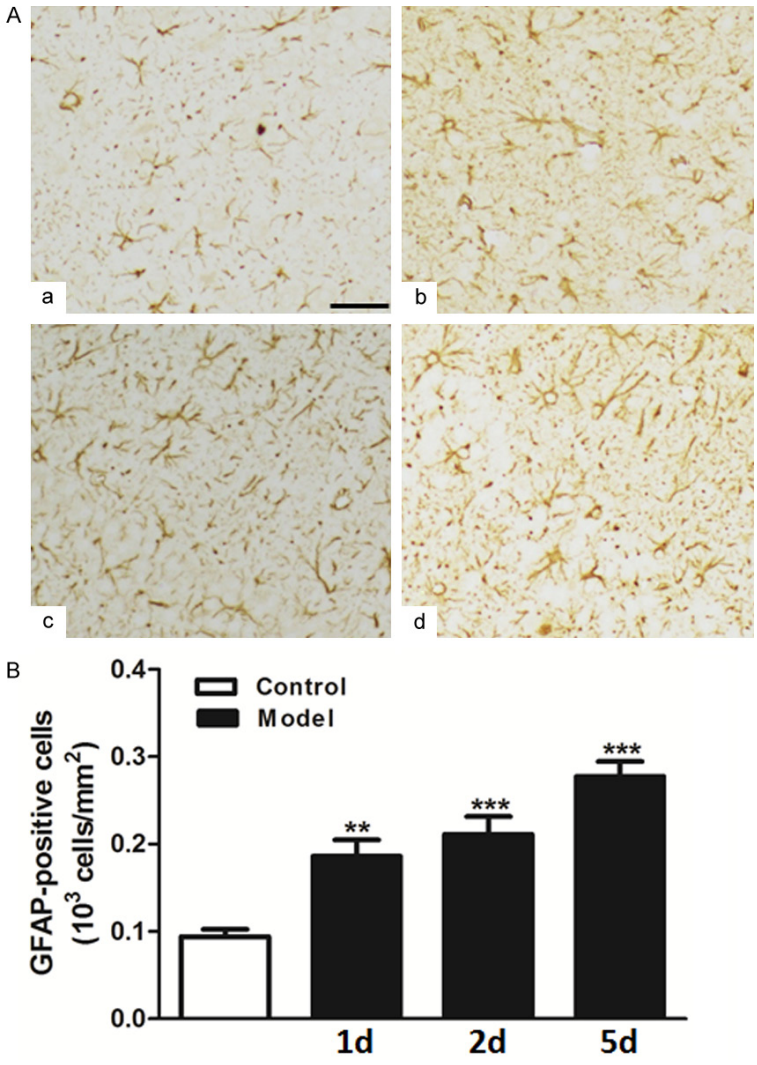

Figure 4. Effect of bacterial meningitis on astrocyte proliferation in rats. (A) Representative photomicrographs presenting GFAP immunopositive astrocytes in the cerebral cortex at 1, 2 and 5 days post-infection (scale bar, 50 μm) and (B) Quantification of results. (a) Control group, (b) 1 d group, (c) 2 d group, (d) 5 d group. n = 7-8 rats; **P < 0.01 and ***P < 0.001 relative to control group.

system score, weight loss of rats and cerebrospinal fluid culture. Histopathologic analysis revealed that inflammatory exudation into the subarachnoid space was consistent with the pathologic characteristics of human bacterial meningitis [27] and indicated neuronal damage in the cerebral cortex and hippocampus. The injury of hippocampal neurons can cause serious damage to memory and intelligence [28], and this could be the major contributing factor to the high incidence of learning and cognitive impairment in *S. pneumoniae* meningitis survivors.

Evidence from previous studies on human cases and bacterial meningitis animal models indicates that cerebral blood flow is diminished, and vascular volume status determines the degree of cerebral ischemia in meningitis [29-32]. Clinical data have indicated that cerebral ischemia is strongly associated with major neurological sequelae or death in children with intracranial infection [33]. This study revealed that the expression of $CysLT_1R$ and $CysLT_2R$ in the brain of rats is up-regulated following focal cerebral ischemia. The increased expression levels of these receptors were found to be mostly in the injured neurons, activated microglia, and proliferative astrocytes [34, 35]. The $CysLT_1R$ regulates microglial activation by agonists that induce ischemia [36, 37]. It also influences astrocyte proliferation in response to mild ischemic stimuli *in vitro* [38]. The previously mentioned evidence suggests that $CysLT_1R$ and $CysLT_2R$ mediate microgliosis, astrocytosis, and neuronal injury following focal cerebral ischemia. The findings of the current study indicated that the protein and mRNA expression levels of $CysLT_1R$ and $CysLT_2R$ change considerably following meningitis injury in rats. Our findings also suggested that *S. pneumoniae* stimulation activates proliferation of astrocytes and microglia. Morphologic changes of glial cells were accompanied by a time-dependent release of elevated levels of TNF-α. Pneumococcal meningitis disease progression is a complex process, involving a series of molecular and cellular events [39]. Mounting evidence has shown that TNF-α plays a role in the development of brain injury following infection with bacterial meningitis [22, 39]. Tumor necrosis factor *(TNF-α)* is released and synthesized by microglia, astro-

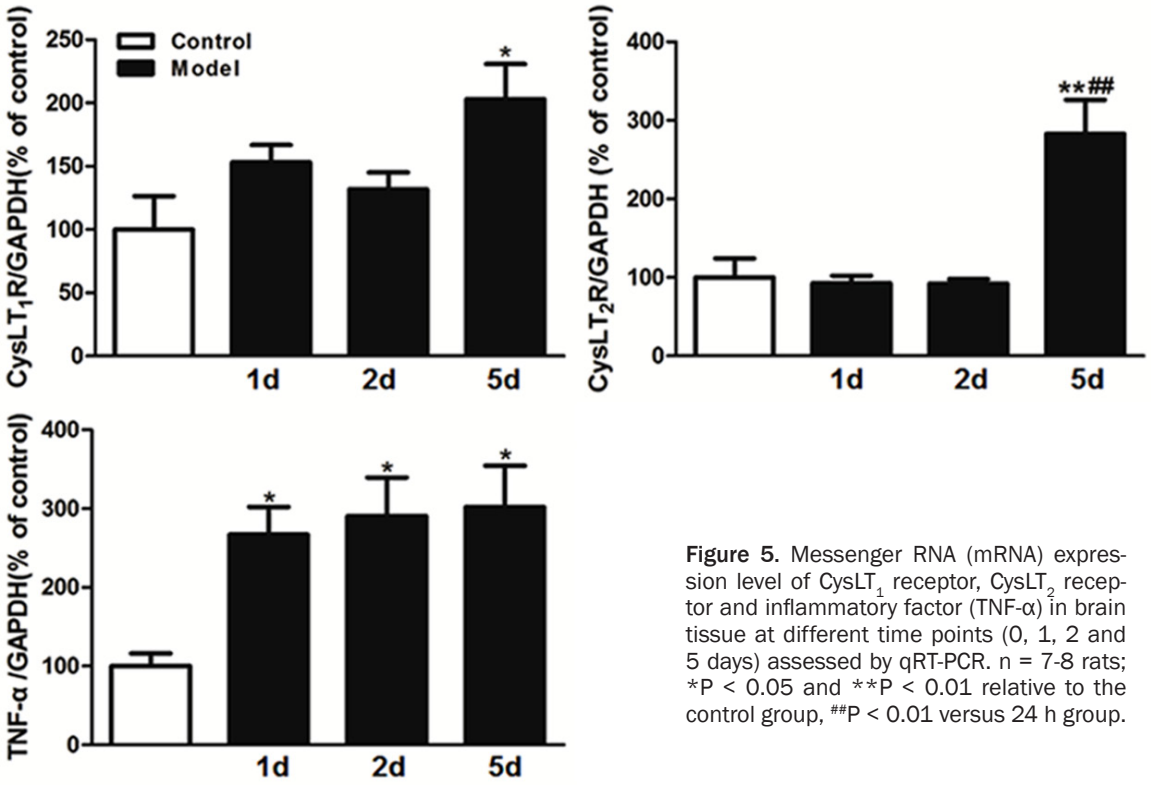

Figure 5. Messenger RNA (mRNA) expression level of $CysLT_1$ receptor, $CysLT_2$ receptor and inflammatory factor (TNF-α) in brain tissue at different time points (0, 1, 2 and 5 days) assessed by qRT-PCR. n = 7-8 rats; *P < 0.05 and **P < 0.01 relative to the control group, ##P < 0.01 versus 24 h group.

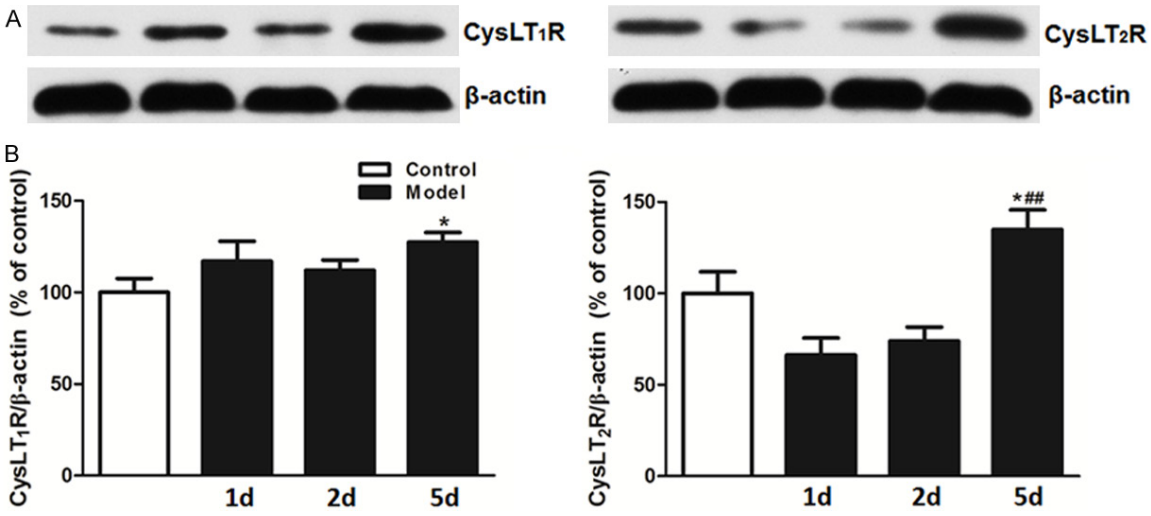

Figure 6. Protein expression level of $CysLT_1$ receptor and $CysLT_2$ receptor in brain tissue at different time points (0, 1, 2 and 5 days) assessed by western blotting. A. Representative western blotting for $CysLT_1$ receptor and $CysLT_2$ receptor expression. B. Quantitative analysis of the relative intensity of $CysLT_1$ receptor and $CysLT_2$ receptor protein in different groups. Data are reported as mean ± S.E.M; n = 7-8 rats; *P < 0.05 compared with the control group, ##P < 0.01 compared with the 24 h group, analyzed by one-way ANOVA.

cytes, and part of neurons [40]. These findings are in principle consistent with our studies. The expression level of TNF-α in brain tissue increased in the early stages of nervous system infection. This study, therefore, selected TNF-α as the standard marker to determine the extent of brain damage. The activation of microglia is usually the first step in the reaction of glial cells caused by pneumococcal products [41]. *In vitro* experiments confirmed that microglial activa-

tion modulates proliferation and hypertrophy of astrocytes and could even induce neuronal death [41, 42]. Following activation, microglia triggers the release of inflammation-promoting factors and cytokines, such as Interleukin 1 beta (IL-1β), TNF-α, Interleukin 6 (IL-6). The receptors of these molecules can be expressed in astrocytes, and the binding of the inflammatory factors promotes proliferation and hypertrophy of astrocytes [43]. Astrocytes also secrete some factors to facilitate either self-regulation or feedback regulation of microglia, which forms a feedback loop and the interaction between astrocytes and microglia. In addition, the regulation of microglia on astrocytes also includes inhibitory effects. Inhibition of microglial activation can also reduce the number of astrocytes [44], so microglia are one of the important regulatory factors of astrocyte activation. Taken together, it can be concluded that CysLTs involved in the process of pneumococcal meningitis may, through mediation of their receptors, participate in neuronal injury and glial cell proliferation.

On the other hand, in meningitis caused by *Escherichia coli* and group B streptococcus, $CysLT_1$ has been confirmed to induce invasion of human brain microvascular endothelial cells and penetration of the blood-brain barrier by bacteria [45, 46]. The findings of *in vivo* studies suggest that $CysLT_1$ expression is upregulated in the brain capillaries after *C. neoformans* infection and CysLTs contribute to *C. neoformans* penetration into brain [21]. A compelling body of evidence directly identifies CysLTs as novel host factors that play a vital role in the pathologic progression of meningitis. Our subsequent results showed (to be published) that intraperitoneal injection of CysLTR antagonists could effectively reduce the loss of neurons and the number of microglial/astrocyte after meningitis and improve the score of neurological symptoms in rats. The results of this study therefore further confirmed that CysLTR is related to neuronal injury, microglial inflammation and astrocyte proliferation in pneumococcal meningitis. However, the precise roles of $CysLT_1R$ and $CysLT_2R$, as well as the detailed mechanisms in pneumococcal meningitis, need further exploration.

In summary, the present preliminary results have for the first-time documented evidence on the expression characteristics of CysLTR in pneumococcal meningitis and provided a molecular biological basis for the pharmacological effects of CysLTR antagonists. These results form the basis for understanding the mechanism associated with the pathogenesis of pneumococcal meningitis and the search for new therapeutic drugs.

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Disclosure of conflict of interest

None.

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