

Hippocampal β 2-microglobulin mediates sepsis-induced cognitive impairment

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Abstract. Acute brain dysfunction is a frequent complication in sepsis patients and is associated with long-term neurocognitive consequences and increased mortality, yet the underlying mechanism remains unclear. Emerging evidence has suggested that β 2-microglobulin [a component of major histocompatibility complex (MHC) class I molecules] is involved in cognitive dysfunction in various neurological diseases. Therefore, the present study tested the hypothesis that β 2-microglobulin in the brain also mediates sepsis-induced cognitive impairment. In the present study, wild-type and antigen processing 1 (*Tap1*)-deficient mice (*Tap1*^{-/-}) were subjected to cecal ligation and puncture (CLP). Survival rate, cognitive function, and biochemical analysis were performed at the indicated time points. The data revealed that CLP induced anxiety-like behavior and impaired hippocampal-dependent contextual memory in wild-type mice, which was accompanied by hippocampal microglial activation, increased level of interleukin-1 β , and decreased concentrations of brain derived neurotrophic factor and postsynaptic density protein 95. Notably, it was demonstrated that *Tap1*^{-/-} mice with reduced cell surface expression of MHC I protected mice from anxiety-like behavior and impaired hippocampal-dependent contextual memory and reversed most of these biochemical

parameters following sepsis development. In summary, the results of the present study suggest that β 2-microglobulin negatively regulates cognitive impairment in an animal model of sepsis induced by CLP.

Introduction

It is reported that up to 70% of patients with severe sepsis exhibit symptoms of encephalopathy, including consciousness disturbance, impaired cognitive function, personality changes, and lack of concentration or somnolence (1-3). Although some patients can be resolved during hospitalization, sepsis-associated encephalopathy can cause long-term consequences, including prolonged length of hospital stay, long-term cognitive and functional decline, and increased morbidity and mortality (1-3). Various potential mechanisms, including oxidative stress, inflammation, neurotransmission disturbance, mitochondrial dysfunction, and cell death, have been proposed to be involved in the pathogenesis of sepsis-induced cognitive impairment (2-4), yet the precise mechanism remains largely to be determined.

β 2-microglobulin, a component of major histocompatibility complex class I (MHCI) molecules, is a low molecular weight protein (11,800 Da) and is considered to be a surrogate marker of putative middle-molecule uremic toxins (5). Notably, increased β 2-microglobulin in the systemic milieu is implicated in age-related decline in adult neurogenesis, and impairments in synaptic plasticity and cognitive function observed during ageing, as antigen processing 1 (*Tap1*)-deficient mice with reduced cell surface expression of MHC I mitigated these abnormalities (6,7). Moreover, it is reported that increased systemic soluble β 2-microglobulin levels are associated with cognitive impairments associated with chronic hemodialysis (8). In the brain, β 2-microglobulin can act independently of their canonical immune function to regulate neuronal signaling and activity-dependent changes in synaptic connectivity (9,10). These findings are in line with previous studies demonstrating that higher β 2-microglobulin levels are observed in the cerebrospinal fluid of patients with Alzheimer's disease or human immunodeficiency

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virus-associated dementia (11,12). However, the functional role of β 2-microglobulin in mediating sepsis-induced cognitive impairments has not yet been investigated.

In the present study, we therefore hypothesized that β 2-microglobulin negatively regulates memory and learning in a mouse model of sepsis induced by cecal ligation and puncture (CLP). Moreover, we explore the underlying molecular mechanisms.

Materials and methods

Animals. Thirty male wild-type C57BL/6 mice (22–25 g) were purchased from Nanjing University of Chinese Medicine and forty-two male transporter associated with antigen processing 1 (*Tap1*^{-/-}) mutant mice (22–26 g) were from the Jackson Laboratory (Bar Harbor, ME, USA). All studies were approved by the Institutional Animal Care and Use Committee of Nanjing University of Chinese Medicine, China and all experimental procedures and protocols used in the present study were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals from the National Institutes of Health. In the present study, only male mice were used. The animals were housed under a 12-h light/dark cycle in a temperature-controlled room of 22–24°C and 40–50% relative humidity with free access to food and water.

Animal model of sepsis. The sepsis was established by CLP as we previously described (4,13). Each mouse was anesthetized with 2% sodium pentobarbital in saline (40 mg/kg, intraperitoneally; Sigma-Aldrich, St. Louis, MO, USA). The cecum was isolated carefully and then ligated with 4.0 silk below the ileocecal junction, approximately 0.6 cm from the distal end. The cecum was then perforated twice with a sterile 22-gauge needle and was gently squeezed to extrude the fecal contents into the peritoneal cavity. The cecum was then returned to the peritoneal cavity and the laparotomy was closed with 4.0 silk sutures. For the animals that served as sham controls, the cecum was exposed in the same manner as CLP, but was neither ligated nor punctured. All mice received subcutaneous normal saline resuscitation (20 ml/kg of body weight), and antibiotic therapy (ertapenem, 20 mg/kg; Merck Research Laboratory, USA) begun immediately after the surgery and once daily for a total of 3 days. All mice were returned to their cages with free access to food and water. The flow chart for the experimental protocol was summarized in Fig. 1A.

Behavioral and cognitive tests. All behavioral tests were performed at 8:00 a.m. –11:00 a.m. in a sound-isolated room and subsequently analyzed by using a video-tracking system (Shanghai Mobile Datum Information Technology Company, Shanghai, China). All behavioral data were recorded by the same investigator who was blinded to the animal grouping as described in our previous studies (13,14).

Open field tests. On day 5 after operation, mice were gently placed in the center of a white plastic chamber (40x40x40 cm) for 5 min while exploratory behavior was automatically recorded by a video tracking system. The total distance and total time traveled in the open field arena were recorded. After each test, the arena was cleaned with 75% alcohol to avoid olfactory cues.

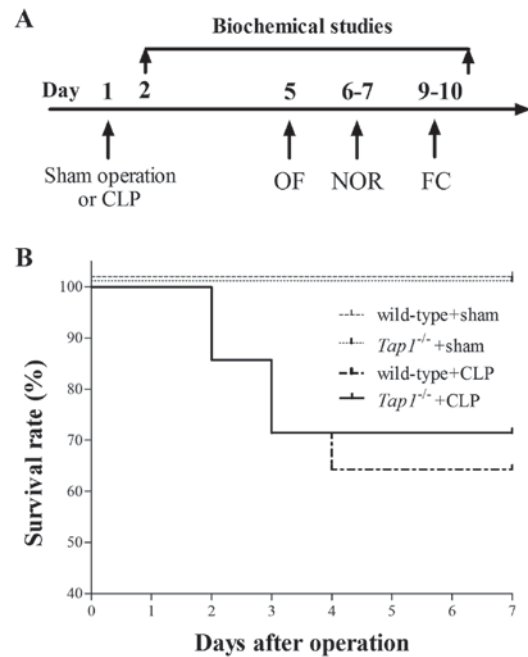


Figure 1. (A) Schematic timeline of the experimental procedure. (B) *Tap1*^{-/-} mice did not confer increased survival rate after CLP (n=12–14 per group). *Tap1*^{-/-}, antigen processing 1-deficient mice; CLP, cecal ligation and puncture; OF, open field; NOR, novel object recognition; FC, fear conditioning.

Novel object recognition test. Novel object recognition test was conducted on days 6–7 after operation to evaluate retention or intact memory as previously detailed (15). This test consisted of two trials. In the training trial, two familiar objects were presented. The testing trial included one familiar object and one novel object present in the respective zones of the open field, with 60-min intervals between trials, during which the animals were placed back to their home cages. The time spent with each object was recorded, and the cognitive outcomes were determined by the ‘discrimination index’ for the testing trial, which was calculated using the formula: % discrimination index = time spent in novel object zone x 100 / (time spent in familiar object zone + time spent in novel object zone).

Fear conditioning test. The fear conditioning tests were performed on days 9 and 10 after operation as previously described (13). In the training section, each mouse was allowed to explore the fear conditioning test chamber for 3 min before the onset of a 30-sec tone (70 db, 3 kHz), followed by a 2-sec footshock (0.7 mA). Then, the mice remained in the chamber for another 30 sec and were then returned to their home cages. After 24 h, the animals were placed in the same chamber in which they were trained and were observed for 5 min without tone or footshock presentation. The auditory-cued fear test was performed 2 h later. The mice were placed in an altered chamber (i.e., a different shaped chamber, odor, no grid floor) and allowed to explore for 3 min. After that, the tone was delivered, and their freezing behavior was scored for an additional 3 min. Freezing behavior was defined as the absence of all visible movement, excluding respiration. Cognitive impairment was assessed by measuring the amount of time the mouse demonstrated ‘freezing behavior’, which is defined as a completely immobile posture except for respiratory efforts.

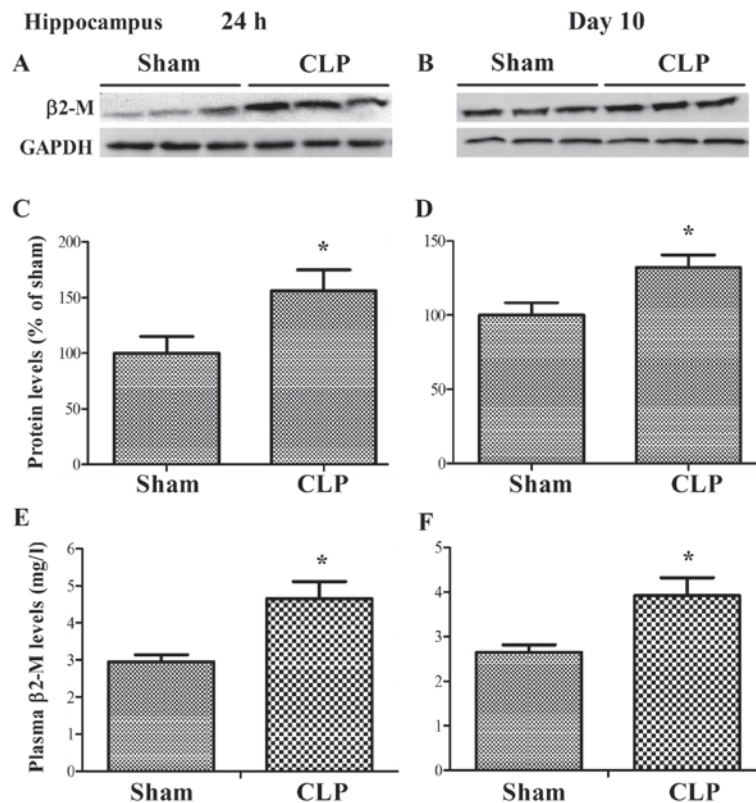


Figure 2. Sepsis increased hippocampal and plasma levels of $\beta 2$ -microglobulin. (A and B) CLP caused a sustained increase of $\beta 2$ -microglobulin expression in the hippocampus in wild-type mice at 24 h and 10 days after CLP. (C and D) Quantification of hippocampal $\beta 2$ -microglobulin expression. (E and F) Plasma levels of $\beta 2$ -microglobulin also significantly increased in CLP mice as compared with sham mice at 24 h and 10 days after CLP. Data are shown as mean \pm SEM (n=5-6 per group), *P<0.05 vs. wild-type + sham group. CLP, cecal ligation and puncture.

Measurement of plasma level of $\beta 2$ -microglobulin. The plasma levels of $\beta 2$ -microglobulin were determined at a laboratory by standard antibody-based multiplex immunoassays on the basis of the principles of immunoassay as described by the manufacturers in our institution (XieHe, Beijing, China).

Western blot analysis. Mouse hippocampi were dissected after perfusion of animals, snap frozen and lysed in RIPA lysis buffer (500 mM Tris, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS, and complete protease inhibitors; Roche, Basel, Switzerland). Tissue lysates were mixed with 4xNuPage LDS loading buffer and loaded on a 4-12% SDS polyacrylamide gradient gel (both Invitrogen, Carlsbad, CA, USA) and subsequently transferred onto a nitrocellulose membrane. Membranes were blocked with 5% skim milk in Tris-buffered saline tween for 1 h and then incubated with anti- $\beta 2$ -microglobulin (1:2,000; cat. no. ab75853; Abcam, Cambridge, UK), anti-BDNF (1:1,500; Santa Cruz Biotechnology Inc., Dallas, TX, USA), anti-PSD95 (1:1,000), and anti-GADPH (1:5,000; both Cell Signaling Technology, Boston, MA, USA) overnight at 4°C temperature room. Horseradish peroxidaseconjugated secondary antibodies and an enhanced chemiluminescence (ECL) kit (GE Healthcare, Uppsala, Sweden) were used to detect protein signals. The bands were detected with Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Rockford, IL, USA) and semiquantified with image J software (version 1.50i; National Institutes of Health, Bethesda, MD, USA).

Enzyme-linked immunosorbent assay (ELISA). The hippocampus was then separated, weighed and placed in a homogenizer. The tissue was homogenized with 1 ml ice-cold physiological saline per 100 mg brain tissue. Hypothermal centrifugation was performed at 5,000 \times g for 10 min and the supernatant was obtained. Standard curves for all cytokines (in duplicates) were generated using the reference cytokine concentrations supplied. The quantifications of tumor necrosis factor α (TNF- α), interleukin (IL)-1 β , IL-6, and BDNF were done by the instructions of the manufacturers (JianCheng Biotechnology, Nanjing, China). The readings were normalized to the amount of standard protein.

Immunohistochemistry. The brains were histologically analyzed using paraffin-embedded sections. Microglia in the hippocampus were evaluated by immunohistochemical staining 24 h and 10 days after operation. The sections were deparaffinized, washed and incubated with IBA1 antibody (1:1,000; Abcam) and biotinylated secondary antibody. The IBA1-positive cells in the mouse hippocampus were counted manually in five randomly selected areas by an investigator who was blinded to the animal grouping. Six brains from each group were used for immunohistochemistry analysis and six brain sections of 5 μ m thickness were examined in each brain.

Statistical analysis. Statistical analysis was performed using the SPSS 16.0 software for Windows (SPSS, Inc., Chicago, IL, USA). Data are expressed as mean \pm S.E.M. Means between two groups were compared with independent student's t-test.

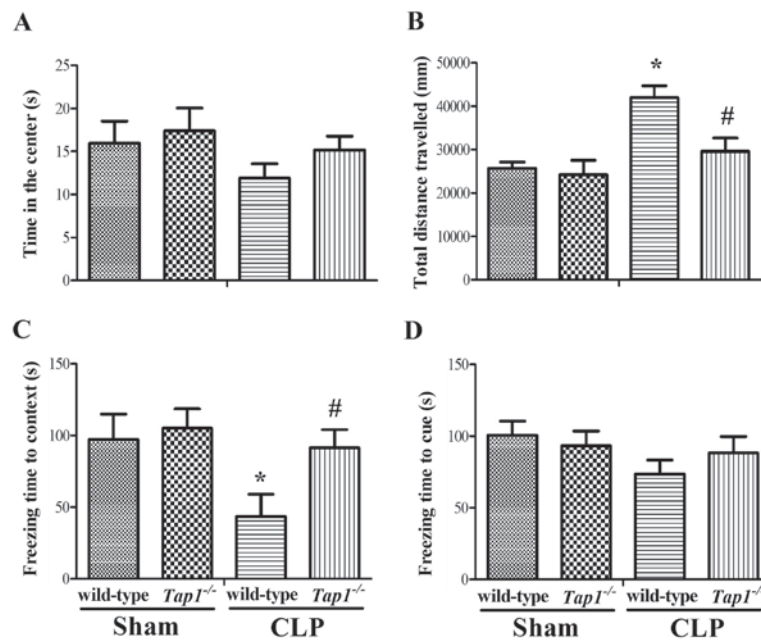


Figure 3. *Tap1*^{-/-} mice protected sepsis-induced contextual memory impairment. (A) No significant difference was observed in time spent in the center among the four groups. (B) Wild-type mice had significantly increased total distance traveled in the open field test as compared with *Tap1*^{-/-} mice subjected to CLP. (C) Wild-type mice displayed decreased freezing time in the contextual fear conditioning compared with *Tap1*^{-/-} mice after CLP. (D) No difference in the freezing time in the hippocampal-independent cued test was observed among groups. Data are shown as mean \pm SEM (n=10–12 per group), *P<0.05 vs. wild-type + sham group, #P<0.05 vs. *Tap1*^{-/-} + CLP group. *Tap1*^{-/-}, antigen processing 1-deficient mice; CLP, cecal ligation and puncture.

Comparisons of means from multiple groups were assessed by one-way analysis of variance (ANOVA) followed by a Tukey test. The 7-day survival rate was compared by the log-rank test by a researcher who was blinded to the group assignments. Bivariate relationships were evaluated using Pearson correlation coefficients. P<0.05 was considered to indicate a statistically significant difference.

Results

Tap1^{-/-} mice did not confer increased survival rate after CLP. There were five animals in the wild type and four animals in the *Tap1*^{-/-} mice subjected to CLP died within 7 days after operation. Deficient in *Tap1*^{-/-} that result in lower expression of β 2-microglobulin did not increase the survival rate after sepsis development (P>0.05, Fig. 1C).

Sepsis increased plasma and hippocampal levels of β 2-microglobulin. As shown in Fig. 2, CLP caused a sustained increase of β 2-microglobulin expression in the hippocampus in wild-type mice at 24 h and 10 days after CLP (24 h: t=-2.313, P=0.049; 10 days: t=-2.667, P=0.029, independent t test). Likewise, plasma levels of β 2-microglobulin also significantly increased in CLP mice as compared with sham mice at 24 h and 10 days after CLP (24 h: t=-3.357, P=0.007; 10 days: t=-2.87, P=0.017, independent t test).

Tap1^{-/-} mice protected sepsis-induced cognitive impairment. Next, we determined whether β 2-microglobulin is functionally involved in sepsis-induced cognitive impairment after CLP. Wild-type and *Tap1*^{-/-} mice that had previously been subjected to CLP or sham operation were analyzed sequentially by open-field, novel object recognition test, and fear conditioning tests.

As shown in Fig. 3A, there was no significant difference in time spent in the center among the four groups (one-way ANOVA; F (3, 40)=1.014, P=0.396). However, we found that wild-type mice had significantly increased total distance traveled in the open field arena as compared with *Tap1*^{-/-} mice subjected to CLP (one-way ANOVA; F (3, 40)=5.742, P<0.001), suggesting sepsis induced anxiety-like behavior.

The fear conditioning test was performed to assess whether β 2-microglobulin deficiency could improve the ability of mice to learn and remember an association between environmental cues and aversive experiences. The wild-type mice exhibited decreased freezing time in the contextual fear conditioning compared with *Tap1*^{-/-} mice after CLP (F (3, 40)=3.066, P=0.039, Fig. 3C), suggesting increased β 2-microglobulin level induced by sepsis might contribute to memory dysfunction. However, there was no significant difference in the freezing time in the hippocampal-independent cued test among groups (F (3, 40)=1.188, P=0.326, Fig. 3D).

The social interaction test is used to assess working memory. There was a general tendency of the *Tap1*^{-/-} mice to spend more time with the novel object as compared with wild-type mice subjected to CLP (familiar, F (3, 40)=0.849; novel, F (3, 40)=1.827; discrimination index, F (3, 40)=0.703, P=0.556; Fig. 4), although this phenomenon did not reach the level of statistical significance and also was not influenced by CLP.

Tap1^{-/-} Mice decrease sepsis-induced inflammatory response in the hippocampus. We determined proinflammatory cytokines including TNF- α , IL-1 β , and IL-6 in the hippocampus. Wild type mice subjected to CLP had significantly increased levels of IL-1 β in the hippocampus (F (3, 20)=9.364, P<0.001, Fig. 5C), while *Tap1*^{-/-} mice did not show a similar increase. Supporting the ELISA results, CLP induced significantly

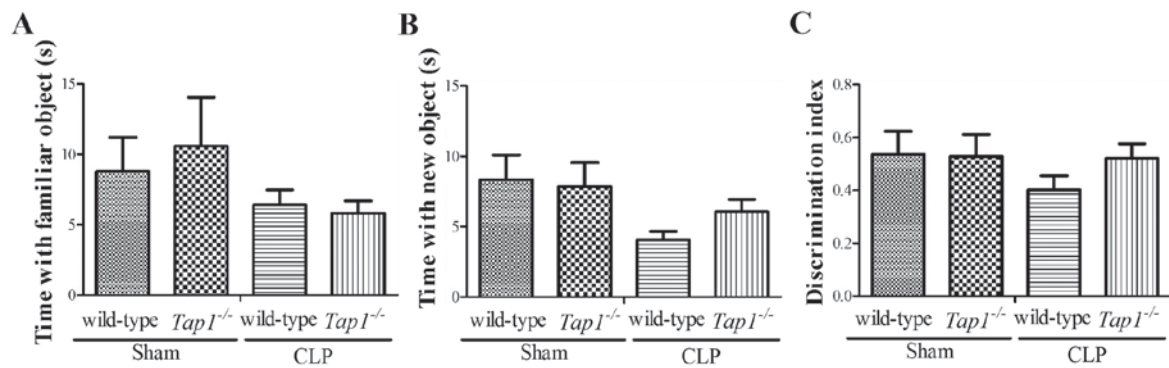


Figure 4. *Tap1*^{-/-} mice or CLP did not affect working memory performance. Neither *Tap1*^{-/-} deficiency nor CLP affected the time with the familiar (A), novel object (B), or discrimination index (C) among the four groups. Data are shown as mean \pm SEM (n=10-12 per group). *Tap1*^{-/-}, antigen processing 1-deficient mice; CLP, cecal ligation and puncture.

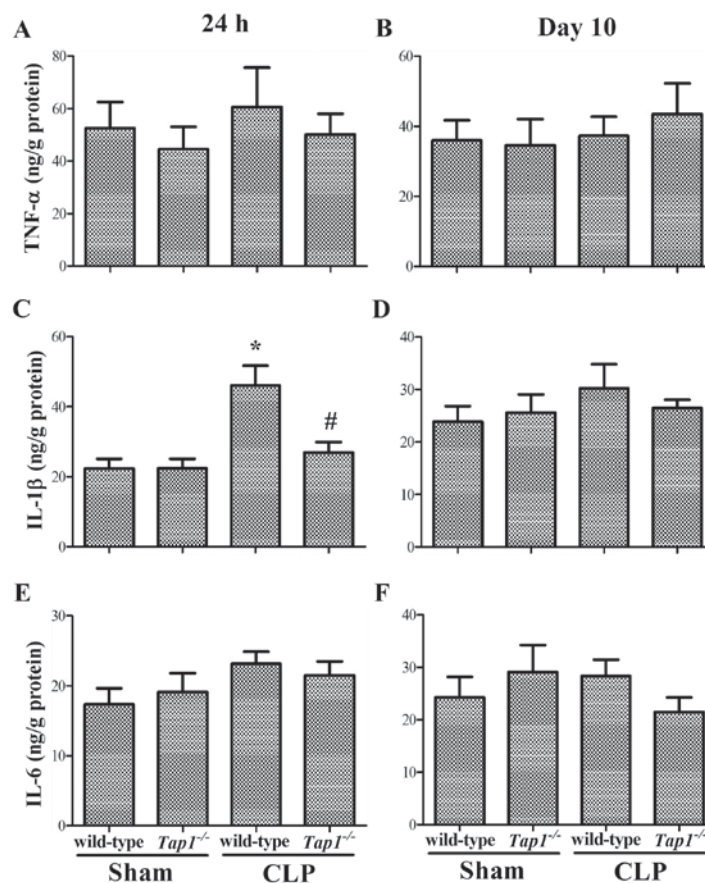


Figure 5. *Tap1*^{-/-} mice decrease sepsis-induced IL-1 β level in the hippocampus. (A and B) Neither *Tap1*^{-/-} deficiency nor CLP affected hippocampal levels of TNF- α at 24 h or 10 days. (C and D) CLP induced significant increase levels of IL-1 β in the hippocampus at 24 h but not 10 days in wild-type mice after CLP, which was not observed in *Tap1*^{-/-} mice. (E and F) Neither *Tap1*^{-/-} deficiency nor CLP affected hippocampal levels of IL-6 at 24 h or 10 days. Data are shown as mean \pm SEM (n=6 per group), *P<0.05 vs. wild-type + sham group, #P<0.05 vs. *Tap1*^{-/-} + CLP group. IL, interleukin; *Tap1*^{-/-}, antigen processing 1-deficient mice; CLP, cecal ligation and puncture.

microglia activation in wild-type mice, which was significantly attenuated in *Tap1*^{-/-} mice at 24 h (CA1: $F_{(3,20)}=1.91$, $P=0.160$; CA3: ($F_{(3,20)}=12.612$, $P<0.001$; DG: ($F_{(3,20)}=11.087$, $P<0.001$, Fig. 6) but not 10 days (data not shown) after CLP, indicating that $\beta 2$ -microglobulin might contribute to the neuroinflammatory reaction after sepsis development. However, we did not detect any difference in hippocampal levels of TNF- α and IL-6 among groups ((TNF- α , 24 h: ($F_{(3,20)}=0.387$, $P=0.764$, Fig. 5A; 10 days: ($F_{(3,20)}=0.317$, $P=0.813$),

Fig. 5B; IL-1 β , 10 days: ($F_{(3,20)}=0.652$, $P=0.591$), Fig. 5D; IL-6, 24 h: ($F_{(3,20)}=1.368$, $P=0.281$, Fig. 5E); 10 days: ($F_{(3,20)}=0.876$, $P=0.470$, Fig. 5).

Tap1^{-/-} mice protected sepsis-induced BDNF and PSD-95 loss in the hippocampus. We further analyzed changes of synaptic related proteins in the hippocampus, known to be critical for spatial memory formation. Wild-type mice subjected to CLP had lower hippocampal levels of BDNF and PSD-95

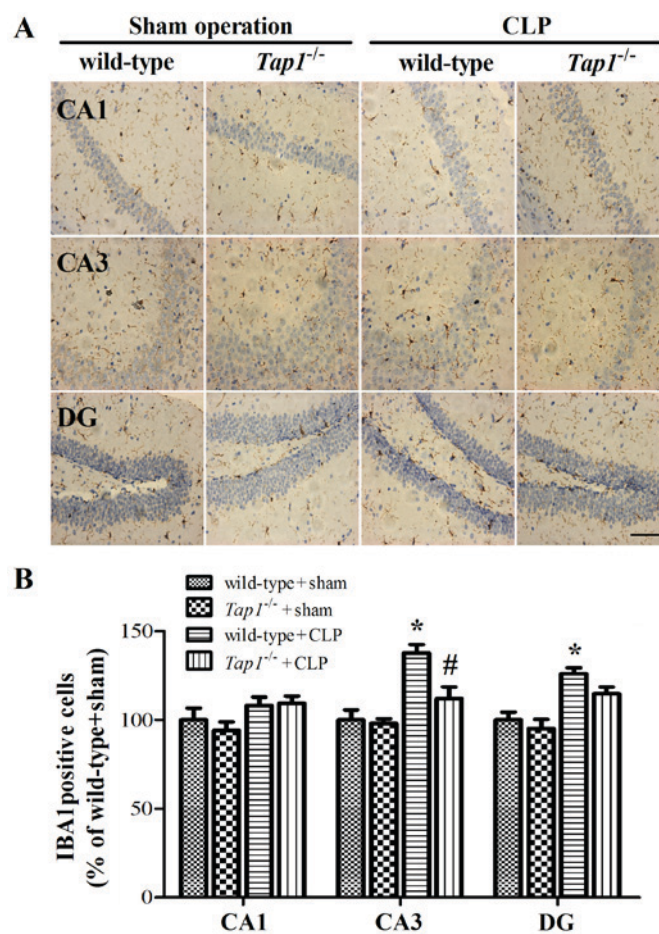


Figure 6. (A) *Tap1*^{-/-} mice decrease sepsis-induced microglia activation in the hippocampus. (B) CLP induced significantly microglia activation in CA3 and DG in the hippocampus in wild-type mice, which was significantly attenuated in *Tap1*^{-/-} mice at 24 h after CLP. Data are shown as mean \pm SEM (n=6 per group). Scale bar, 50 μ m. *P<0.05 vs. wild-type + sham group, #P<0.05 vs. *Tap1*^{-/-} + CLP group. *Tap1*^{-/-}, antigen processing 1-deficient mice; CLP, cecal ligation and puncture.

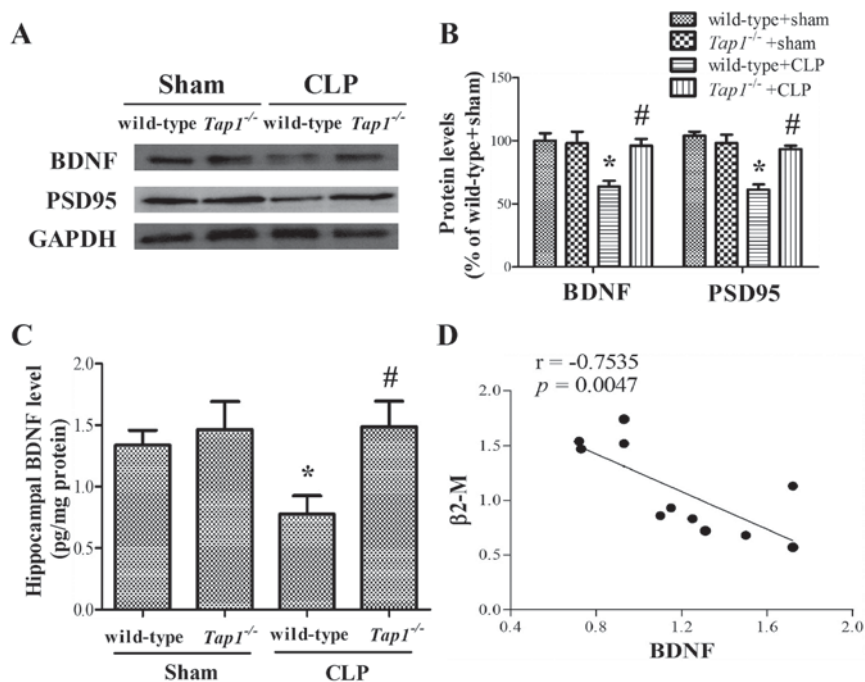


Figure 7. *Tap1*^{-/-} mice protected sepsis-induced synaptic related protein loss in the hippocampus. (A and B) Wild-type mice subjected to CLP decreased the BDNF and PSD-95 levels in the hippocampus, whereas the reduction of the BDNF and PSD-95 levels was not observed in *Tap1*^{-/-} mice with the same stimulus. (C) Sepsis-induced decrease of hippocampal level of BDNF was more pronounced in wild-type than in *Tap1*^{-/-} mice. (D) correlation analysis showed that the expression of BDNF was negatively correlated with β 2-microglobulin expression in the hippocampus. *P<0.05 vs. wild-type + sham group, #P<0.05 vs. *Tap1*^{-/-} + CLP group. *Tap1*^{-/-}, antigen processing 1-deficient mice; CLP, cecal ligation and puncture.

levels (BDNF: ($F_{(3,17)}=3.454$, $P=0.046$; PSD-95: ($F_{(3,17)}=4.75$, $P=0.014$), whereas the reduction of the BDNF and PSD-95 was not observed in *Tap1*^{-/-} mice with the same stimulus. Consistently, ELISA results showed that the sepsis-induced decrease of hippocampal level of BDNF was more pronounced in wild-type than in *Tap1*^{-/-} mice ($F_{(3,28)}=3.368$, $P=0.032$, Fig. 7C). In addition, correlation analysis showed that the expression of BDNF was negatively correlated with $\beta 2$ -microglobulin expression in the hippocampus ($r=-0.7535$, $P=0.0047$, Fig. 7C).

Discussion

It has been demonstrated that the brain is one of the first organs affected during sepsis, which leads to neurological complications, such as sepsis-associated encephalopathy (2-4). Compared to the widely used lipopolysaccharide model, the CLP model is clinically relevant because it has a feature shared with human sepsis-induced cognitive impairment (4,13,14). Although many mechanisms, including oxidative stress, endothelial dysfunction, inflammation, unbalanced neurotransmission, mitochondrial dysfunction, and cell death have been implicated in the pathogenesis of sepsis-associated cognitive impairment (16-20), the precise mechanism remains to be elucidated. In this study, we showed that $\beta 2$ -microglobulin deficiency protected mice from sepsis-induced neurobehavioral and biochemical abnormalities, suggesting $\beta 2$ -microglobulin may serve as a therapeutic target for sepsis-associated cognitive impairment.

Class I MHC molecules, known to be important for immune responses to antigen, are also expressed by neurons that undergo activity-dependent, long-term structural, and synaptic modifications (9). Accumulating evidence has demonstrated increased $\beta 2$ -microglobulin level in the systemic milieu is associated with age-related decline in adult neurogenesis, and impairments in synaptic plasticity and cognitive function observed during ageing (6,7). In dialysis patients, $\beta 2$ -microglobulin is considered to be a potential marker of inflammation and could be served as a predictor of mortality (5,21). Moreover, higher serum $\beta 2$ -microglobulin level may be a result of increased inflammation such as in cardiovascular diseases in end-stage renal disease patients (22). Based on the observation that higher $\beta 2$ -microglobulin level is negatively associated with cognitive performance, we take advantage of *Tap1*^{-/-} mice with reduced cell surface expression of MHC I to address the functional role of $\beta 2$ -microglobulin in sepsis-induced cognitive impairment. In our study, the detrimental effects of $\beta 2$ -microglobulin on cognition were confirmed by anxiety-like behavior and impaired hippocampal-dependent contextual memory. Although we did not observe that $\beta 2$ -microglobulin deficiency protected working memory dysfunction after CLP, our results collectively support the hypothesis that $\beta 2$ -microglobulin can negatively affect sepsis-induced cognitive impairment. However, the mechanisms by which $\beta 2$ -microglobulin negatively induced cognitive impairments remain unclear. Previous studies suggest that $\beta 2$ -microglobulin is a marker of low-grade inflammation (21-23). This notion is supported by our data that analysis of the brain innate immune system revealed a subtle but sustained activation of microglia that was detected in wild-type mice but not in *Tap1*^{-/-} mice, which was accompanied by a distinct regulation of cytokine levels including IL-1 β . Due to the reported negative impact of IL-1 β on learning and

memory function, as well as on LTP (24,25), the reduced level of IL-1 β in *Tap1*^{-/-} mice may partly explain the observed protection.

Given the important role of $\beta 2$ -microglobulin in regulation of the activity-dependent remodeling and plasticity of connections in the developing and mature mammalian central nervous system, we next studied the effects of CLP and $\beta 2$ -microglobulin deficiency on the regulation of neurotrophic factor BDNF and synaptic related protein PSD-95, which have key functions for learning and memory (26-28). It has been demonstrated that LPS induced significant reduction of BDNF and PSD-95 contribute to memory deficits (29,30). In line with the findings presented in this study, wild-type mice with decreased PSD-95 and BDNF expressions after sepsis development showed impaired memory performance (31,32). Importantly, $\beta 2$ -microglobulin deficiency protected from PSD-95 and BDNF loss. In addition, we can not rule out that other mechanism are also involved in the detrimental effects of $\beta 2$ -microglobulin in sepsis. Therefore, future studies are required to elucidate the specific mechanism by which $\beta 2$ -microglobulin exerts its effects in sepsis-associated cognitive impairment.

In conclusion, our study suggests that the detrimental role of the $\beta 2$ -microglobulin in cognitive impairment after sepsis development, and possibly the usefulness of $\beta 2$ -microglobulin as a therapeutic target for sepsis-induced long-term cognitive impairment. However, future studies should evaluate whether these results can be replicated in more heterogeneous samples and in different clinical settings.

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Competing interests

The authors declare that they have no competing interests

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