Molecular Medicine

HMGB1 Mediates Cognitive Impairment in Sepsis Survivors

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Severe sepsis, a syndrome that complicates infection and injury, affects 750,000 annually in the United States. The acute mortality rate is approximately 30%, but, strikingly, sepsis survivors have a significant disability burden: up to 25% of survivors are cognitively and physically impaired. To investigate the mechanisms underlying persistent cognitive impairment in sepsis survivors, here we developed a murine model of severe sepsis survivors following cecal ligation and puncture (CLP) to study cognitive impairments. We observed that serum levels of high mobility group box 1 (HMGB1), a critical mediator of acute sepsis pathophysiology, are increased in sepsis survivors. Significantly, these levels remain elevated for at least 4 wks after CLP. Sepsis survivors develop significant, persistent impairments in learning and memory, and anatomic changes in the hippocampus associated with a loss of synaptic plasticity. Administration of neutralizing anti-HMGB1 antibody to survivors, beginning 1 wk after onset of peritonitis, significantly improved memory impairments and brain pathology. Administration of recombinant HMGB1 to naïve mice recapitulated the memory impairments. Together, these findings indicate that elevated HMGB1 levels mediate cognitive decline in sepsis survivors, and suggest that it may be possible to prevent or reverse cognitive impairments in sepsis survivors by administration of anti-HMGB1 antibodies.

INTRODUCTION

Severe sepsis, the clinical syndrome that complicates infection and injury, has an incidence of more than 750,000 cases per year, and kills more than 210,000 individuals in the United States annually (1–3). Although it is the leading cause of hospitalized death, the therapeutic choices are limited, and the only FDA-approved therapy, "activated protein C," recently was pulled from the market. Moreover, the significance of this problem extends long after hospitalization, because survivors of severe sepsis develop complications of nonresolving inflammation that persist following discharge from the hospital (4). Survivors are frequently unable to return to work, and develop significant cognitive impairment, immunological dysfunction and poor quality of life indicators (1,5). More than half of the 540,000 severe sepsis survivors discharged annually from the hospital are dead within 5 years (3). There is clearly a significant need to understand mechanisms that can mediate cognitive impairment in severe sepsis survivors.

The pathogenesis of severe sepsis and associated organ damage is attributable to cytokines and other pathogenic mediators. Inflammation in tissues can persist long after the inciting trauma or infection (a phenomenon termed "nonresolving inflammation") that significantly impairs organ function (6). There has been a long-standing interest in targeting specific molecular mechanisms for therapeutic benefit. Indeed, since the demonstration that monoclonal anti-tumor necrosis factor (anti-TNF) antibodies can prevent lethal septic shock in bacteremic baboons, there have been more than 11,500 publications addressing the role of cytokines in causing organ dysfunction during severe sepsis. A major limitation of this antibody approach in the clinic is that the excessive production of cytokines is established early after the onset of infection or injury (7). Indeed, by the time severe sepsis is recognized, the majority of patients do not have elevated TNF levels; rather, levels of HMGB1, and other late mediators of sepsis are elevated significantly. Importantly, serum HMGB1 levels remained elevated in sepsis survivors at the time of hospital discharge (8), and, until now, the impor-

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tance of elevated HMGB1 levels in sepsis survivors has not been studied.

In considering the hypothesis that HMGB1 mediates cognitive decline in sepsis survivors, previous studies indicate that TNF, IL-1 and HMGB1 occupy critical roles in modulating neuronal activity underlying cognition and behavior. TNF modulates the expression of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) and *N*-methyl-D-aspartate receptors (NMDARs) on neurons, which are critical in learning and memory (9). IL-1β and HMGB1 also modulate NMDARs, with functional consequences that can contribute to altered memory (10,11). HMGB1 regulates neuronal and dendritic growth, and, as do TNF and IL-1, induces sickness behavior associated with fever, anorexia, withdrawal and impaired cognition. Administration of anti-TNF antibody and anti-IL-1 antibody in acute murine injury and infection models significantly improves cognitive abnormalities (12,13). Clinical studies of serum cytokine levels in severe sepsis survivors indicate that HMGB1 levels are increased significantly at the time of hospital discharge, whereas TNF and IL-1 levels are not (8,14).

Accordingly, here we established severe sepsis in mice to study the role of HMGB1 in mediating cognitive dysfunction in the survivors. The results show that serum HMGB1 levels are elevated significantly for weeks after sepsis. Whereas animals exhibit outwardly normal behavior, cognitive testing reveals significant impairments in learning and memory. As administration of neutralizing anti-HMGB1 monoclonal antibody to sepsis survivors confers significant protection against memory impairment, and administration of rHMGB1 to naïve mice induces cognitive impairment, these results implicate a pathogenic role of HMGB1 in sepsis survivors.

MATERIALS AND METHODS

Animals

Experiments with male BALB/c mice (6–8 wks old, Charles River Laboratories,

Wilmington, MA, USA) were performed in accordance with the National Institutes of Health (NIH) Guidelines under protocols approved by the Institutional Animal Care and Use Committee (IACUC) Committee of the Feinstein Institute for Medical Research, North Shore-LIJ Health System, Manhasset, New York, USA.

Surgery for Cecal Ligation and Puncture (CLP)

Severe polymicrobial sepsis was induced by CLP (15). Mice were anesthetized using ketamine (100 mg/kg) and xylazine (8 mg/kg) administered intramuscularly (i.m.). A midline incision was made to expose the cecum. After ligation with a 4-0 silk ligature below the ileocecal valve, the cecum was punctured once with a 22-gauge needle and stool (approximately 1 mm) was extruded from the hole. The perforated cecum was placed back into the abdominal cavity, and the incision was closed with two layers of 6-0 Ethilon running sutures. All animals received normal saline resuscitation (20 mL/kg of body weight, injected subcutaneously [s.c.]), and a single dose of antibiotics (Primaxin, 0.5 mg/mouse in 200 μL sterile saline, injected s.c.) (Merck & Co. Inc., Whitehouse Station, NJ, USA) immediately after CLP. Sham-operated animals had the cecum isolated and then returned to the peritoneal cavity, without being ligated or punctured. These animals also received an antibiotic treatment and resuscitative fluid as described above.

Serum HMGB1 Determination

Mice were subjected to CLP surgery or sham surgery as described above, and the survivors were monitored for 12 wks. To determine levels of HMGB1 in the serum, mice were euthanized at indicated time points, and blood was collected by cardiac puncture. Serum HMGB1 concentrations were determined by Western immunoblotting analysis as described previously (16). In brief, serum samples (100–200 μL) were ultrafiltered with Centricon 100 (EMD Millipore Corporation, Billerica, MA, USA). The eluate was fractionated by SDS-PAGE, transferred to a

poly(vinylidene difluoride) immunoblot membrane (Bio-Rad Laboratories, Hercules, CA, USA), and probed with either specific anti-HMGB1 antiserum (1:250 dilution) or purified IgG from anti-HMGB1 antiserum (5 μg/mL) for Western blot analysis. Polyclonal anti-HMGB1 IgG was purified by using protein-A agarose, according to the manufacturer's instructions (Pierce Biotechnology Inc., Rockford, IL, USA). Western blots were scanned with a Bio-Rad image analyzer (Bio-Rad), and the levels of HMGB1 were determined by reference to standard curves generated with purified HMGB1.

Administration of Anti-HMGB1 Monoclonal Antibody

For antibody administration, animals subjected to CLP surgery were randomized into two groups after 7 d. Anti-HMGB1 monoclonal antibody (IgG2b) or isotype control antibody (50 μg/mouse, intraperitoneally [i.p.]) was administered once a day on 3-d intervals (d 7, 9 and 11 after CLP). Animals were followed for 4 wks, and subjected to behavioral assessment.

HMGB1 Administration to Naïve Mice

Recombinant HMGB1 was prepared as described previously (15). Contaminating lipopolysaccharide (LPS) from protein preparations was removed by Triton X-114 extraction as described previously (16). Residual LPS is less than 1 pg/μg protein. Purified recombinant HMGB1 or vehicle was administered to 6–8 wk-old healthy BALB/c mice (500 μg diluted in 350 μL of PBS 1 × every day, i.p.) for 3 wks. Animals were subjected to behavioral assessment 1 wk later.

Behavioral Assessment

The behavioral procedures have been described previously (17). All testing occurred between 10:00 and 18:00 h and the mice were between 10 wks and 6 months old. Each mouse was subjected to a multistage assessment consisting of a primary screen, adapted from Irwin (1968) (18) and the first stage of the SHIRPA procedure (18,19), an open-field test that measured spontaneous locomotion, a ro-

Table 1. Primary screen variables contributing to the evaluation of specific functions.

Function	Variables		
1. Muscle and spinal function	Abdominal tone Body position Body tone Contact righting Defecation Gait	Grip strength Limb grasping Limb tone Pelvic elevation Positional passivity Righting reflex	Spontaneous activity Tail elevation Trunk curl Urination Visual placing Wire maneuver
2. Spinocerebellar function	Abdominal tone Body position Body tone Contact righting Gait	Grip strength Limb grasping Limb tone Pelvic elevation	Righting reflex Tail elevation Trunk curl Visual placing
3. Sensory function	Corneal reflex Gait Negative geotaxis Pinna reflex	Provoked biting Righting reflex Toe pinch	Touch escape Transfer arousal Visual placing
4. Neuropsychiatric function	Abnormal behavior Aggressivity Body position Body tone Contact righting Fear Irritability	Latency to move Locomotion Negative geotaxis Positional passivity Provoked biting Righting reflex Spontaneous activity	Startle response Touch escape Transfer arousal Tremor Vocalizations Wire maneuver
5. Autonomic function	Defecation Heart rate Lacrimation Palpebral closure	Piloerection Respiratory rate Salivation Skin color	Startle response Tail elevation Urination

tarod test that measured motor coordination, a black-and-white alley test that measured anxiety (20) and a navigational test that measured spatial working memory, modified from Deacon and Rawlins (21). Each of these tests was separated by at least 1 d.

The primary screen (Table 1) started with anatomical parameters (coat length, hair length and hair morphology), followed by observation in a cylindrical glass flask (height 15 cm, diameter 11 cm), which measured body position, spontaneous activity, respiratory rate, tremor occurrence, defecation and urination. Transfer to an arena (55 cm × 33 cm) allowed for measuring of transfer arousal, latency to move in the arena and locomotion in the arena. This was continued with manipulations for measuring piloerection, palpebral closure, startle response, gait, pelvic elevation, tail elevation, touch escape, positional passivity, trunk curl, limb grasping, visual placing, grip strength, body tone, pinna reflex, corneal reflex, toe pinch, body length, tail length, lacrimation, whisker morphology, provoked biting, salivation, heart rate, abdominal tone, skin color and limb tone. Measuring several reflexes (wire maneuver, righting reflex, contact righting, negative geotaxis) completed the screen. Throughout the screen, incidences of fear to the experimenter, irritability, aggressivity to the experimenter, vocalizations and abnormal behavior were recorded. Finally, body weight was measured. The observed parameters were grouped according to five functional categories, which were: muscle and spinal function; spinocerebellar function; sensory function; neuropsychiatric function; and autonomic function (19). The summed scores for each function were averaged across mice with

similar treatment (CLP or sham surgery) and these were then subjected to statistical analysis.

For the open-field test, each mouse was placed in an empty chamber (30 cm × 50 cm) with 15-cm high walls made of opaque white acrylic for 1 min under dim red light. Movement was recorded from a centrally placed video camera using automated video tracking software to record the distance traveled (EthoVision, Noldus, Attleboro, MA, USA). For the rotarod test, mice were placed individually on a rotating drum (ENV-576M, Med Associates Inc, St. George, VT, USA), which accelerated from 4 to 40 rpm over a course of 5 min. The time at which the mouse fell off the drum was recorded. The test was repeated 10 times for each mouse with an interval of at least 1 h between trials. The room was illuminated with low-level white lights. The black-and-white alley test occurred in two stages with a habituation alley (painted gray, 120 cm long, 9 cm wide, 30 cm high) and a testing alley (same dimensions, one half-painted black and the other white, with 1-cm high wire barrier separating the two halves). The purpose of the habituation alley was to familiarize the mouse, immediately before testing, to an environment structurally similar to the testing alley, potentially exacerbating the black-and-white contrast in the latter. Each mouse was put in the habituation alley for 30 s, then directly transferred into the test alley for a 5-min testing trial. The animal was placed near the closed end of the white half, facing the wall. The initial latency to enter (all four paws) into the dark half, and the total time spent in each half were recorded by software (Ethovision, Noldus). Between each mouse, feces and urine were carefully removed and the test alley thoroughly wiped with a damp tissue.

The navigational test that measured spatial working memory was modified from Deacon and Rawlins (21). The apparatus (termed "clock maze") was a circular base platform (diameter, 85 cm) surrounded by a clear wall (30 cm high),

sealed to the base by aquarium sealant to make it waterproof. Cold water (20° C ± 1° C) was added to a depth of 2 cm, sufficient to wet the underside of the belly of mice. The perimeter wall was pierced by 12 holes, 4 cm in diameter, arranged equidistantly around the circumference so that they were 23 cm apart, like the 12 h on a clock face. The lower edge of each hole was 3 cm above the maze floor, that is, at mouse head level. Eleven of these tubes were sealed with black plugs, flush with the internal pool wall surface; one was open and led to an escape pipe, which was 4 cm in diameter, made of black flexible plastic. Thus, from within the clock maze, the true exit looked similar to the decoys, even to the human eye. The pool was surrounded by distal unmoving cues (face masks, large curtain, a bench), which were illuminated by focal white lights within a darkened testing room. Besides the clock maze, there was a pretraining box (28 cm long × 8 cm wide × 30 cm high), which was made of black wood, open on the top and with one short side (made of an acrylic sheet) containing an escape tube of black plastic (diameter, 4 cm, length, 4 cm), with its lower edge at 3 cm above the base, at mouse head height. The escape pipe, as used on the clock maze, was fitted onto the tube. There was also a pretraining tunnel (rectangle, 39 cm long × 24 cm wide × 30 cm high), made of clear plastic, and was filled with water to 2.5 cm. It had two black plastic escape tubes set in diagonally opposite corners of the short sides, centered 5 cm above the base and 5 cm from the adjacent longer wall. One tube was open and leads to the escape pipe, while the other was sealed with a black plug. For each apparatus, the task was to escape into the open tube and connected pipe; the pipe was then removed and the mouse was transported to the nearby home cage. For pretraining, each mouse was first placed in the far end of the pretraining box to learn the principle of escaping into the tube. The mice received four trials in 1 d with each trial lasting a maximum of 60 s. The next day, mice received four trials in the pre-

training tunnel. The animal was placed facing one of the long sides in a semi-random fashion (not more than three consecutive times the same way) and the time taken to enter the open tube was recorded by software (Ethovision, Noldus). Errors were defined as approaching the closed tube (within a head's length). Maximum trial length was 60 s. For the working memory task, a mouse underwent four trials per day in the clock maze for 3 consecutive days. The target remained in a fixed location during the initial four trials, but it was switched to a different location during the next four trials, and yet another location for the final four trials. Therefore, each mouse was required to learn three different targets during the test. The latency to reach the target was recorded by software (Ethovision, Novus) with a maximum of 60 s for each trial. A learning score (LS) for each animal was obtained from the latencies ("Lat") for each trial (Lat2 to Lat12), using the following equation;

$$LS = ([(1/Lat2) + (1/Lat3) + (1/Lat4) + (1/Lat6) + (1/Lat7) + (1/Lat8) + (1/Lat10) + (1/Lat11) + (1/Lat12)]/9),$$

which averaged the inverses of the latencies in nonnovel trials.

Histology

Brain circulation was replaced with heparinized normal saline, followed by 4% paraformaldehyde. Brains were removed, postfixed for 1 h, and infiltrated with 30% sucrose overnight at 4°C. A 5-mm coronal brain slab was isolated in a brain mold, mounted on a freezing stage, and sectioned by microtome at 40 μm. Sections were collected in 0.1 mol/L PB (pH7.4). Every fourth section was mounted in 0.05 mol/L PB on gelatin-coated slides, air-dried and stained with cresyl violet (22).

Golgi staining was performed with the FD Rapid Golgi Stain kit (FD Neuro Technologies, Ellicott, City, MD, USA). Mice were saline perfused and brains were immersed for 2 wks in an impregnation solution, and then cryoprotected for 48 h to 1 wk and cut on a cryostat; 100 μm sections were transferred to gelatin-coated slides.

After drying, slides were stained and cover slipped. Quantitation of apical dendritic spines in neurons in which the cell body was in the CA1 region and which were whole and unbroken into the projection field was performed as described (23), except that the images of the neurons were acquired at 100 × oil with a Zeiss Axio imager. A Z-stack of the neuron cell body in the CA1 region and distal dendritic field was captured in 0.5μm steps. The images were transferred to an automated program for counting and analysis (Neurolucida, MBF Bioscience, Williston, VT, USA.)

Statistical Analysis

Data are presented as mean ± SEM, mean ± SD or median (25% quartile/75% quartile) as indicated. We used factorial analysis of variance (ANOVA), repeated measures ANOVA, Student t test, Mann-Whitney test and Mood's median test to examine statistical significance, which was defined as $P < 0.05$.

RESULTS

Serum HMGB1 Levels are Increased Significantly in Sepsis Survivors

As compared with prototypical early cytokine mediators of sepsis, for example, TNF and IL1, which increase early during the syndrome but then return to normal levels, HMGB1 levels increase later (24). To investigate whether HMGB1 levels remained elevated in sepsis survivors, BALB/c mice were subjected to cecal ligation and puncture, a clinically relevant animal model of severe sepsis characterized by polymicrobial infection or to sham surgery (15). Animals were monitored for 12 wks, and serum samples collected for determination of HMGB1 levels by quantitative Western blot analysis. Serum HMGB1 levels were elevated significantly for weeks in sepsis-surviving mice, only returning to baseline after 12 wks (Figure 1).

Sepsis Survivors Exhibit Persistent Learning and Memory Impairment

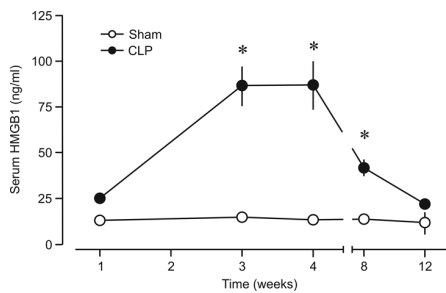There is an improved understanding that interactions between innate immune

Figure 1. Serum HMGB1: Serum samples were harvested from murine sepsis survivors or sham surgery survivors at each indicated time point (*n* = 4 to 6 mice per time point), and assayed for HMGB1 by Western blot. Densitometry and a standard curve were used for quantitation. Data: mean ± SE. *$p < 0.05$.

mediators and brain underlie normal cognitive function, as well as the cognitive impairment that develops after injury or infection. Accordingly, we reasoned that sustained elevation of HMGB1 levels following sepsis might contribute to cognitive or behavioral impairment. We subjected mice to CLP or sham surgery (*n* = 20 in each group), and assessed behavior and cognitive function. Both groups behaved comparably across the measurement parameters (Table 1). Memory function assessed in a clock maze task, in which mice use visual cues placed around the maze to navigate to an exit, differed between the groups. Proper performance of this task depends on a functional hippocampus. As shown in Figure 2A, sepsis survivors exhibited significant impairment in this task as compared with sham surgery mice at 4 wks following CLP or the sham procedure. This impairment persisted for at least 4 months following the onset of severe sepsis (Figure 2B).

To test whether this was an isolated neurological defect or whether there were additional cognitive or behavioral impairments, CLP and sham-operated mice were subjected to further behavioral assessment. We found that the two groups had comparable performance on all the tasks (Table 2 and Figures 2C–F), indicating that the memory impairment in the clock maze task was selective.
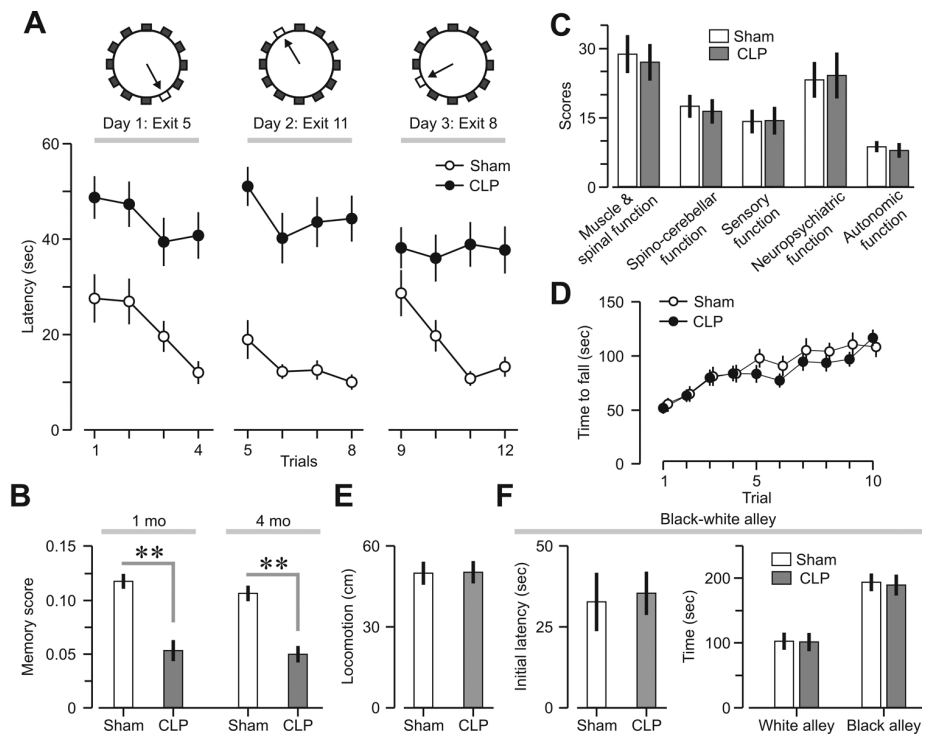

Figure 2. Cognitive and behavioral assessments of sepsis surviving mice. (A) Mice (CLP, *n* = 20, sham surgery, *n* = 20) were assessed for their ability to find the exit (indicated by a white box in the top view of the clock maze) in the spatial task. (B) CLP-surviving mice needed significantly more time to exit the maze at 1 month and 4 months following insult (**$P < 0.01$). (C) Sham surgery and CLP mice behaved equivalently in the primary screen as shown by their scores in muscle and spinal function, spinocerebellar function, sensory function, neuropsychiatric function and autonomic function at 1 month following CLP or sham surgery (see Methods for details). Sham surgery and CLP mice behaved (D) equivalently on the rotarod task, (E) exhibited equivalent locomotion and (F) displayed a similar level of anxiety in the black-white alley.

Administration of Anti-HMGB1 Antibodies Improves Cognitive Impairment in Sepsis Survivors

We next explored whether neutralization of circulating HMGB1 improved cognitive impairment. The rationale to target HMGB1 with a neutralizing monoclonal antibody was based on the following considerations. First, we wished to avoid the possibility that antibodies to TNF or IL-1 might delay the clearance of microorganisms and potentially increase the risk of infection. Second, TNF and IL-1 levels return to baseline by 24 to 72 h after the acute insult, a very limited therapeutic window, and we did not wish to inhibit cytokine levels in the normal range. In contrast, HMGB1 levels remain signifi-

cantly elevated for weeks, a therapeutic window that can be exploited readily. Neutralizing anti-HMGB1 antibody or isotype control antibody was administered to sepsis survivors on d 7, 9 and 11 after sepsis. The antibodies were unmodified IgG2b which has a half-life of 21 d. Spatial memory was assessed in these animals at 4 wks after sepsis. As shown in Figure 3, mice receiving anti-HMGB1 antibody exhibited significantly reduced serum HMGB1 levels, and significant improvement in spatial memory function as compared with mice that received isotype control antibody. This indicates that high levels of circulating HMGB1 in sepsis survivors either directly or indirectly mediate the after sepsis cognitive impairment.

Table 2. Primary screen reveals unaltered behavioral variables in sepsis-surviving mice.[a]

No.	Variable	Scale range	Sham (n = 20)	CLP (n = 20)	Chi-square	d.f.	P
1	Coat length	0–4	0 (0/0)	0 (0/0)	0	1	1
2	Hair length	0–3	0 (0/0)	0 (0/0)	0	1	1
3	Hair morphology	0–2	0 (0/0)	0 (0/0)	1.03	1	0.3112
4	Body position	0–8	5 (3/5)	5 (3/5)	3.24	1	0.0717
5	Spontaneous activity	0–8	3 (3/5)	5 (3/5)	2.56	1	0.1097
6	Respiratory rate	0–3	2 (2/2)	2 (2/2)	0	1	1
7	Tremor	0–2	0 (0/0)	0 (0/0)	0	1	1
8	Defecation	0–13	4 (2/7.5)	4 (2/5.5)	0.42	1	0.5186
9	Urination	0–1	0 (0/0)	0 (0/1)	0.53	1	0.4652
10	Transfer arousal	0–6	5 (4/5)	5 (4/5)	0	1	1
11	Latency to move	0–10	2 (1/2.5)	2 (1/3)	0.13	1	0.7233
12	Locomotion in arena	0–26	11 (7/14.5)	10.5 (9/12.75)	0.42	1	0.5186
13	Piloerection	0–1	0 (0/0)	0 (0/0)	0	1	1
14	Palpebral closure	0–2	0 (0/0)	0 (0/0)	0	1	1
15	Startle response	0–3	1 (1/1)	1 (1/1)	0	1	1
16	Gait	0–3	0 (0/0)	0 (0/0)	0	1	1
17	Pelvic elevation	0–3	2 (2/2)	2 (2/2)	0	1	1
18	Tail elevation	0–2	1 (1/1)	1 (1/1)	1.03	1	0.3112
19	Touch escape	0–3	1 (0/2)	1 (0/2)	0.11	1	0.7357
20	Positional passivity	0–4	1.5 (0/4)	3 (0.5/4)	0.91	1	0.3422
21	Trunk curl	0–1	0 (0/0)	0 (0/0)	1.03	1	0.3112
22	Limb grasping	0–1	1 (0/1)	1 (0/1)	0	1	1
23	Visual placing	0–4	2 (1.25/2)	2 (2/2)	0	1	1
24	Grip strength	0–4	2 (2/2.75)	2 (1.25/2.75)	0	1	1
25	Body tone	0–2	0 (0/1)	0 (0/1)	0.44	1	0.5073
26	Pinna reflex	0–2	1 (0/1)	1 (1/1)	0	1	1
27	Corneal reflex	0–3	1 (1/1)	1 (1/1)	0.36	1	0.5483
28	Toe pinch	0–4	1.5 (1/2.25)	1 (0/2)	0.4	1	0.525
29	Body length	0–100	90 (90/95)	90 (85.5/95)	0	1	1
30	Tail length	0–100	90 (90/90)	90 (85/90)	0	1	1
31	Lacrimation	0–1	0 (0/0)	0 (0/0)	0	1	1
32	Whisker morphology	0–1	0 (0/0)	0 (0/0)	1.03	1	0.3112
33	Provoked biting	0–1	0 (0/0)	0 (0/0.5)	1.56	1	0.2119
34	Salivation	0–2	0 (0/0)	0 (0/0)	2.11	1	0.1468
35	Heart rate	0–2	1 (1/1)	1 (1/1.5)	0.63	1	0.4292
36	Abdominal tone	0–2	1 (0/1)	0 (0/1)	1.62	1	0.2036
37	Skin color	0–2	1 (1/1)	1 (1/1)	0	1	1
38	Limb tone	0–4	1.5 (0.75/2)	1 (0/1.75)	2.56	1	0.1098
39	Wire maneuver	0–4	0 (0/1)	0 (0/0.5)	0.48	1	0.4902
40	Righting reflex	0–3	0 (0/0)	0 (0/0)	2.11	1	0.1468
41	Contact righting	0–1	1 (1/1)	1 (1/1)	0	1	1
42	Negative geotaxis	0–4	0 (0/0)	0 (0/0)	0.17	1	0.6773
43	Fear to experimenter	0–1	0 (0/0)	0 (0/0)	0.78	1	0.3758
44	Irritability	0–1	0 (0/0)	0 (0/0)	0	1	1
45	Aggressivity	0–1	0 (0/0)	0 (0/0)	1.03	1	0.3112
46	Vocalizations	0–1	0 (0/1)	0 (0/0)	4.8	1	0.0285
47	Abnormal behavior	0–1	0 (0/0)	0 (0/0)	0	1	1
48	Body weight	grams	27.8 (26.725/ 31.975)	27.75 (26.45/ 30.025)	0	1	1

[a]The table lists the medians (25%/75% interquartile) for the 48 variables in the order they were measured. The nonparametric Mood's median test was used to examine statistical significance. Chi-square, degrees of freedom (d.f.) and probability (P) values are shown. We found that only the *vocalizations* variable was different at the P < 0.05 level.
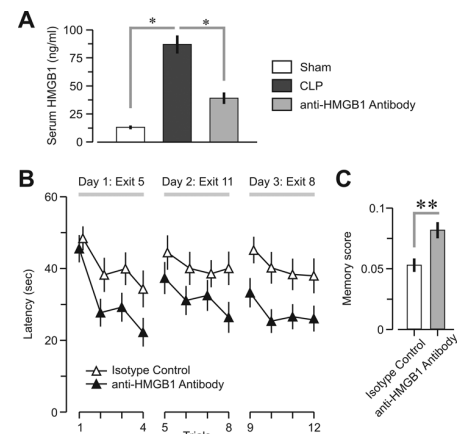
Figure 3. Preservation of memory function by neutralizing HMGB1 (A) Neutralizing anti-HMGB1 antibody was administered to CLP mice at 1 wk after sepsis. Serum levels of HMGB1 were analyzed at 4 wks after CLP (*P < 0.05). (B) CLP mice were given anti-HMGB1 monoclonal antibody or iso-type control antibody and subjected to the clock maze task. (C) Mice receiving anti-HMGB1 antibody performed significantly better than those receiving control antibody (**P < 0.01).

Systemic Administration of HMGB1 Causes Memory Impairment

To explore the critical role of HMGB1 in memory impairment in sepsis-surviving mice, we administered rHMGB1 or vehicle daily for a period of 3 wks to naïve mice and assessed memory function 1 wk later. Mice receiving HMGB1 developed significantly impaired memory function in the clock maze task (Figure 4). Thus, exposure to systemically elevated levels of HMGB1 for a period of 3 wks is sufficient to mediate cognitive impairment by 1 month after the initial inflammatory insult.

Altered Hippocampal Structure is Present in Sepsis-Surviving Mice

To assess whether HMGB1 mediated structural changes in the hippocampus, brains were sectioned and prepared for histopathological examination. Sections from sepsis survivors and sham surgery mice appeared grossly normal, with no obvious loss of hippocampal neurons at 4 wks after onset of sepsis, a time at which
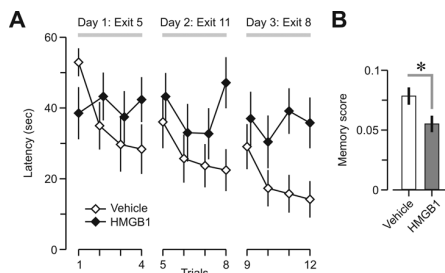
Figure 4. Memory impairment in mice receiving HMGB1. (A) Mice were given daily injections of HMGB1 or saline and subjected to training on the clock maze task. (B) HMGB1 caused a significant decline in memory function (*$P < 0.05$).

the mice exhibited memory impairment (Figure 5A). However, as demonstrated in Figures 5B and C, spine density on dendritic processes of CA1 neurons was reduced significantly in sepsis survivors as compared with controls. This abnormal pattern persisted for at least 4 months following the onset of sepsis. Importantly, it was not present at 2 wks following the onset of sepsis, indicating that dendritic degeneration develops progressively over time in sepsis survivors, and is not caused by the onset of acute sepsis. Administration of neutralizing HMGB1 conferred significant protection against the loss of dendritic spines (Figure 5D). Together with prior studies suggesting that a loss of dendritic processes is associated with synaptic plasticity and memory function, our results suggest that the mechanism of impaired cognition in sepsis survivors mediated by HMGB1 is, at least in part, a loss of hippocampal spine density.

DISCUSSION

This study demonstrates that mice surviving severe sepsis following cecal ligation and puncture develop significant memory impairment similar to the clinical problem observed in patients that have survived severe sepsis. While there are other minor functional limitations in patients following sepsis, the major impairment is cognitive. Multiple cytokines are released into the circulation as a consequence of polymicrobial sepsis (25). The

Figure 5. Neuroanatomical assessments in the hippocampus. (A) Representative cresyl violet–stained sections showed no gross abnormalities in the hippocampus of CLP mice when compared with sham surgery mice. (B) Golgi staining of CA1 neurons in the hippocampus, 4 wks after CLP or sham surgery (bar, 500 μm). (C) Quantification of CA1 apical dendritic spine density (spines per μm, mean ± SD) at 2 wks, 4 wks and 4 months after CLP or sham surgery. The sham group had significantly more spines at the later time points (*$P < 0.05$, three to four mice in each group; three to six dendritic arbors from each animal). (D) Mice that were treated with anti-HMGB1 antibody (Ab) following CLP exhibited a density of dendritic spines comparable to sham animals, 4 wks after surgery (three animals, six dendritic arbors from each animal). At right, Golgi–stained section of CA1 neuron from a CLP animal that was treated with anti-HMGB1 Ab (bar, 50 μm).

early cytokine response includes IL-1 and TNF, both of which have been implicated in acute memory impairment in models of sterile inflammation. Neutralizing these cytokines during infection is potentially hazardous, and indeed there are warnings against the use of these immunosuppressive agents in the context of infection. In sepsis survivors, IL-1 and TNF levels have returned to baseline levels, but HMGB1 levels are high. The role of HMGB1 in me-

diating memory deficits had not been previously been explored, and here we show that sustained elevations in HMGB1 mediate memory impairment.

Administration of neutralizing HMGB1 beginning 1 wk after CLP improved memory function, indicating that HMGB1 was necessary for pathogenesis, and administration of rHMGB1 induced memory impairment, indicating that HMGB1 is sufficient for pathogenesis. HMGB1 lev-
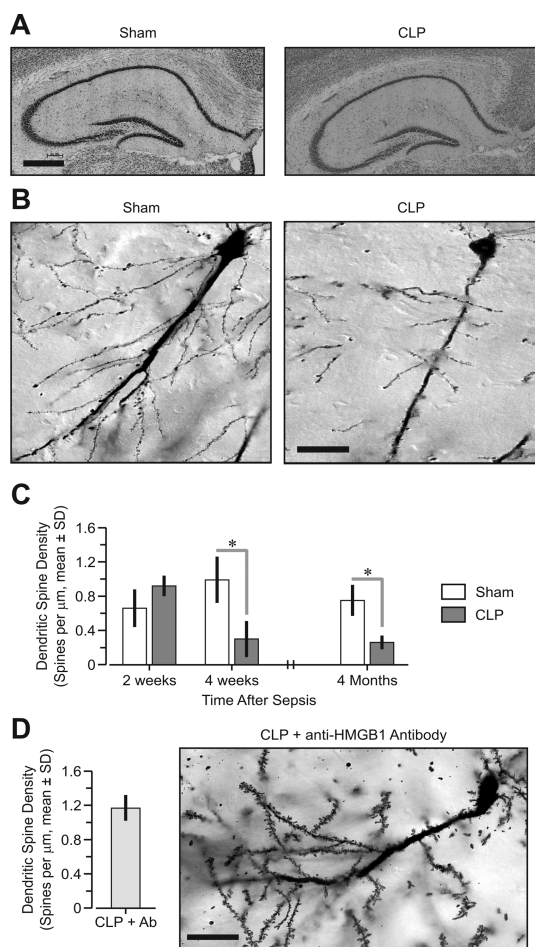
els increase after the onset of infection or sterile injury, so it is plausible to study further the role of this cytokine in mediating cognitive impairment and memory dysfunction in other disease models.

How systemic elevations in HMGB1 cause loss of dendritic spines and associated memory dysfunction is unknown. Indeed, how systemic inflammation, in general, is communicated to the brain is not well understood. It is possible that HMGB1, acting on RAGE receptors or Toll-like receptors (TLR) 2, 4, 7 or 9, directly activates peripheral nerves and an inflammatory circuit is transduced into neural signaling. Alternatively, HMGB1 may act on brain microvasculature endothelial cells either to breach blood-brain barrier integrity and expose the brain to neurotoxic substances in the circulation or to activate brain endothelial cells to alter the abluminal milieu with release of cytokines and other inflammatory mediators such as prostaglandins into brain parenchyma. HMGB1 also may penetrate porous regions of brain microvasculature, binding directly to receptors on glial cells and neurons. It also is plausible that high systemic levels of HMGB1 lead to increased levels of another, as yet unidentified, inflammatory mediator that then leads to brain dysfunction through any of the mechanisms described above.

CONCLUSION

Our findings indicate that neutralizing HMGB1, beginning 1 wk after the onset of sepsis, confers significant protection against memory impairment. This impairment evolves over time in sepsis survivors, but additional work is needed to reveal the mechanism and kinetics that relate elevated HMGB1 levels to the development of functional hippocampal lesions. The loss of dendritic processes is associated with impaired synaptic plasticity, which is the functional substrate of memory. It is likely that loss of dendritic processes is mediated by macroautophagy. That this might be stimulated by HMGB1 is the focus of ongoing investigation. Indeed, studies are underway to determine which inflammatory cyto-

kines, if any, are present in the brain of CLP-surviving mice and which are produced within the brain.

The data presented here indicate that elevated HMGB1 levels in serum impair the structure and function of hippocampal neurons that underlie normal cognition. Of potential clinical importance, this study demonstrates that there may be a window of opportunity following sepsis in which neural protection can be achieved by administration of anti-HMGB1 antibodies to prevent or reverse cognitive impairment.

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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