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Implantation of Brain-Derived Extracellular Matrix Enhances Neurological Recovery after Traumatic Brain Injury

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Abstract

Scaffolds composed of extracellular matrix (ECM) are being investigated for their ability to facilitate brain tissue remodeling and repair following injury. The present study tested the hypothesis that the implantation of brain-derived ECM would attenuate experimental traumatic brain injury (TBI) and explored potential underlying mechanisms. TBI was induced in mice by a controlled cortical impact (CCI). ECM was isolated from normal porcine brain tissue by decellularization methods, prepared as a hydrogel, and injected into the ipsilesional corpus callosum and striatum 1 h after CCI. Lesion volume and neurological function were evaluated up to 35 d after TBI. Immunohistochemistry was performed to assess post-TBI white matter integrity, reactive astrogliosis, and microglial activation. We found that ECM treatment reduced lesion volume and improved neurobehavioral function. ECM-treated mice showed less post-TBI neurodegeneration in the hippocampus and less white matter injury than control, vehicle-treated mice. Furthermore, ECM ameliorated TBI-induced gliosis and microglial pro-inflammatory responses, thereby providing a favorable microenvironment for tissue repair. Our study indicates that brain ECM hydrogel implantation improved the brain microenvironment that facilitates post-TBI tissue recovery. Brain ECM offers excellent biocompatibility and holds potential as a therapeutic agent for TBI, alone or in combination with other treatments.

Keywords

astrogliosis, microglia, neurobehavioral function, neurodegeneration, white matter injury, concussion

Introduction

Although traumatic brain injury (TBI) is a major public health problem with a deep socioeconomic impact, no effective therapies have been developed to date.[1] The primary mechanism underlying TBI-induced brain injury is the physical disruption of brain tissue by powerful mechanical forces. A number of secondary pathophysiological events occur thereafter, including oxidative stress, apoptosis, inflammatory responses, and neurovascular dysfunction, all of which may last for months to years and impede tissue recovery.[2] Both gray matter and white matter are affected during severe TBI, leading to profound sensorimotor and cognitive deficits.[3,4] Pro-inflammatory responses further deteriorate neurological function and contribute to permanent tissue loss.[5,6] Therefore, therapeutic strategies may need to target each of these heterogeneous pathophysiological responses in order to achieve long-lasting protection after TBI.

The extracellular matrix (ECM) is a highly dynamic, heterogeneous accumulation of structural and functional

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molecules present in all tissues and organs. The ECM provides 3-dimensional physical support, segregates cells and tissues, and transmits biological signals for cell proliferation, adhesion, and migration.[7] The ECM plays a major role in intercellular communication and cellular differentiation. Secreted by cells within the local microenvironment, the ECM is considered an ideal substrate for the maintenance of tissue-specific cell types. Thus, scaffold materials composed of (acellular) ECM have been developed to promote the constructive and functional remodeling of various tissues and organs, such as the esophagus, urinary tract, tendon, and myocardium.[8–11] Precisely how ECM scaffolds facilitate tissue remodeling is not yet fully understood, although several possible mechanisms have been implicated. For example, the degradation products from urinary bladder matrix (UBM) have been shown to possess chemotactic and mitogenic activities for endogenous multipotent progenitor cells.[12] Furthermore, biologic scaffolds can exert a profound influence on innate immune responses by modulation of the macrophage phenotypic profile, thereby regulating the remodeling process.[13] Finally, ECM supports the survival of stem cells and has been successfully used as a delivery vehicle in stem cell transplantation. In a previous study, ECM harvested from porcine urinary bladder tissue supported the proliferation and differentiation of neural stem cells (NSCs) after transplantation into mice and mitigated the effects of TBI in vivo.[14] The molecular composition of the ECM varies across tissues and is likely to be particularly complex in heterogeneous structures such as the brain. Thus, an injectable hydrogel derived from brain ECM has also recently been developed.[15] However, it is not known whether brain ECM facilitates tissue repair and functional recovery after TBI in animal models.

In the present study, we tested the therapeutic potential of brain-derived ECM in experimental TBI and explored possible underlying mechanisms. Our data indicate that brain ECM offers long-lasting histological protection and functional improvements via multiple mechanisms, including preservation of white matter integrity and attenuation of pro-inflammatory responses, and may be a promising therapeutic candidate for TBI.

Materials and Methods

Animals

Male C57 black 6 (C57BL/6J) mice (8 to 10 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in a temperature- and humidity-controlled animal facility with a 12-h light–dark cycle. Food and water were available ad libitum. All animal procedures used in this study were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC) and performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and the number of animals used. Animal group assignments for TBI or sham operation or for ECM or vehicle treatment were randomized using a lottery-drawing box. All outcome assessments were performed by investigators who were blinded to experimental group assignments. Animals that died during or after the surgery were excluded from further study.

Preparation of Brain-Derived ECM

ECM derived from porcine brains was prepared as described previously.[16] Briefly, porcine brain tissue was obtained from animals (approximately 120 kg) at a local abattoir (Thoma's Meat Market, Saxonburg, PA). Tissues were frozen at −80 °C for at least 16 h, thawed completely, and separated from all non-central nervous system (CNS) tissues. Dura mater was removed. Tissues were decellularized by passing through a series of agitated baths, as described previously.[16] The decellularized brain tissue was then lyophilized and stored in dry form until use.

Traumatic Brain Injury

TBI was induced by a controlled cortical impact (CCI) as described previously.[17] Briefly, mice were anesthetized with 1.5% isoflurane (Butler Schein Animal Health, Dublin, OH) in a 30% O_2/68.5% N_2O mixture under spontaneous breathing conditions. Mice were then stabilized in a stereotaxic frame, and an approximately 4-mm craniotomy was performed over the right parietotemporal area using a motorized drill. The CCI was centered 2.0 mm lateral to midline and 2.0 mm anterior to bregma and was produced with a pneumatically driven CCI device (Precision Systems and Instrumentation, Fairfax, VA) using a 3-mm flat-tip impounder (velocity, 3.75 m/sec; duration, 150 ms; depth, 1.5 mm). Immediately after the injury, the bone flap was removed, and the scalp incision was closed. The core body temperature was monitored by a rectal thermistor probe and maintained at 37.5 °C ± 0.5 °C during surgery and up to 30 min after CCI with a heating pad. Sham surgery consisted of all aspects of the protocol (anesthesia, craniotomy, and recovery) except for the CCI itself.

Injection of ECM into the Injured Brain

One hour after TBI, mice were anesthetized and placed in a stereotaxic frame. The surgical site was reopened, and the burr hole was reexposed. Each injection contained 1 μL of ECM (5 μg/mL in phosphate-buffered saline [PBS]) and was administered through a 10-μL Hampton syringe at a rate of 0.5 μL/min controlled by a Micro 4 Microsyringe Pump Controller (World Precision Instruments, Sarasota, FL). Each mouse received 2 injections into the right corpus callosum (CC) (anterior–posterior: 1.10 mm; medial–lateral: 1.0 mm; dorsal–ventral: 1.5 mm) and the striatum (anterior–posterior: −0.80 mm; medial–lateral: 1.5 mm; dorsal–ventral: 3.5 mm). The syringe was left in place for 5 min to allow diffusion from the tip. PBS was used as the vehicle control.

Examination of ECM Distribution after Injection

To examine the distribution of the ECM after injection, 1 μL of a mixture of Evans blue (0.5 mg/mL in water; Sigma-Aldrich, St. Louis, MO) and ECM (5 μg/mL in PBS) at a volume of 1:1 was injected into the brain at 1 h after CCI as described above. Twenty-four hours after injection, mice were transcardially perfused with 4% paraformaldehyde (PFA). Thick coronal sections (1 mm) were cut to view the anatomical distribution of the Evans blue dye.

Neurobehavioral Tests

Rotarod test. The rotarod test was performed, as described previously,[18] by placing the mouse on a rotating drum with a speed accelerating from 0 to 10 rpm for a 5-min period. The time at which the animal fell off the drum was recorded as the latency to fall. On each testing day, mice were tested for 3 trials with intervals of 15 min, and the mean latency to fall was calculated.

Wire hang test. The wire hang test was performed as described previously.[17] A stainless steel bar (length: 50 cm; diameter: 2 mm) rested on 2 vertical supports and was elevated 37 cm above a flat surface. Mice were placed in the middle of the bar and were observed for 30 s in 4 trials. The amount of time spent hanging was recorded and scored according to the following criteria: 0, fell off; 1, hung onto the bar with 2 forepaws; 2, hung onto the bar with added attempt to climb onto the bar; 3, hung onto the bar with 2 forepaws and 1 or both hind paws; 4, hung onto the bar with all 4 paws and with tail wrapped around the bar; 5, escaped to 1 of the supports.

Corner test. The corner test was performed as described previously.[19] Briefly, the mouse entered a corner made by 2 black board pieces placed at an angle of 30° in front of the nose. After TBI, mice preferentially turned toward the nonimpaired (right) side. Ten trials were performed on each testing day. Performance score was calculated as (right − left)/(right + left).

Morris water maze test. The Morris water maze test was performed on day 29 to 34 after TBI to evaluate long-term cognitive functions, as described previously.[20] Briefly, a circular platform (diameter: 11 cm) was submerged in a pool of opaque water (diameter: 109 cm). In the "learning phase" of the test, mice were placed into the pool from one of the 4 locations and allowed 60 s to locate the hidden platform. The time at which the animal found the platform (escape latency) was recorded for each trial. At the end of each trial, the mouse was placed on the platform or allowed to remain on the platform for 30 s with prominent spatial cues displayed around the room. Four trials were performed on each day for 5 consecutive days. In the "memory phase" of the test, a single, 60-s probe trial was performed in which the platform was removed after the last day of the hidden platform test. The number of crossings made by the mouse through the goal quadrant (where the platform was located previously) was recorded. Swim speed was also recorded to assess gross locomotor function.

Immunohistochemistry and Quantification

At 35 d after TBI, mice were deeply anesthetized and transcardially perfused with 0.9% NaCl followed by 4% PFA in PBS. Brains were cryoprotected in 30% sucrose in PBS, and frozen serial coronal brain sections (25 μm) were cut on a cryostat (CM1900, Leica, Blenheim, Germany). Immunohistochemistry was performed on free-floating sections as we described previously.[21] Sections were blocked with 5% donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS for 1 h, followed by overnight incubation (4 °C) with the following primary antibodies: rabbit antimicrotubule-associated protein 2 (MAP2; Santa Cruz Biotechnology, Dallas, TX), mouse antinonphosphorylated neurofilaments (SMI-32; Abcam, Cambridge, MA), rabbit antimyelin basic protein (MBP; Abcam), rabbit anti-Iba1 (Wako, Richmond, VA), rabbit antiglial fibrillary acidic protein (GFAP; Dako, Carpentaria, CA), and rat anti-CD16/32 (BD Biosciences, Franklin Lakes, NJ). After washing, sections were incubated for 2 h at 37 °C with the appropriate donkey secondary antibodies conjugated with DyLight 488 or DyLight 594 (Jackson ImmunoResearch Laboratories). Alternate sections from each experimental condition were incubated in all solutions except the primary antibodies to assess nonspecific staining. Sections were then counterstained with 4′,6-diamidino-2-phenylindole (Thermo Fisher Scientific, Pittsburgh, PA) for 2 min at room temperature, mounted, and coverslipped with Fluoromount-G (Southern Biotech, Birmingham, AL). Fluorescence images were captured with an Olympus Fluoview FV1000 confocal microscope using FV10-ASW 2.0 software (Olympus America, Center Valley, PA).

Images were processed in a blinded fashion for automated analysis with ImageJ, Version 1.48 (NIH, Bethesda, MD) as described previously.[17] Cell numbers were calculated per square millimeter from 3 random microscopic fields on 3 sections (9 images total) cut through the CC, cortex, and striatum. For fluorescence quantification, a region of interest was drawn by a blinded investigator in TBI or sham brains. White matter injury was expressed as the ratio of SMI-32 to MBP immunostaining relative to sham animals. Tissue loss after TBI was measured on 8 equally spaced MAP2-stained sections encompassing the CCI territory (from −1.58 mm to +1.42 mm bregma) using ImageJ. Tissue loss was calculated as the volume of the contralateral hemisphere minus the ipsilateral hemisphere.

Nissl Staining and Cell Counting of Cornu Ammonis 3 (CA3) Neurons

Cresyl violet staining was performed as we described previously.[20] Free-floating coronal sections were mounted

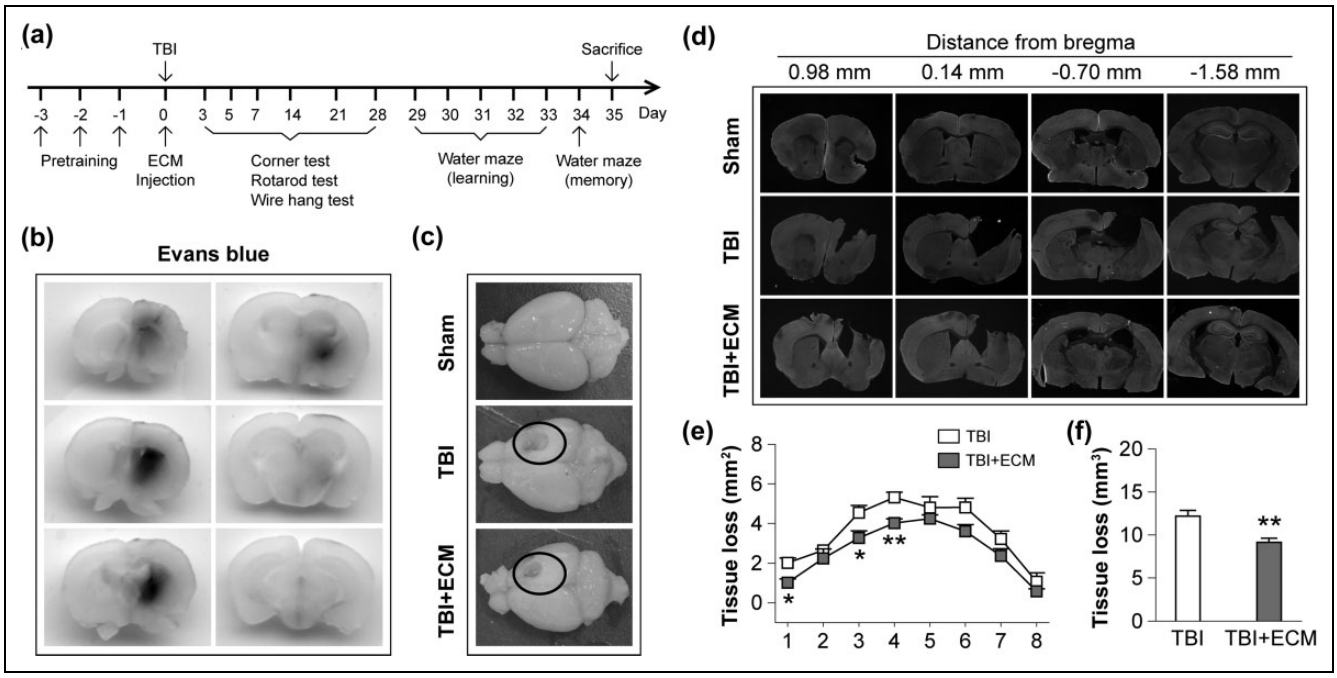

Figure 1. Extracellular matrix (ECM) treatment reduces traumatic brain injury (TBI) lesion volume. (a) Illustration of the experimental design. Mice were pretrained for the corner, rotarod, wire hang, and Morris water maze tests for 3 d before TBI or sham operation. One hour after TBI, mice received ECM or vehicle treatment. Sensorimotor functions were evaluated up to 28 d after TBI by the corner, rotarod, and wire hang tests. Long-term cognitive functions were assessed by the Morris water maze test at 29 to 34 d after TBI. Mice were sacrificed 35 d after TBI for histological examinations. (b) A mixture of ECM and Evans blue was injected into the striatum and corpus callosum (CC) 1 h after TBI as described in Methods. Brains were harvested 1 d after injection. The anatomical distribution of Evans blue in the brain is shown on 6 consecutive thick coronal sections near the injection site. (c) Representative brain images illustrate less tissue loss (black circle) in ECM-treated mice compared to vehicle controls at 35 d after TBI. (d) Representative rabbit antimicrotubule-associated protein 2 (MAP2)-stained coronal sections at different levels from the bregma show significant tissue loss 35 d after TBI in both ECM- and vehicle-treated mice. However, ECM treatment significantly reduced TBI-induced tissue loss compared to vehicle controls. (e,f) Tissue loss was quantified on 8 equally spaced MAP2-stained sections as described in Methods section. The area of tissue loss on each section (e) and total volume of tissue loss (f) is shown. n = 4 to 5 mice/group (*$P \leq 0.05$, **$P \leq 0.01$ TBI + ECM vs. TBI).

onto color frost/plus slides. Sections were sequentially processed through solutions as described[20] and were then coverslipped with neutral balata. Images of the CA3 subfield were captured with an Olympus BX51 microscope (Olympus America) and a color CCD camera (Diagnostic Instruments, Sterling Heights, MI), and viable CA3 neurons were quantified by stereology as previously described.[20]

Statistical Analyses

All data are expressed as mean ± standard error of the mean. The statistical difference between the means of 2 groups was analyzed by the Student *t* test. The differences between means of multiple groups were assessed by 1- or 2-way analysis of variance followed by the Bonferroni/Dunn post hoc test. The Pearson product linear regression analysis was used to correlate the number of CA3 neurons with spatial memory. A *P* value less than or equal to 0.05 was considered statistically significant.

Results

Implantation of Brain-Derived ECM Reduces TBI Lesion Volume

First, we examined the distribution of brain ECM after intracerebral implantation. One hour after the CCI surgery (Fig. 1a), the mixture of ECM and Evans blue was injected into the ipsilateral CC and striatum. During the next 24 h, the injected material diffused readily to adjacent areas surrounding the CCI injury site, including the vulnerable hippocampus (Fig. 1b). These data demonstrate the maintenance of injected ECM in the targeted brain tissue and validate our selection of injection sites and the amount of ECM injected.

We hypothesized that the ECM hydrogel would provide a biological scaffold and facilitate cell survival, proliferation, and migration, thereby promoting tissue repair in the injured area following TBI. Treatment with ECM significantly reduced the lesion volume at 35 d after TBI (Fig 1c). On MAP2-stained coronal sections (Fig. 1d and e), ECM-treated brains showed a 25% reduction in tissue loss at 35 d

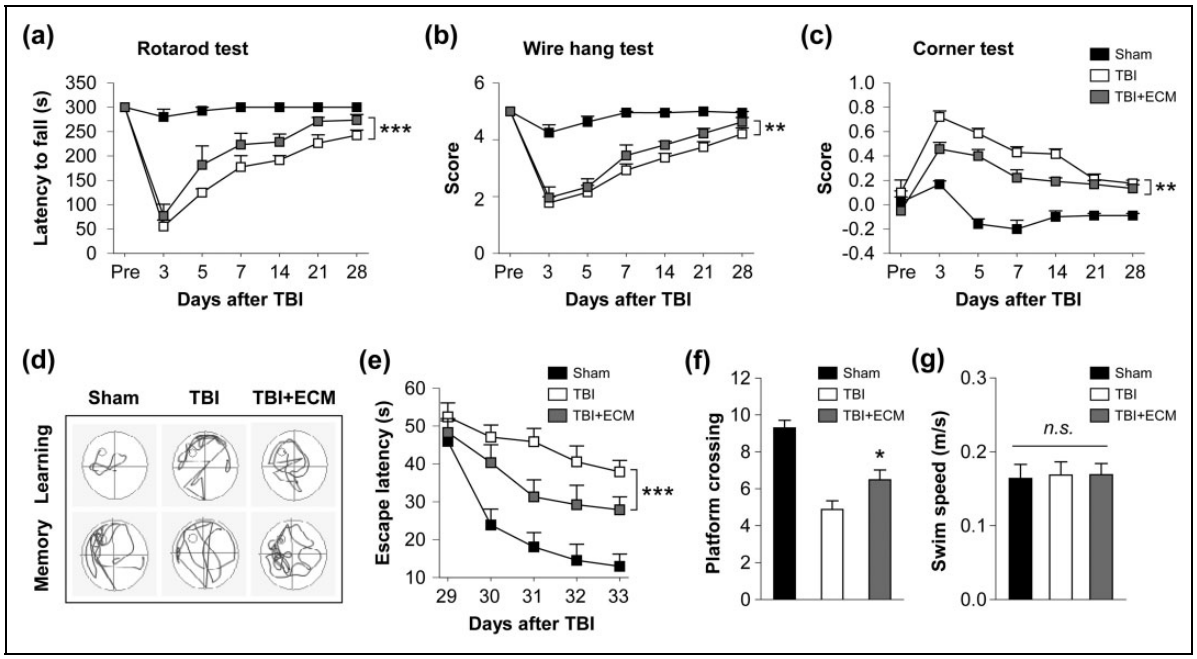

Figure 2. Extracellular matrix (ECM) treatment confers long-term protection against traumatic brain injury (TBI)-induced neurobehavioral deficits. (a-c) The rotarod, wire hang, and corner tests were performed before TBI or sham operation and up to 28 d after TBI to assess sensorimotor functions. ECM treatment attenuated TBI-induced motor deficits, as shown by improved performance in the rotarod test at 21 d after TBI (a) and in the wire hang test at 14 d after TBI (b). (c) Asymmetric sensorimotor deficits were also ameliorated by ECM treatment in the corner test at 3 to 14 d after TBI. (d-g) Long-term cognitive deficits were assessed by the Morris water maze. (d) Representative images of the swim paths of mice in each group when the submerged platform was present (learning phase) and after it was removed (memory phase) are shown. (e) Time to locate the submerged platform was measured from 29 to 33 d after TBI or sham surgery. (f) Spatial memory of the location of the previously submerged platform was measured at 34 d after TBI or sham surgery and expressed as the number of crossings made by the mouse through the goal quadrant when the platform was removed. (g) Swim speed was measured 34 d after TBI and showed no significant difference among groups. n = 8 to 9 mice/group. *$P \leq 0.05$, **$P \leq 0.01$ TBI + ECM versus TBI by 2-way analysis of variance (ANOVA; in a, b, c, and e) or 1-way ANOVA (in f) and Bonferroni/Dunn post hoc test. NS represents not significant.

after TBI (Fig. 1f, 9.14 $\pm$ 0.49 mm^3 vs. 12.17 $\pm$ 0.68 mm^3, $P \leq 0.01$).

ECM Treatment Ameliorates TBI-induced Neurobehavioral Deficits

Brain ECM implantation not only reduced tissue loss but also improved sensorimotor and cognitive functions after TBI. Relative to vehicle-treated mice, ECM-treated mice exhibited superior performance in the rotarod test (Fig 2a, $P \leq 0.001$) and in the wire hang test (Fig 2b, $P = 0.009$) up to 28 d after TBI. In the corner test, TBI-induced asymmetric deficits were attenuated by ECM implantation compared to vehicle controls (Fig. 2c, $P = 0.004$). Long-term cognitive functions were also improved with ECM treatment as revealed by the Morris water maze test (Fig. 2d). ECM-treated mice exhibited lower escape latencies in the learning task (Fig. 2e, $P \leq 0.001$) and improvements in spatial memory (Fig. 2f), with similar gross locomotor function (Fig. 2g). These data suggest that brain ECM treatment provides long-lasting improvements in functional recovery after TBI.

ECM Treatment Preserves Hippocampal CA3 Neurons after TBI

Next, we characterized ECM-afforded protection at the cellular level by examining changes in the number of viable neurons upon treatment. TBI induced prominent neurodegeneration in the ipsilesional hippocampal CA3 region (Fig. 3a). ECM treatment preserved viability in CA3, with a 36% increase in the number of viable neurons compared to vehicle-treated TBI brains (Fig. 3b, 74.84 $\pm$ 4.09% vs. 54.85 $\pm$ 3.85%, $P \leq 0.05$). There was a positive correlation between the animals' spatial memory performance in the Morris water maze and the number of viable CA3 neurons in sham, vehicle-, and ECM-treated groups (Fig. 3c, Pearson product–moment correlation, $r = 0.799$, $P < 0.001$). These data suggest that ECM-mediated improvements in spatial memory might reflect greater preservation of viable neurons in the CA3 region.

ECM Treatment Attenuates White Matter Injury after TBI

White matter injury contributes significantly to the neurological deficits in TBI patients.[2] The integrity of white matter

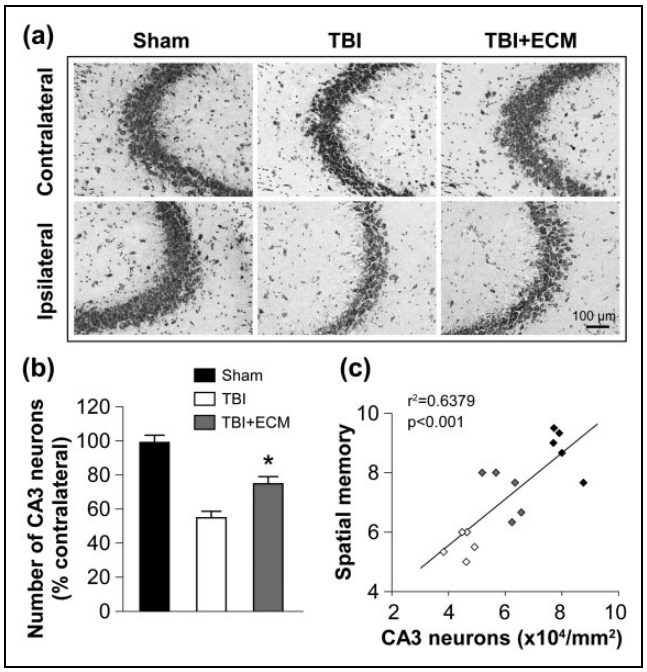

Figure 3. Extracellular matrix (ECM) treatment reduces hippocampal *Cornu Ammonis* 3 (CA3) neurodegeneration after traumatic brain injury (TBI). Mice were subjected to TBI and received ECM implantation 1 h after TBI. (a) Nissl staining reveals viable neurons from both ipsilateral and contralateral CA3 regions of the hippocampi in sham, vehicle-, or ECM-treated mice at 35 d after TBI. (b) The number of CA3 neurons was counted by stereology and expressed relative to the sham contralateral side. (c) Correlation between spatial memory and number of viable neurons in ipsilateral CA3 of the hippocampus. n = 5 mice/group. * $P \leq 0.05$ TBI + ECM versus TBI.

in the ipsilesional hemisphere was severely compromised at 35 d after TBI, including in the CC and striatum. As shown in Fig. 4a, TBI induced the loss of MBP and increased the expression of abnormally dephosphorylated neurofilament protein (detected using the SMI-32 antibody). As a result, the ratio of SMI-32 to MBP immunofluorescence was robustly elevated after TBI (~14-fold in the CC and ~18-fold in the striatum, Fig. 4b), as an index of white matter injury.[17] In the ECM-treated brains, SMI-32 fluorescence was significantly reduced compared to vehicle-treated brains, and the SMI-32/MBP ratio was lowered (by 49% in the CC and 60% in the striatum, $P \leq 0.01$). These data suggest that ECM implantation preserves white matter integrity after TBI, either by protection against white matter destruction or by promotion of white matter repair.

ECM Treatment Inhibits Reactive Astrogliosis and Glial Scar Formation after TBI

TBI induces rapid astrocyte responses at acute stages (Fig. 5a) and promotes the formation of glial scars at chronic stages (Fig. 5b), both of which may impede tissue recovery.[4,22] At 7 d after TBI, a large number of GFAP$^+$

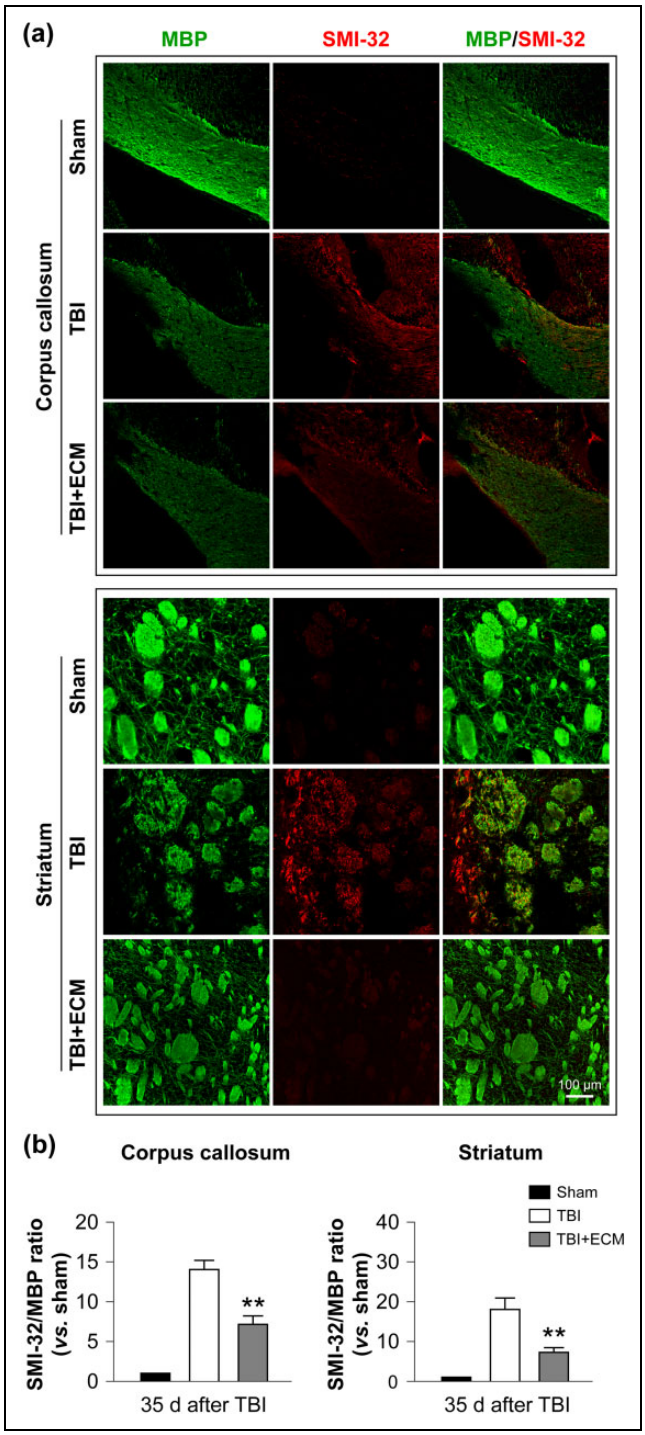

Figure 4. Extracellular matrix (ECM) implantation ameliorates white matter injury after traumatic brain injury (TBI). Mice were subjected to TBI and received ECM implantation 1 h after TBI. Brain sections were dual-stained for rabbit antimyelin basic protein (MBP; green) and nonphosphorylated neurofilament H (mouse antinonphosphorylated neurofilaments [SMI-32]; red) 35 d after TBI. (a) Representative immunofluorescent images of MBP and SMI-32 staining in the corpus callosum (CC) and striatum. (b) The degree of white matter injury was quantified by the ratio of SMI-32 to MBP and expressed relative to sham controls. n = 4 mice/group. ** $P \leq 0.01$ TBI + ECM versus TBI.

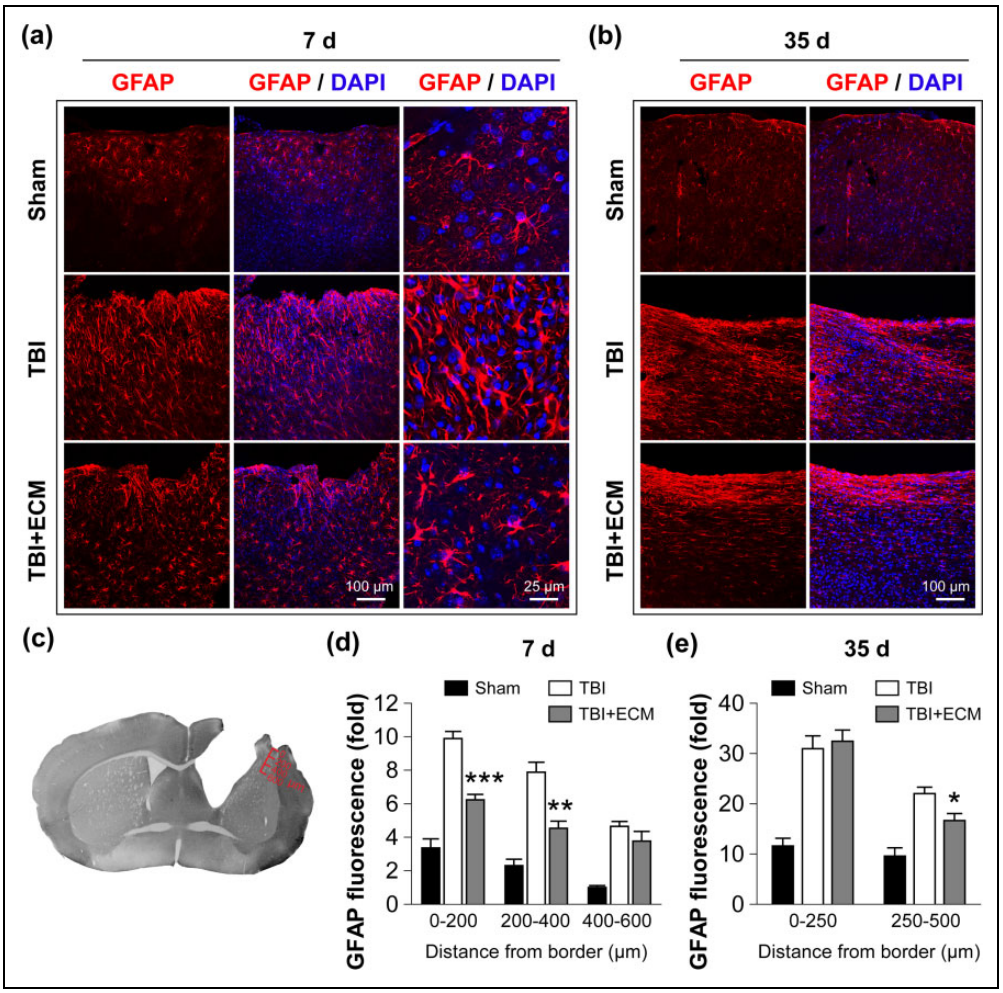

Figure 5. Extracellular matrix (ECM) implantation reduces reactive astrogliosis and glial scar formation after traumatic brain injury (TBI). Mice were subjected to TBI and received ECM implantation 1 h after TBI. Brain sections were stained for the astrocyte marker glial fibrillary acidic protein (GFAP) at 7 and 35 d after TBI. (a,b) Representative immunofluorescence images of rabbit GFAP (red) staining in peri-lesion tissues at 7 d (a) or 35 d (b) after TBI. Sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; blue) for nuclear labeling. TBI induced prominent astrogliosis in the peri-lesion tissues at 7 d and glial scar formation at 35 d after TBI. (c) Illustration of the ipsilateral peri-lesion area where the gradient of GFAP fluorescence intensity was quantified. GFAP fluorescence intensity was measured at different distances from the lesion border at 7 d (d) and 35 d (e) after TBI and expressed relative to the sham group at 400 to 600 μm. n = 4 mice/group. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ TBI + ECM versus TBI.

reactive astrocytes were observed in the ipsilateral peri-lesion area, with more astrocytes in the areas closer to the lesion site (Fig. 5a to c). ECM reduced reactive astrogliosis, as reflected by reduced GFAP immunofluorescence, especially in the area closer to the TBI lesion site, that is, at 0 to 200 μm and 200 to 400 μm from the lesion border (Fig. 5c to d). At 35 d after TBI, a prominent $GFAP^+$ glial scar was formed in the peri-lesion area (Fig. 5b to c). Interestingly, ECM treatment reduced GFAP fluorescence at 250 to 500 μm but not at 0 to 250 μm from the lesion border, perhaps because the injury was too severe at this location (Fig. 5e). These data suggest that ECM inhibits glial scar formation, specifically, the thickness of the scar, at chronic stages after TBI, which may facilitate tissue remodeling.

ECM Treatment Ameliorates Pro-inflammatory Microglial Responses Induced by TBI

Finally, we performed immunohistochemistry to determine the effect of ECM implantation on microglial activation. Ten days after CCI, dramatic microglial activation was detected in the ipsilesional CC, cortex, and striatum as visualized by Iba1 immunostaining (Fig. 6a). Furthermore, most cells demonstrated a pro-inflammatory M1 phenotype,[23] as manifested by the expression of the M1 marker CD16/32 (Fig 6a and b, $P \leq 0.001$ TBI vs. sham in CC, cortex, and striatum). ECM implantation inhibited microglial pro-inflammatory activation after TBI (Fig. 6b). The numbers of $CD16/32^+$/$Iba1^+$ cells were significantly reduced by ECM treatment in the CC ($160.0 \pm 24.3/mm^2$ vs. $316.8 \pm 38.7/mm^2$, $P \leq 0.01$) and striatum ($307.2 \pm 56.9/mm^2$ vs. $505.6 \pm 58.0/$

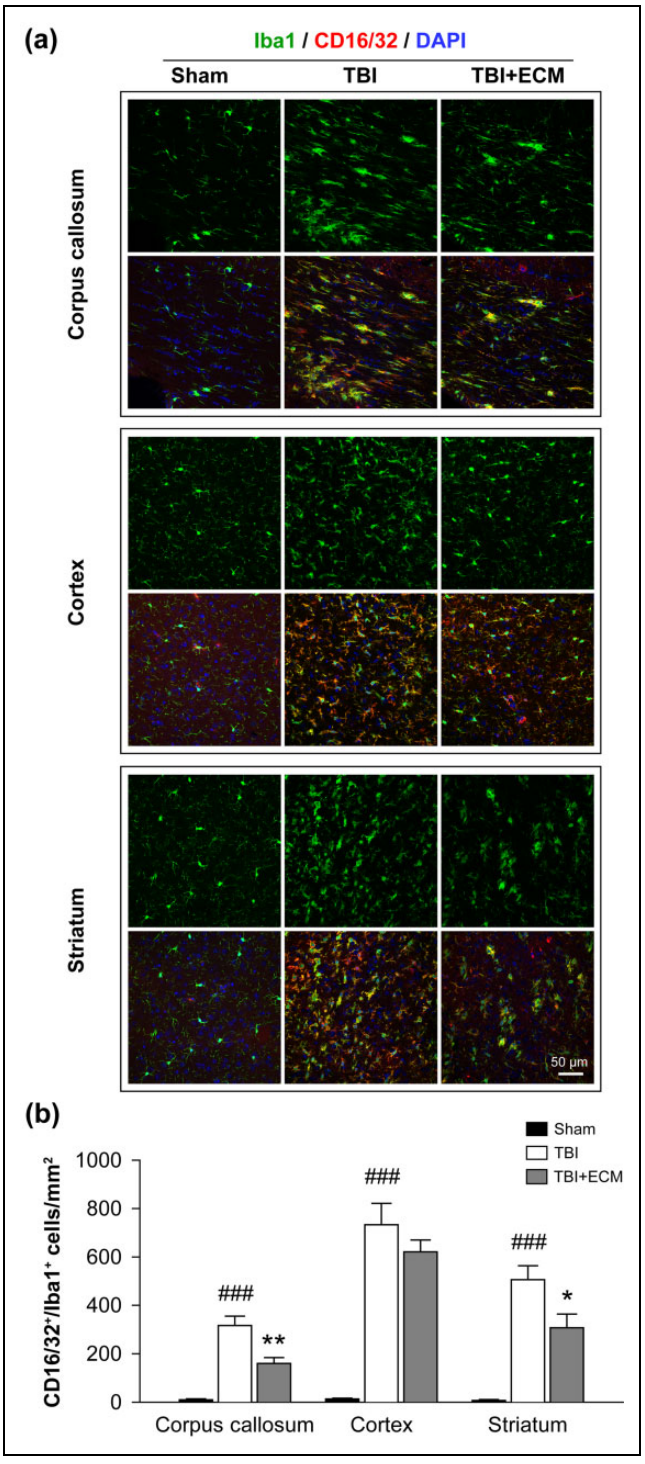

Figure 6. Extracellular matrix (ECM) implantation suppresses pro-inflammatory microglial/macrophage activation after traumatic brain injury (TBI). Mice were subjected to TBI and received ECM implantation 1 h after TBI. Brain sections were dual-stained for microglial/macrophage marker Iba1 and M1 microglial/macrophage marker CD16/32 at 10 d after TBI. (a) Representative immunofluorescence images of CD16/32 (red) and Iba1 (green) staining in the corpus callosum (CC), cortex, and striatum. Sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; blue) for nuclear labeling. (b) Quantification of $CD16/32^+/Iba1^+$ cells. n = 5 mice/group. $^{###}P \leq 0.001$ TBI versus sham. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ TBI + ECM versus TBI.

mm^2, $P \leq 0.05$) at 10 d after TBI. In the cortex, $CD16/32^+/Iba1^+$ cells were not significantly reduced by the ECM treatment (Fig. 6b), possibly due to the lower content of ECM in the cortex compared to the striatum (Fig. 1b). As described in Methods, ECM was delivered to the mice by 2 intracerebral injections, into the right CC and striatum, respectively. These injections resulted in diffusion of the ECM into adjacent areas, including the ipsilateral cortex and hippocampus (Fig. 1b). However, compared to the striatum, there was much less ECM in the cortex (Fig. 1b), likely because the ECM had to diffuse from the injection sites in the CC and striatum to reach the cortex. In summary, these results suggest that implanted ECM modulates microglial polarization by inhibiting the detrimental M1 phenotype in post-TBI brains.

Discussion

The present study is the first to examine the protective effects of brain-derived ECM implantation on post-TBI recovery and to investigate potential underlying mechanisms. ECM treatment reduced the size of the TBI lesion and improved long-term functional outcomes, likely through multiple mechanisms including amelioration of white matter injury and inhibition of gliosis and microglial pro-inflammatory activation.

The ECM is a dynamic and complex 3-dimensional microenvironment secreted by locally residing cells. ECM consists of various tissue-specific structural and functional molecules that affect resident cells, including collagen, glycosaminoglycans, proteoglycans, and growth factors.[24] Viable cells release the appropriate factors into the ECM that facilitate their communication with neighboring cells and continue to support their survival and function in a reciprocal loop. Since tissue repair is severely impeded after destruction of the ECM, native ECM represents an ideal scaffold for tissue-specific reconstruction. Bioengineered scaffold materials composed of ECM have been shown to accelerate post-injury remodeling and repair in various tissues and organs, through mechanisms involving the recruitment of endogenous progenitor cells[12,25] and modulation of innate immune responses.[13] Brain ECM possesses tissue-specific effects in promoting CNS remodeling compared to ECM derived from other tissues. In cultured N1E-115 neuroblastoma cells, brain ECM was shown to promote neurite growth, an effect that was not evident in cells treated with spinal cord ECM or UBM.[15] ECM derived from the CNS also facilitates the differentiation of NSCs into neurons, while UBM does not have a significant impact.[26] In the present study, we demonstrate the therapeutic effects of brain ECM in an animal model of TBI, with some long-term histological protection and functional improvements. Aside from its physical lesion-filling function,[27] brain ECM treatment was able to modulate the brain microenvironment toward a favorable state of promoting post-TBI repair, as discussed further below.

Previous research on brain injuries largely focused on the pathological changes in neurons within gray matter, but few effective therapies have been translated from such unidimensional studies into the clinic. The present study has a more expansive focus on both gray matter and white matter and investigates the rather underappreciated role of ECM in health and disease. Recent studies have highlighted the importance of white matter integrity in long-term recovery after stroke and TBI.[17,28] White matter, consisting of myelin-ensheathed axons and myelin-producing oligodendrocytes, is known to play a critical role in signal transduction and communication across brain regions.[29] White matter destruction is an integral component of most human TBI cases[30,31] and accounts for profound sensorimotor and cognitive impairments.[3] In the present study, ECM treatment preserved white matter integrity and improved long-term neurological function after TBI. Two potential mechanisms might underlie these improvements. First, ECM may directly promote axonal growth and myelination. Multiple components of the ECM, for example, tenascin-C, laminin, and integrins, have been shown to facilitate oligodendrocyte progenitor cell proliferation and migration, as well as their differentiation into mature myelinating oligodendrocytes.[32]

The second explanation for the white matter protection afforded by brain ECM might be an indirect effect from microglia. Microglia are among the most potent regulators of brain repair and regeneration. Microglia undergo phenotypic polarization in response to brain injury.[33,34] Classically activated (M1) microglia release pro-inflammatory mediators and are neurotoxic. In contrast, alternatively activated (M2) microglia produce protective trophic factors and phagocytose cellular debris, thereby facilitating postinjury tissue repair.[23] After TBI, there is a transient M2 activation of microglia followed by a shift to the neurotoxic M1 phenotype,[34] thereby propelling progressive tissue damage. Importantly, ECM implantation reduced microglial M1 activation after TBI, which would be expected to mitigate toxic inflammatory responses and provide a favorable microenvironment for tissue repair.[35] Furthermore, the shift of microglial phenotype might also promote post-TBI white matter repair.[17,28] Our results are consistent with previous findings that ECM scaffold materials induce a change in macrophages phenotype from a predominantly pro-inflammatory population immediately following implantation to an enriched M2-like, regulatory population by 7 to 14 days after implantation.[36] The exact mechanism that controls microglial/macrophage polarization has not yet been identified, and future studies on this topic are warranted.

The heterogeneity of TBI pathogenesis demands that novel therapies target multiple aspects of injury progression in order to elicit true, long-lasting protection of both gray matter and white matter. Our study suggests that brain ECM is a candidate for such multifaceted protection, as it regulates post-TBI white matter repair, microglial polarization, reactive gliosis, and improves functional outcomes. ECM treatment also holds great promise for potential use as a delivery vehicle for other therapeutic agents. Notably, treatment with brain ECM alone conferred limited protection against the TBI lesion volume (Fig 1d to f). This was perhaps because ECM alone was not sufficient to provide sustained support of bioactive substances to facilitate postinjury tissue repair for an extended period of time. While we are aware of this limitation, the present study provides important information that ECM itself achieved significant improvements in post-TBI histological and functional outcomes and modulated the postinjury microenvironment toward a favorable state for tissue remodeling. Although transplantation of neural stem/progenitor cells promotes neural regeneration after brain injury, the survival and functional integration of transplanted cells is severely limited, and a large tissue cavity remains within the injury site.[37–39] Combination of ECM with other treatments, for example, transplantation of NSCs, may boost the beneficial effects of either treatment alone. Our previous study demonstrated that transplantation of UBM carrying NSCs significantly reduced TBI lesion and improved long-term sensorimotor and cognitive deficits.[14] Given the beneficial tissue-specific effects of brain ECM on CNS remodeling and its excellent biocompatibility, future studies on cell therapies administered in combination with brain ECM implantation are highly warranted.

Conclusion

The present study demonstrates long-lasting structural and functional protection offered by brain-derived ECM against TBI-induced tissue loss and neurological deficits. ECM ameliorated TBI-induced neurodegeneration and white matter injury. Furthermore, ECM treatment mitigated glial scar formation and pro-inflammatory microglial responses, thereby promoting tissue remodeling and repair. In summary, brain ECM may be an ideal scaffold material for local transplantation and injection in future studies to achieve a more complete, full-scale recovery after TBI.

Author Contributions

J.C., L.C., J.Z., and Z.L. designed the research. Y.W., J.W., Y.S., H.P., and A.K.F.L. performed the research. S.F.B contributed special reagents and assisted with editing of the manuscript. Y.S. and R.K.L. analyzed the data. Y.W., Y.S., L.C., and J.C. wrote the manuscript. All authors reviewed and approved the manuscript. Y.W., J.W., and Y.S. contributed equally to this research.

Ethical Approval

The protocols in this study were approved by the relevant ethics committee (see Materials and Methods).

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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