

High-mobility Group Box 1 Protein Initiates Postoperative Cognitive Decline by Engaging Bone Marrow–derived Macrophages

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ABSTRACT

Background: Aseptic trauma engages the innate immune response to trigger a neuroinflammatory reaction that results in postoperative cognitive decline. The authors sought to determine whether high-mobility group box 1 protein (HMGB1), an ubiquitous nucleosomal protein, initiates this process through activation and trafficking of circulating bone marrow–derived macrophages to the brain.

Methods: The effects of HMGB1 on memory (using trace fear conditioning) were tested in adult C57BL/6J male mice; separate cohorts were tested after bone marrow–derived macrophages were depleted by clodrolip. The effect of anti-HMGB1 neutralizing antibody on the inflammatory and behavioral responses to tibial surgery were investigated.

Results: A single injection of HMGB1 caused memory decline, as evidenced by a decrease in freezing time ($52 \pm 11\%$ vs. $39 \pm 5\%$; $n = 16-17$); memory decline was prevented when bone marrow–derived macrophages were depleted ($39 \pm 5\%$ vs. $50 \pm 9\%$; $n = 17$). Disabling HMGB1 with a blocking monoclonal antibody, before surgery, reduced postoperative memory decline ($52 \pm 11\%$ vs. $29 \pm 5\%$; $n = 15-16$); also, hippocampal expression of monocyte chemoattractant protein-1 was prevented by the neutralizing antibody ($n = 6$). Neither the systemic nor the hippocampal inflammatory responses to surgery occurred in mice pretreated with anti-HMGB1 neutralizing antibody ($n = 6$).

Conclusion: Postoperative neuroinflammation and cognitive decline can be prevented by abrogating the effects of HMGB1. Following the earlier characterization of the resolution of surgery-induced memory decline, the mechanisms of its initiation are now described. Together, these data may be used to preoperatively test the risk to surgical patients for the development of exaggerated and prolonged postoperative memory decline that is reflected in delirium and postoperative cognitive dysfunction, respectively. (*ANESTHESIOLOGY* 2014; 120:1160-7)

ASEPTIC surgical trauma provokes a neuroinflammatory response, presumably, to defend the organism from further injury.^{1,2} When this homeostatic response is dysregulated, detrimental consequences can follow, including postoperative cognitive decline that can persist in up to 10% of surgical patients over the age of 65 yr.^{3,4} Although it is possible that the cognitive response to surgery may also include enhancement (if the surgery “cures” a process that interferes with cognition) or no change (short-lived initiation and resolution of aseptic trauma-induced inflammation), we have explored, in rodent models, the process that mediates persistent postoperative cognitive decline.^{1,2,5} After tissue injury the innate immune response is engaged, resulting in penetration of bone marrow–derived macrophages (BM-DM) into the brain through a disrupted blood–brain barrier.² Within the hippocampus these activated macrophages release proinflammatory cytokines that are capable of attenuating long-term potentiation that is the neurobiologic correlate of learning and memory.^{6,7} These processes are reversed within days through inflammation-resolving mechanisms involving both neural and humoral pathways.² Failure to resolve the neuroinflammatory response results in exaggerated and persistent

What We Already Know about This Topic

- Surgical trauma induces a neuroinflammatory response that contributes to cognitive dysfunction in rodent models
- Penetration of bone marrow–derived macrophages into the brain with release of proinflammatory cytokines plays a major role in this neuroinflammatory response to injury

What This Article Tells Us That Is New

- Administration of the alarmin high-mobility group box 1 protein produced memory dysfunction in mice
- A neutralizing antibody to high-mobility group box 1 protein reduced memory dysfunction and prevented the inflammatory response after tibial surgery, indicating a major initiating role for this mediator in postoperative memory dysfunction

postoperative cognitive decline.^{1,8,9} In an attempt to devise strategies that can detect and mitigate this risk, the most vulnerable patients need to be identified; in pursuit of this goal we sought to precisely define the initiating processes in order to devise a preoperative functional assay that is predictive of the patient’s likely immune response to aseptic trauma.

Alarmins, a family of damage-associated molecular patterns, are capable of activating the innate immune response

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through their interaction with pattern recognition receptors on circulating monocytes.¹⁰ In particular, high-mobility group box 1 protein (HMGB1) is an alarmin that is passively released into the circulation from traumatized necrotic cells; also, HMGB1 can be rapidly secreted by stimulated leukocytes and epithelial cells.^{10,11} We previously demonstrated that circulating HMGB1 increases after surgery in humans and also in a murine aseptic trauma model^{12,13}; furthermore, we reported that this species of alarmin is required for trauma-induced exacerbation of the morphological and functional consequences of stroke.¹²

Now we describe data from experiments designed to test the hypothesis that the early release of HMGB1 triggers the neuroinflammatory and behavioral responses to trauma. These data set the stage for the development of a functional assay that assesses the initiation and resolution of inflammatory processes that are pivotal in postoperative cognitive decline.

Materials and Methods

Animals

All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco, San Francisco, California, and conformed to the National Institutes of Health Guidelines. All animals were fed standard rodent food and water *ad libitum*, and were housed (five mice per cage) in

sawdust-lined cages in an air-conditioned environment with 12-h light/dark cycles. Wild-type male mice (C57BL/6J, 12–14 weeks old) were purchased from Jackson Laboratory (Bar Harbor, ME) for the behavior tests (fig. 1, A and B) and for the cytokine expression (fig. 1C).

Animals were tagged and randomly allocated to each group before any treatment or procedure. Researchers were blinded to the group assignment, which was revealed only after the analysis phase.

Body weight was measured before any procedure or treatment and 3 days later, after assessment of freezing behavior.

Surgical Trauma

Under aseptic conditions, groups of mice were subjected to an open tibia fracture of the left hind paw with an intramedullary fixation as previously described.^{2,12,14,15} Briefly, mice received general anesthesia with 2% isoflurane and analgesia was achieved with buprenorphine 0.1 mg/kg administered subcutaneously, immediately after anesthetic induction and before surgical insult. Warming pads and temperature-controlled lights were used to maintain body temperature at $37^{\circ} \pm 0.5^{\circ}\text{C}$. The entire procedure from induction of anesthesia to end of surgery lasted 12 ± 5 min.

Trace-fear Conditioning

Fear conditioning is used to assess learning and memory in rodents, which are trained to associate a conditional stimulus, such as a tone, with an aversive, unconditional stimulus,

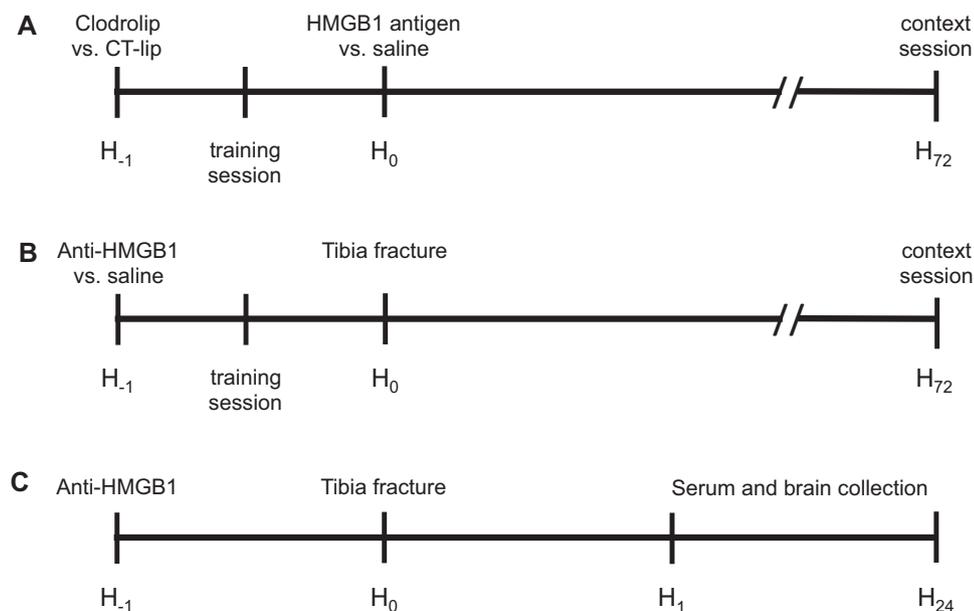


Fig. 1. Study design. (A) First experiment: mice were divided in five groups treated with intraperitoneal injection of control liposome *versus* clodrolip 1 h before high-mobility group box 1 protein (HMGB1) Ag *versus* saline injection. Control animals received saline injections. The training session of the memory test was performed 30 min after the clodrolip/control liposome injection and 30 min before HMGB1 Ag/saline injection; and the context session was performed 72 h later. (B) Second experiment: mice were divided in four groups treated with anti-HMGB1 *versus* saline 1 h before tibia fracture. The training session of the memory test was performed 30 min after the intraperitoneal injection and 30 min before tibia fracture. (C) Third experiment: mice were divided in four groups treated with anti-HMGB1 *versus* saline 1 h before tibia fracture and sacrificed 1 and 24 h after the tibia fracture. Anti-HMGB1 = neutralizing HMGB1 monoclonal antibody; CT-lip = control liposome; HMGB1 Ag = HMGB1 antigen.

such as a foot-shock.¹⁶ Freezing behavior is an indicator of aversive memory that is measured when subjects are reexposed to the conditional stimulus.

For this study we used a previously published paradigm.^{1,5,12,15,17} In brief, the behavioral study was conducted by using a conditioning chamber (Med. Associates Inc., St. Albans, VT) and an unconditional stimulus (two periods of 2-s foot-shock of 0.75 mAmp). Behavior was captured with an infrared video camera (Video Freeze; Med. Associates Inc.). Thirty minutes after a particular intervention, animals underwent the training session after which they were returned to their housing cage. Three days after training, mice underwent a context test, during which no tones or foot-shocks were delivered. Freezing behavior, recognized as lack of movement, was recorded by video and analyzed by software. A decrease in the percentage of time spent freezing indicated impairment of memory.

Systemic Inflammatory Response to Surgery

One hour and then 24 h after aseptic surgical trauma, blood was collected transcardially after thoracotomy under terminal isoflurane anesthesia and placed into heparin-coated syringes. Samples were centrifuged at 3,400 rotations per minute for 10 min and plasma was collected and stored at -80°C until these were assayed. Blood samples taken from animals without intervention served as controls. Plasma interleukin (IL)-6 and HMGB1 were measured using commercially available enzyme-linked immunosorbent kits, according to the manufacturer's instructions (Invitrogen, Grand Island, NY, and IBL International, Toronto, Ontario, Canada, respectively).

Neuroinflammatory Response to Surgery

Twenty-four hours after surgery, mice were perfused with saline and the hippocampus was then rapidly extracted, placed in RNAlater™ solution (Qiagen, Valencia, CA) and stored at 4°C overnight. Total RNA was extracted using RNeasy Lipid tissue Kit (Qiagen). Extracted RNA was treated with recombinant DNase I by using a RNase-Free Dnase set™ (Qiagen). Messenger RNA (mRNA) concentrations were determined with a ND-1000 Spectrophotometer (NanoDrop®; Thermo Fisher Scientific, Wilmington, DE) and mRNA was reverse transcribed to complementary DNA with a High Capacity RNA to-cDNA Kit (Applied Biosystems, Carlsbad, CA).

TaqMan Fast Advanced Master Mix (Applied Biosystems) and specific gene-expression assays were used for quantitative polymerase chain reaction as follows: actin beta (NM_007393.1), IL-6 (Mm00446190_m1), tumor necrosis factor- α (Mm00443258_m1), IL-1 β (Mm01336189_m1), and monocyte chemoattractant protein-1 (MCP-1) (Mm00441242_m1). Quantitative polymerase chain reaction was performed using StepOnePlus™ (Applied Biosystems). Each sample was run in triplicate, and relative gene expression was calculated using the comparative threshold cycle $\Delta\Delta\text{Ct}$ and normalized to β -actin. Results are expressed as fold increases relative to controls.

Interventions

Depletion of BM-DM. Clodrolip is a liposomal formulation of clodronate (dichloromethylene bisphosphonic acid), a non-toxic bisphosphonate. Liposomes (lipid vesicles consisting of concentric phospholipid bilayers surrounding aqueous compartments) encapsulate clodronate, which are then ingested and digested by phagocytes, followed by an intracellular release and accumulation of clodronate. At a certain intracellular concentration, clodronate induces apoptosis of the phagocytes. Clodrolip was obtained from clodronateliposomes.org (Vrije Universiteit, Amsterdam, The Netherlands) at 7 mg/ml concentration and prepared as previously described.^{18,19} Clodrolip (200 μl , about 100 mg/kg) was injected intraperitoneally 60 min before aseptic surgical trauma. Control animals received 200 μl of control liposomal solution.

Administration of Reagents to Simulate or Block the HMGB1 Response to Trauma. Fifty microgram per kilogram (100 μl) of recombinant HMGB1 (R&D System, Minneapolis, MN) was administered intraperitoneally. To neutralize trauma-released HMGB1, 50 μg of anti-HMGB1 neutralizing monoclonal antibody (2G7, mouse IgG2b supplied by Dr. Tracey's Laboratory, Manhasset, NY) in 100 μl of saline was administered intraperitoneally, 60 min before bone fracture. Control animals received the same volume (100 μl) of the vehicle (saline).

Statistical Analysis

Data are presented as mean \pm SD. Normality was tested with the Kolmogorov-Smirnov normality test. Equality of variances was tested with the F test. We applied a log transformation ($\ln(X)$) to the response of HMGB1 and IL-6 blood concentrations and mRNA expression before performing analyses to better adhere to ANOVA model's assumptions of normally distributed residuals and homogeneity of variance.

For comparisons of more than two groups, means were compared using one-way ANOVA followed by t tests with a Bonferroni-corrected α level. We used the two-way ANOVA procedure to determine whether or not time and antibody treatment were significant factors in predicting HMGB1 and IL-6 concentrations in the serum; this was followed by Bonferroni *post hoc* analyses.

For our study, the primary outcome was the percentage of freezing time during the context session observed in anti-HMGB1 neutralizing monoclonal antibody and control groups. On the basis of previous freezing time data,¹⁵ we estimated that a sample of 13 C57BL/6J surgical mice per group was necessary to demonstrate a 20% increase in percentage freezing time, with 80% power at the 0.0125 α level (after adjusting for four comparisons) to reach a significant difference.

A two-tailed P value less than 0.05 was considered statistically significant for two-group comparisons and the significance threshold was adjusted for multiple comparisons with a Bonferroni correction. Prism 6 (GraphPad Software Inc, La Jolla, CA) was used to conduct the statistical analyses.

Results

HMGB1 Antigen Is Sufficient to Cause Cognitive Decline through the Participation of BM-DM

A single administration of HMGB1 produced cognitive decline as evidenced by a significant reduction in freezing time ($52 \pm 11\%$ vs. $39 \pm 5\%$, $n = 16$ in control group, $n = 17$ in HMGB1 antigen group; $P = 0.012$; fig. 2).

Recently, we showed that depletion of BM-DM by clodrolip exposure blocks surgery-induced neuroinflammation and cognitive decline.¹⁵ To determine whether HMGB1-induced cognitive decline requires the participation of BM-DM, mice were exposed to clodrolip or its vehicle before administration of HMGB1. Training was unaffected by the clodrolip exposure (data not shown). We lost one animal in the clodrolip group and another in the control liposome group on day 2. According to the manufacturer this may happen if there is transference of microorganisms from the skin or by injection of a nonhomogeneous suspension of liposome. We did not experience any death in the surgical groups. The HMGB1-induced decline in contextual freezing time was prevented by previous clodrolip exposure ($39 \pm 5\%$ vs. $50 \pm 9\%$, $n = 17$; $P = 0.039$; fig. 2).

HMGB1 Antibody Prevented Postsurgical Cognitive Decline

During the preoperative training period, learning was similar in the anti-HMGB1-exposed group and the control (nonexposed) groups (data not shown). Surgery significantly decreased the percentage of freezing time when compared with the control group ($52 \pm 11\%$ vs. $29 \pm 5\%$, $n = 16$ in control group, $n = 15$ in surgical group; $P < 0.001$); preoperative exposure to anti-HMGB1 attenuated the surgery-induced freezing behavior, rendering the response to be no different from that of the nonsurgical control group ($52 \pm 11\%$ vs. $47 \pm 11\%$, $n = 16$ in control group, $n = 15$ in anti-HMGB1 + surgery group, ns; fig. 3).

HMGB1 Antibody Reduces Systemic and Neuroinflammatory Response to Surgery

As expected,¹² we observed a significant decrease of the early increase of HMGB1 blood level between the first hour and 24 h after surgery (two-way ANOVA; $P = 0.002$ for the time effect; fig. 4). However, the neutralizing anti-HMGB1 reduced the systemic levels of HMGB1 (two-way ANOVA; $P = 0.005$ for the treatment effect and $P = 0.010$ for interaction between time and treatment), and this reduction was significant 1 h after the trauma ($P = 0.04$ with Bonferroni's *post hoc* analysis; fig. 4A). With regard to IL-6 increase after surgery, we observed a significant decrease within the first day (two-way ANOVA; $P = 0.015$ for the time effect) HMGB1 neutralizing antibody was also a significant predicting factor for IL-6 concentration (two-way ANOVA; $P < 0.001$ for treatment effect). Using HMGB1 neutralizing antibody, we observed a reduction in IL-6 concentration 1 h after trauma ($P = 0.006$ with Bonferroni's *post hoc* analysis; fig. 4B).

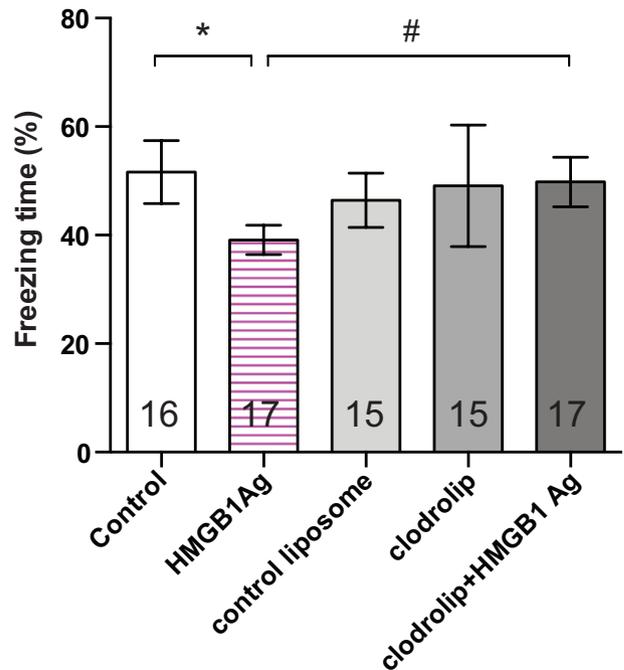


Fig. 2. Effects of high-mobility group box 1 protein (HMGB1) on cognitive decline. Contextual fear response reveals hippocampal-dependent memory impairment at postoperative day 3 (Arm A). Quantification of the freezing time percentage according to the five groups ($n = 15$ – 17 ; * $P = 0.012$ control vs. HMGB1 Ag, and # $P = 0.039$ HMGB1 Ag vs. clodrolip + HMGB1 Ag, with one-way ANOVA and Bonferroni *post hoc* analysis). HMGB1 Ag = HMGB1 antigen.

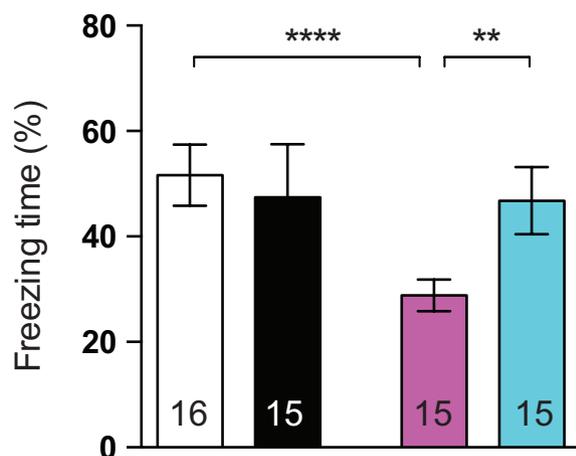
Twenty-four hours after surgery, the increase in hippocampal mRNA expression of IL-6 ($n = 6$; $P = 0.009$) and tumor necrosis factor- α ($n = 6$; $P < 0.001$) was blocked by exposure to the neutralizing anti-HMGB1 antibody. Treatment did not significantly change mRNA transcription of IL-1 β ($n = 6$; $P = 0.085$; fig. 5, A–C).

Circulating chemokine C-C motif receptor 2-expressing BM-DM are recruited into the brain by the chemoattractant, MCP-1.¹² After surgery there was an increase of hippocampal mRNA transcription of MCP-1 ($n = 6$; $P = 0.003$); treatment with the anti-HMGB1 neutralizing antibody prevented surgery-induced expression of MCP-1 ($n = 6$; $P = 0.005$; fig. 5D).

Conclusion

This study posits that HMGB1, when released from a sterile traumatic injury, plays a pivotal role in postoperative memory dysfunction. Together with the detection of the cell type involved in the initiation of the surgery-induced inflammatory cascade these findings establish both the precise elements of the immune response that need to be interrogated for establishing risk of dysregulated trauma-induced inflammation as well as putative targets for interventions designed to limit or reverse persistent postoperative cognitive decline.

The independent role of the alarmin HMGB1 in disrupting cognitive processing was established by the fact that a



Tibia surgery - - + +
Anti-HMGB1 - + - +

Fig. 3. Effects of preoperative administration of high-mobility group box 1 protein (HMGB1) neutralizing monoclonal antibody on postsurgical memory impairment. Quantification of the freezing time percentage on contextual testing according to the four groups at postoperative day 3 (Arm B) ($n = 15-16$; **** $P < 0.001$ control vs. surgery, and ** $P = 0.001$ surgery vs. anti-HMGB1 + surgery and with one-way ANOVA and Bonferroni *post hoc* analysis). Anti-HMGB1 = neutralizing HMGB1 monoclonal antibody.

single injection of HMGB1 was capable of reproducing a deficit in an hippocampal memory test similar to the surgical phenotype that we previously described.¹ Furthermore, its dependence on bone marrow-derived monocytes was

evidenced by the attenuation of HMGB1-induced cognitive decline if clodrolip was first administered to induce apoptosis of these circulating phagocytes (fig. 2); this is similar to findings of a previous report in the setting of surgery.^{1,5}

After the aseptic trauma of elective surgery, either experimentally (fig. 4A) or clinically, HMGB1 is released into the circulation where it can interact with pattern recognition receptors (toll-like receptors 2 and 4 as well as receptor for advanced glycation end products) on immunocytes.¹⁵ By neutralizing the early release of alarmins, HMGB1 antibody both decreased surgery-induced inflammation (figs. 4 and 5) as well as cognitive decline (fig. 3), further establishing the importance of hippocampal inflammation for the development of postoperative memory dysfunction.^{1,5}

After peripheral surgery, chemokine C-C motif receptor 2-expressing cells migrate to the brain, attracted by signaling from hippocampal MCP-1, a chemokine that regulates migration and infiltration of monocytes/macrophages.^{15,20} Interestingly, by depleting BM-DM, the expression of MCP-1 in the surgical model remained unaffected, indicating that BM-DM are not the self-perpetuating source of this chemoattractant for its own recruitment.¹⁵ We now show that treatment with HMGB1 antibody was able to prevent the synthesis of MCP-1 in the hippocampus, establishing the dependence of its increased expression on the release of HMGB1. Taking our previous report¹⁵ and our current findings together, it follows that HMGB1 is signaling to the hippocampus to produce the chemoattractant, MCP-1, through a mechanism that is independent of BM-DM; this HMGB1-dependent hippocampal expression of MCP-1 could involve either a neural or humoral pathway.

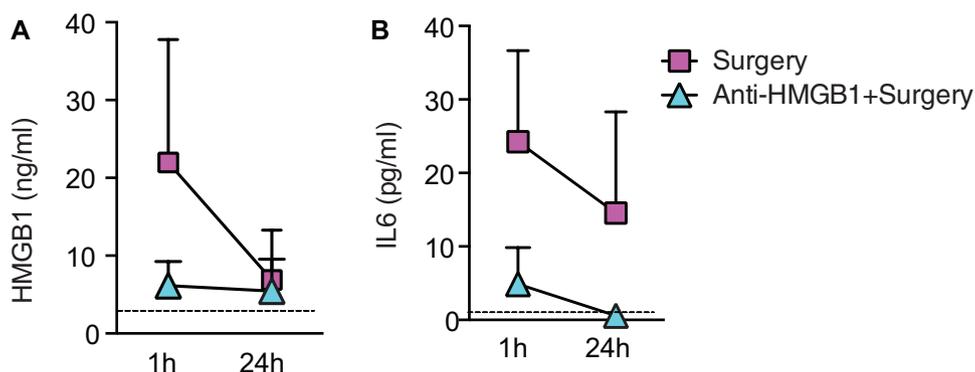


Fig. 4. Effects of preoperative administration of high-mobility group box 1 protein (HMGB1) neutralizing monoclonal antibody on the HMGB1 and interleukin (IL)-6 serum concentration at 1 and 24 h after tibia fracture (Arm C). The dotted lines represent the average values for respectively for HMGB1 and IL-6 concentration of six control mice without any treatment or surgery ($n = 6$). (A) Levels of HMGB1 serum concentration 1 and 24 h after surgery. After log transformation of the raw data, we observed with two-way ANOVA significant time and treatment effects ($P = 0.002$ for the time effect; $P = 0.005$ for the treatment effect, and $P = 0.010$ for interaction between time and treatment) and a significant difference between the two groups at 1 h (22.00 ± 16.54 vs. 5.29 ± 4.13 surgical control vs. anti-HMGB1 + surgery; $P = 0.040$ with two-way ANOVA with Bonferroni's *post hoc* analysis). (B) Levels of IL-6 serum concentration 1 and 24 h after surgery. After log transformation of the raw data, we observed with two-way ANOVA significant time and treatment effects ($P = 0.015$ for the time effect; $P < 0.001$ for the treatment effect; and $P = 0.015$ for interaction between time and treatment) and a significant difference between the two groups at 1 h (24.24 ± 12.87 vs. 4.90 ± 4.47 surgical control vs. anti-HMGB1 + surgery; $P = 0.006$ with two-way ANOVA with Bonferroni's *post hoc* analysis). Anti-HMGB1 = neutralizing HMGB1 monoclonal antibody.

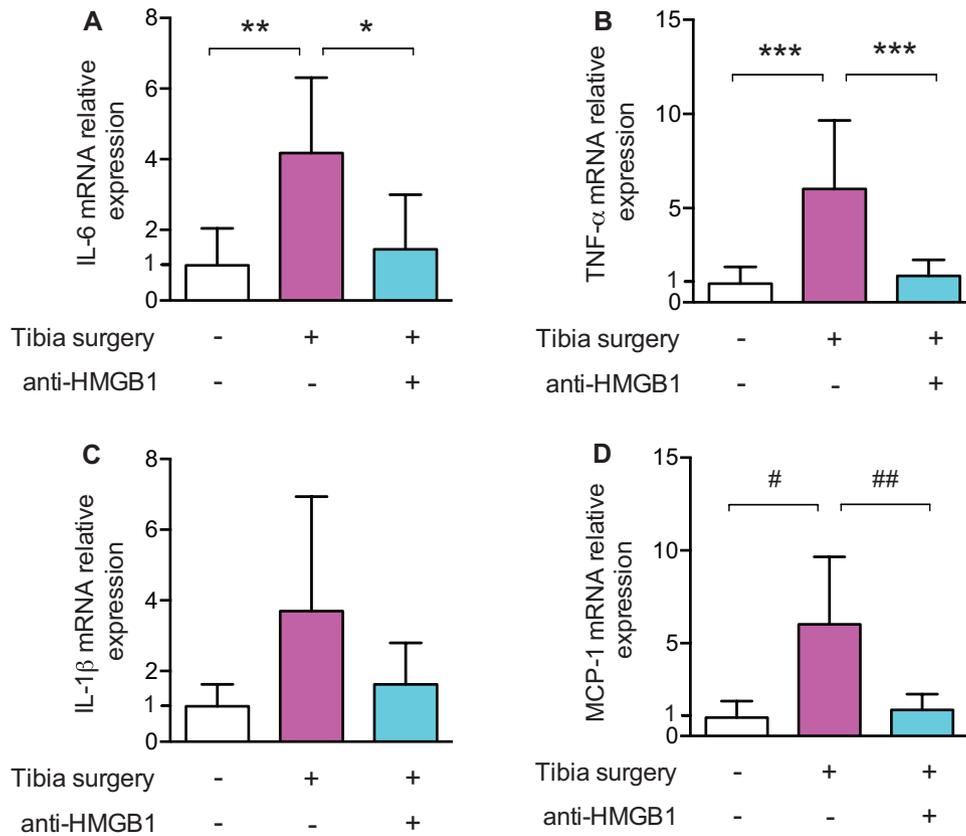


Fig. 5. Effects of high-mobility group box 1 protein (HMGB1) neutralizing monoclonal antibody on hippocampal transcription of interleukin (IL)-6, tumor necrosis factor (TNF)- α , IL-1 β , and monocyte chemoattractant protein (MCP)-1 24 h after tibia surgery (Arm C). (A) IL-6 messenger RNA (mRNA), (B) TNF- α mRNA, (C) IL-1 β and (D) MCP-1 (n = 6; *P = 0.023; **P = 0.009; ***P < 0.001; #P = 0.003; ##P = 0.005 with one-way ANOVA and Bonferroni *post hoc* analysis). Anti-HMGB1 = neutralizing HMGB1 monoclonal antibody.

Accumulating evidence indicates that HMGB1 can stimulate migration of not only monocytes, but also various types of cells including neurite,²¹ smooth muscle cells,²² tumor cells,²³ meso-angioblast stem cells,²⁴ dendritic cells,^{25,26} and neutrophils.^{27,28} This could explain how blocking the effect of the release of HMGB1 may have blocked signaling of other HMGB1-derived factors. Although BM-DM play a necessary role, they may not be sufficient to completely explain the cognitive decline seen after surgery; there may be other cells and factors involved in the genesis of postoperative cognitive dysfunction.

The following caveats apply when interpreting our findings. Our surgery model involves disruption of the bone marrow with an intramedullary pin for internal fixation of the broken bone. The bone marrow itself produces soluble factors that affect immune cells, such as a proliferation-inducing ligand A (APRIL), B-cell activating factor (BAFF), both belonging to the tumor necrosis factor family, CXCL12, IL-6, IL-7, and macrophage inhibitory factor.²⁹ The possibility exists that surgical trauma that does not involve damage to the bone marrow may not use the same panoply of alarmins. In addition to its role as a primary lymphoid organ, the bone marrow can act as a host for various mature lymphoid cell types. Several subsets of bone marrow cells have been shown to support immune cell function.²⁹

Given that HMGB1 resides in the nucleus and functions as an essential nonhistone chromatin-binding protein, there are no HMGB1 knockout animals. For this study we then decided to use a pharmacologic strategy to quickly deplete the pool of systemic macrophages. However, because clodrolip is highly toxic to all phagocytes, it can increase the risk of post-surgical infections, generating a phenotype of its own.³⁰ With a single dose, we did not observe loss of weight or other signs of sickness within 3 days. As this was a short-term study, focusing on the acute exaggeration phase of neuroinflammation, we are unable to extrapolate from these data the long-term effects and did not perform any long-term study with clodrolip. Clodrolip should be considered a tool for mechanistic studies.

In previous reports using this model of surgical trauma, we documented the effectiveness of preoperative administration of interventions such as IL-1 receptor antagonist, anti-tumor necrosis factor monoclonal antibody and activation of the $\alpha 7$ subtype of nicotinic acetylcholine receptor in preventing postoperative memory dysfunction.^{1,2,5} Each of these affect important host defense mediators, and risk of infection needs to be evaluated before considering these agents.

These studies on the initiation of trauma-induced cognitive decline, coupled with our previous reports on the resolution of postoperative cognitive decline,^{1,2,5} sets the stage

for the development of an *ex vivo* bioassay that can test the function of the innate immune response to trauma. Such an assay may be capable of prospectively identifying surgical patients at increased risk for the development of exaggerated and persistent cognitive decline; stratification of a surgical cohort, enriched for the development of cognitive decline, can result in a randomized trial to test efficacy of interventions using fewer surgical patients.

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Competing Interests

The authors declare no competing interests.

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