Chromatographically isolated CD63$^+$CD81$^+$ extracellular vesicles from mesenchymal stromal cells rescue cognitive impairments after TBI

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Extracellular vesicles (EVs) secreted by cells present an attractive strategy for developing new therapies, but progress in the field is limited by several issues: The quality of the EVs varies with the type and physiological status of the producer cells; protocols used to isolate the EVs are difficult to scale up; and assays for efficacy are difficult to develop. In the present report, we have addressed these issues by using human mesenchymal stem/stromal cells (MSCs) that produce EVs when incubated in a protein-free medium, preselecting the preparations of MSCs with a biomarker for their potency in modulating inflammation, incubating the cells in a chemically defined protein-free medium that provided a stable environment, isolating the EVs with a scalable chromatographic procedure, and developing an in vivo assay for efficacy of the cells in suppressing neuroinflammation after traumatic brain injury (TBI) in mice. In addition, we demonstrate that i.v. infusion of the isolated EVs shortly after induction of TBI rescued pattern separation and spatial learning impairments 1 mo later.

MSCs | neuroinflammation | exosomes | efficacy assay

Traumatic brain injury (TBI) has devastating effects on the victims and creates a large burden on the healthcare system (1). TBI was originally considered an acute injury syndrome, but it is now recognized to have chronic effects similar to those found in neurodegenerative disorders (2–5). In the acute phase, the trauma destroys tissue, and it also triggers a cascade of events that include excessive neural excitability, oxidative stress, disruption of the blood–brain barrier, and inflammation. The cascade causes additional cell death that occurs through necrosis, apoptosis, and excessive autophagy. The cascade involves astrocytes and microglia, in addition to invading neutrophils, monocytes/macrophages, and T cells. The sequence of events is similar to the sequence seen with sterile injuries to other tissues. Initially, proinflammatory effects predominate and are useful in clearing tissue debris. Thereafter, there is a transition to an antiinflammatory phase, with the microglia and macrophages transiting from "classical" proinflammatory M1 phenotype to multiple alternative M2 phenotypes that suppress the M1 proinflammatory mediators and enhance tissue repair. The chronic effects of TBI occur because the inflammatory phase is not fully suppressed. Instead, the inflammatory responses persist, and they initiate a self-perpetuating cycle of tissue destruction, followed by further inflammation. A similar cycle is now recognized to contribute to the pathology of many chronic diseases.

Multiple strategies have been tested to modulate inflammation in TBI and other CNS disorders (2–4). Among these strategies is the use of mesenchymal stem/stromal cells (MSCs) from bone marrow and other tissues (6–19). The beneficial effects of the MSCs are probably explained by their normal roles as perivascular cells that are among the first responders to tissue injury. One of their responses is to act in concert with other cells as guardians of excessive inflammation because they are activated by proinflammatory cytokines such as TNF-α to secrete modulators of inflammation that include TNF-alpha stimulated gene/protein 6 (TSG-6), PGE-2, STC-1, IL-1 receptor antagonist, and TIMP3 (18, 20–25).

Recently, we have explored the hypothesis that extracellular vesicles (EVs) produced by MSCs may be an effective therapy for TBI because extensive recent reports indicate that EVs may provide a highly efficient means of delivering therapeutic factors to target cells (26–29). As noted by György et al. (29), there are several issues that currently limit therapeutic applications of EVs. In the present report, we have addressed most of these issues. In addition, we demonstrate the efficacy of EVs isolated from MSCs in a mouse model for TBI. As this work was in progress, Zhang et al. (30) reported that exosomes isolated from MSCs improved functional recovery in a rat model for TBI, but they did not characterize the exosomes.

Results

Selection of Optimal MSCs and Culture Conditions for Production of EVs. Preparations of tissue-derived MSCs vary in their characteristics dependent on undefined properties of the human donor of the tissue and the site from which the cells are obtained from the same donor (31–34). Therefore, we selected a preparation of bone marrow MSCs (defined as donor 6015), from our NIH-sponsored center for distribution of MSCs, that met the classical in vitro criteria for MSCs and that ranked among the top 3 of 13 MSC preparations in expression of the biomarker of mRNA for TSG-6 that was highly correlated with the efficacy of the cells in modulating inflammation in three murine models (34). MSCs also vary with culture conditions, such as cell densities, and the culture medium (6). To reduce the variability, we followed a protocol in which the MSCs were consistently plated at 500 cells per cm^2 in a standardized medium (21–24) containing 17% of a pretested batch of FBS [defined as complete culture medium (CCM)]. The CCM was replaced after 2 or 3 d. After 5 d, the medium was

Significance

It has gradually been recognized that the vesicles secreted by cells are a major means by which cells communicate with each other. This recognition has stimulated interest in using vesicles to deliver therapeutic agents. The results presented here address several limitations to progress in the field by developing protocols to produce and isolate large numbers of extracellular vesicles (EVs) from stem-like cells found in the bone marrow. The isolated EVs were found to reduce the adverse effects of traumatic injury to the brain in mice.

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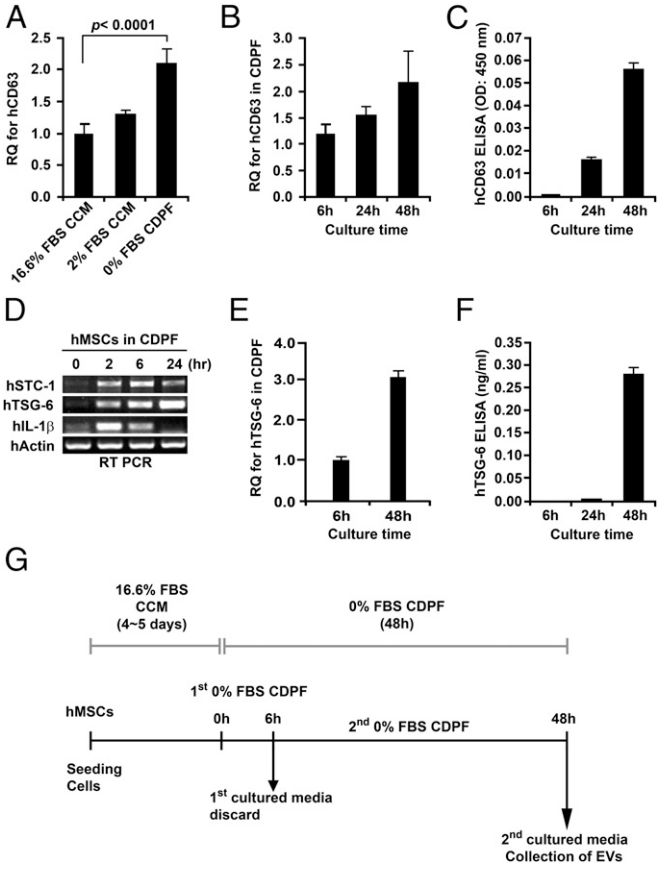

Fig. 1. Defining conditions for production of EVs. Cultures of human MSCs (donor 6015) at 70–80% confluent were transferred to the media indicated and incubated for 6–48 h. (*A*) Expression of mRNA for CD63 was increased by culture for 48 h in CDPF medium compared with culture in CCM with standard concentration of FBS (16.6%) or reduced FBS (2%). Assay by RT-PCR. (*B*) Expression of mRNA for CD63 was increased with time of incubation in CDPF. (*C*) Secretion of $CD63^+$ was increased with time in CDPF. Medium was assayed by ELISA for vesicle-bound protein (Fig. S1). (*D*) RT-PCR assays in MSCs incubated in CDPF indicated that the proinflammatory cytokine IL-1β was expressed for up to 6 h and that expression of the inflammation-modulating protein TSG-6 increased between 2 and 24 h. Expression of the antiapoptotic/calcium-phosphate metabolic protein STC-1 peaked at about 6 h. (*E*) Expression of mRNA for TSG-6 increased with time of incubation in CDPF. (*F*) Secretion of TSG-6 increased with time in CDPF. Assay by ELISA. (*G*) Schematic for protocol developed for production of EVs by MSCs.

changed to a proprietary chemically defined and protein medium (CDPF) that was initially optimized by a commercial supplier for production of recombinant proteins by Chinese hamster ovary cells (Invitrogen). We further supplemented the medium (Table S1) to minimize aggregation of cells secreting TSG-6 by cross-linking hyaluronan on the cell surface. As a convenient marker for EVs, we used assays for CD63 (Fig. S1), a tetraspan protein frequently found in EVs (26–29). Culture of MSCs in the CDPF medium increased the expression of mRNA for CD63 (Fig. 1*A*). The expression of the mRNA increased for at least 48 h and was accompanied by the accumulation of the CD63 protein in the medium (Fig. 1 *B* and *C*). However, the pattern of genes expressed differed during the time of incubation in the CDPF. At 2 h, there was a high level of expression of mRNA for IL-1β, a major proinflammatory cytokine. In contrast, expression of mRNA for the inflammation-modulating protein TSG-6 was low at 2 h and increased progressively at 6, 24, and 48 h (Fig. 1 *D* and *E*). The TSG-6 protein in medium did not increase until about 48 h (Fig. 1*F*). On the basis of these observations, we developed a standardized protocol for production on EVs that might have antiinflammatory properties (Fig. 1*G*). The MSCs did not expand, but there was little evidence of cell death (Fig. 2*A*). Comparison of preparations of MSCs demonstrated that the levels of CD63 protein in the harvested medium were higher in MSCs from donor 6015, the preparation initially selected here, than in three other preparations (Fig. 2 *B*, *i*). As expected, the level of TSG-6 in the harvested medium was the highest in donor 6015 (compare Fig. 2 *B*, *ii*, with figure 4A in ref. 34).

Isolation of EVs with a Scalable Protocol. Most of the published protocols for isolation of EVs involve high-speed centrifugation or other procedures that cannot be readily scaled up for large-scale production (29). To develop a scalable protocol, we isolated EVs from the harvested medium by chromatography. In a small-scale test, we found that most of the protein in the harvested medium bound to an anion exchange resin but that little bound to a cation exchange resin (Fig. 2*C*). Therefore, we developed a

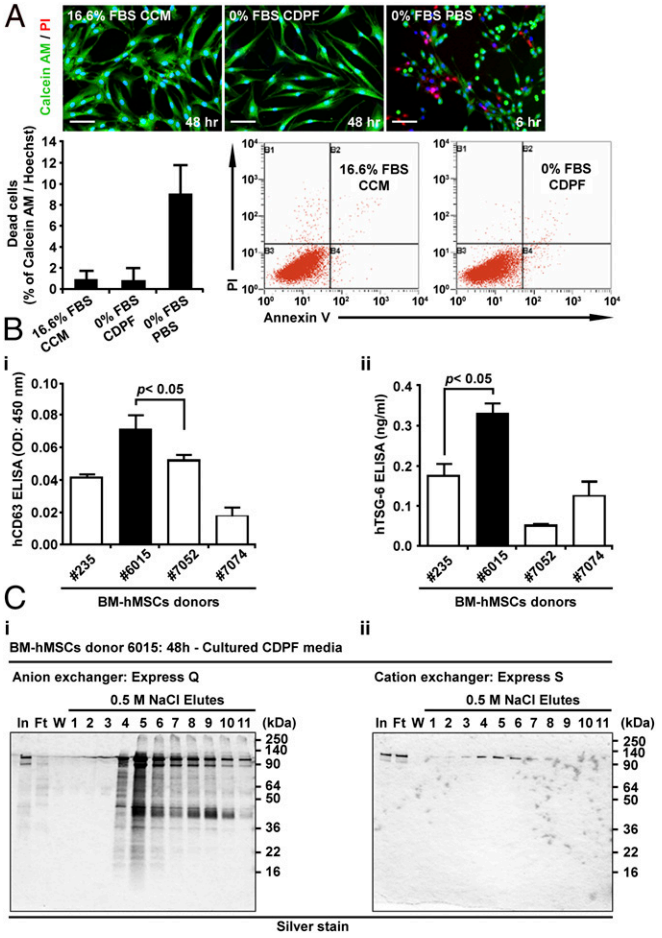

Fig. 2. Survival of the MSCs under the culture conditions, comparisons of four donors, and demonstration that most of the secreted proteins and EVs are anionic. (*A*) Survival of MSCs in CDPF. MSCs were expanded to about 70% confluence and then incubated an additional 48 h in CCM, CDPF, or PBS. (*Top*) Cultures labeled with Hoechst, Calcein AM, and propidium iodide (PI) demonstrate viable cells in CDPF but not PBS. (*Bottom*) Assays of the same cultures by flow cytometry after labeling with PI and Annexin V demonstrated survival in CCM or CDPF medium but not PBS. (*B*) Comparisons of four different preparations of MSCs (donors 235, 6015, 7052 and 7074) after incubation as in Fig. 1*G*. (*i*) $CD63^+$ in medium assayed by ELISA. (*ii*) TSG-6 in medium assayed by ELISA. (*C*) Small-scale assays in SDS-electrophoretic gels demonstrated that most of the medium proteins bound to and were eluted from an anionic resin and not a cationic resin. Gels were stained with silver.

protocol in which the harvested medium was centrifuged at 2,500 × *g* for 15 min and the supernatant was chromatographed on an anion exchange column. The protein eluted with 0.5 M NaCl was recovered as a single broad peak that contained CD63 (Fig. 3 *A*, *i* and *ii*). The recovery of CD63 in the peak ranged from 73% to 81% (n = 3) and was slightly higher than was obtained by centrifuging the harvested medium at 100,000 × *g* for 12 h (Fig. 3*B*). Assay of the peak fractions with a nanoparticle tracking system (Fig. 3*C*) demonstrated that they contained about 0.51 × 10^9 vesicles per microgram of protein. Assays at decreasing concentrations indicated that the mean size of the vesicles was 231 ± 3.2 nm (SEM), 216 ± 2.3 nm, and 207 ± 1.8 nm, respectively. Interestingly, the three peaks observed at the lowest concentration were 85, 165, and 236 nm, the expected sizes of EVs of 85 nm that were also recovered as dimers and trimers.

Surface Epitopes of the Isolated EVs. To map surface epitopes, we used a previously published method (35) whereby EVs are first trapped with a large bead linked to an antibody to CD63, and then additional epitopes on the trapped EVs are assayed with standard protocols for flow cytometry. As expected, the EVs captured with the protocol were positive for CD63 (Fig. S2). They were also about 80% positive for CD81, another epitope frequently found on EVs (26–29). However, they were negative for CD9, a third epitope frequently found on EVs. Also, they were also negative for 13 epitopes found on the surface of MSCs (Table 1). Therefore, they probably correspond to the EVs frequently referred to as exosomes (26–29).

Assay for Efficacy of EVs in Suppressing of Neuroinflammation after TBI. Quantitative assays for efficacy are critical for the development of most therapeutics (29). We elected to develop a quantitative assay for the efficacy of EVs in a model for TBI by ELISAs on brain homogenates. Initial experiments (Figs. S3 and S4) demonstrated that, after TBI, levels for the proinflammatory cytokine IL-1β peaked between 6 and 12 h and that the IL-1β colocalized with GFAP$^+$ astrocytes (Fig. 4*A*). Therefore, we followed a protocol in which TBI was produced in mice and IL-1β levels in brain were assayed 12 h after the TBI. Administration of the EVs decreased the levels of IL-1β in a dose-dependent manner (Fig. 4*B*). The highest dose of EVs was more effective than i.v. infusion of 1 million of MSCs expanded in CCM, apparently because the brains were assayed 12 h after administration of the cells but i.v.-administered MSCs that are trapped in the lung do not express high levels of TSG-6 until 24 h after infusion (36). The dose of EVs that produced the largest effect (30 μg of protein and 15 × 10^9 EVs) was synthesized by about 1 million MSCs under the conditions used here (Fig. 1*G*), but the in vitro and in vivo data are obviously directly comparable. Of special interest was a result in which the dose of EVs that produced the largest effect (30 μg of protein and 15 × 10^9 EVs) contained only 4 ng of TSG-6 whereas administration of 50 μg of

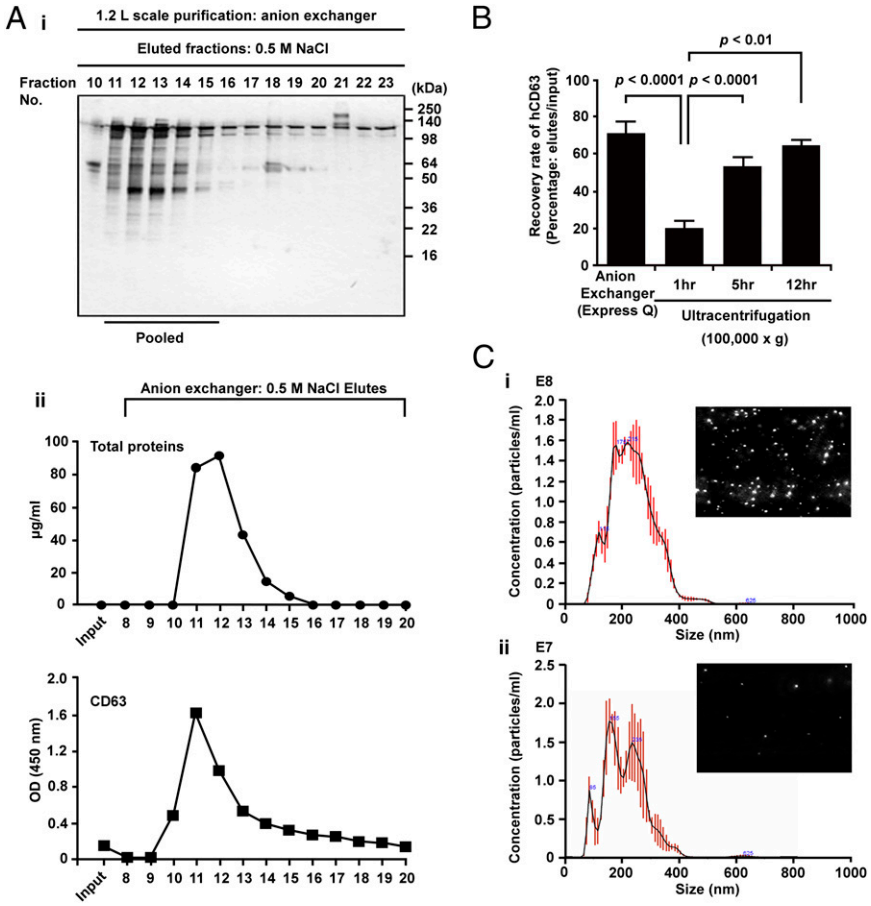

Fig. 3. Chromatographic isolation and characterization of EVs from the medium. (*A*) Preparation and characterization of CD63$^+$ EVs from medium of MSCs incubated as in Fig. 1*G*. (*i*) Assay by SDS-electrophoretic gel of medium eluted from anion exchange column with 0.5 M NaCl. Gel was silver-stained. (*ii*) Assays of eluted fractions for protein and CD63. (*B*) Recovery of CD63$^+$ protein from the column was slightly greater than recovery by centrifuging the same samples at 100,000 × *g* for 12 h. (*C*) Assays of eluted fractions by nanoparticle diffusion analysis demonstrated that the mean size of the vesicles ranged from 209 ± 1.8 nm (SEM) to 231 ± 3.2 nm. The three peaks at the lower concentration (*ii*) were 85, 165, and 236 nm. (*Insets*) Photos of nanoparticles in the instrument.

Table 1. Surface epitopes in hMSCs and EVs

Surface epitopes	hMSCs*		EVs*
	CCM	CDPF	
hMSC markers			
CD29	>99	>99	<1
CD44	>99	>99	<2
CD49c	>99	>99	<1
CD49f	>99	>99	<1
CD59	>99	>99	2.04
CD73	>99	>99	<2
CD90	>99	>99	<1
CD105	>99	>99	<1
CD146	>99	>99	<2
CD147	>99	>99	<1
CD166	>99	>99	<1
HLA-a, b, c	>99	>99	<2
PODXL	95	91	<2
EV markers			
CD9	93	99	<1
CD63	48	85	90.6
CD81	>99	>99	79.9

hMSC, human mesenchymal stem/stromal cell.
*Positively stained cells or EVs (% of total) with antibodies indicated in Table S2.

recombinant TSG-6 i.v. was required previously in four models of induced inflammation in mice (21–23).

After i.v. administration of 30 μg of $CD63^+$ EVs into naive mice, the EVs were detected by ELISAs of plasma after 5 min, but they were not detected after 15 min (Fig. S3). Therefore, they were apparently rapidly distributed to tissues. Low levels of $CD63^+$ EVs were also detected in plasma 5 min after infusion of 1×10^6 MSCs from donor 6015.

Effects of EVs on Pattern Separation Function and Spatial Learning Ability After TBI. Because the isolated EVs decreased inflammation 12 h after TBI, we tested their effects on pattern separation ability and spatial learning and memory function a month after TBI (Fig. 5). Pattern separation is proficiency for discriminating analogous experiences through storage of similar representations in a nonoverlapping manner (37, 38), and the hippocampus neurogenesis plays a major role in maintaining this function. An object-based behavioral test demonstrated preservation of the ability for pattern separation in TBI mice treated with i.v. EVs, in comparison with TBI mice treated with vehicle exhibiting pattern separation deficits (Fig. 5*D*). Furthermore, in a water maze test (Fig. 5*A*), TBI mice treated with i.v. EVs learned to find the concealed platform after three trials as reflected in the decrease in latency time that became similar to the latency time in control mice that received sham operations, implying preservation of spatial learning ability in TBI mice receiving EVs. In contrast, mice that received vehicle after TBI improved only slightly. There were no significant differences in memory retrieval function in a probe test between TBI mice receiving EVs or vehicle because the number of times mice entered the platform zone and the time spent in the platform zone (Fig. 5 *B* and *C*) were similar between the two TBI groups, which contrasted with the behavior of control mice exhibiting greater dwell times in the platform zone.

Discussion

The role of EVs in cellular communication has stimulated renewed interest in using vesicles for the delivery of therapeutic agents (26–29). The strategies have included vesicles prepared from purified components (liposomes), a strategy initiated many years ago, as well as newer strategies with vesicles prepared by fragmentation of cellular membranes and EVs produced by cultured cells. Each strategy is likely to be ideal for different applications. EVs produced by cells have the advantage that they may contain naturally occurring therapeutic cargos and specific cell-targeting

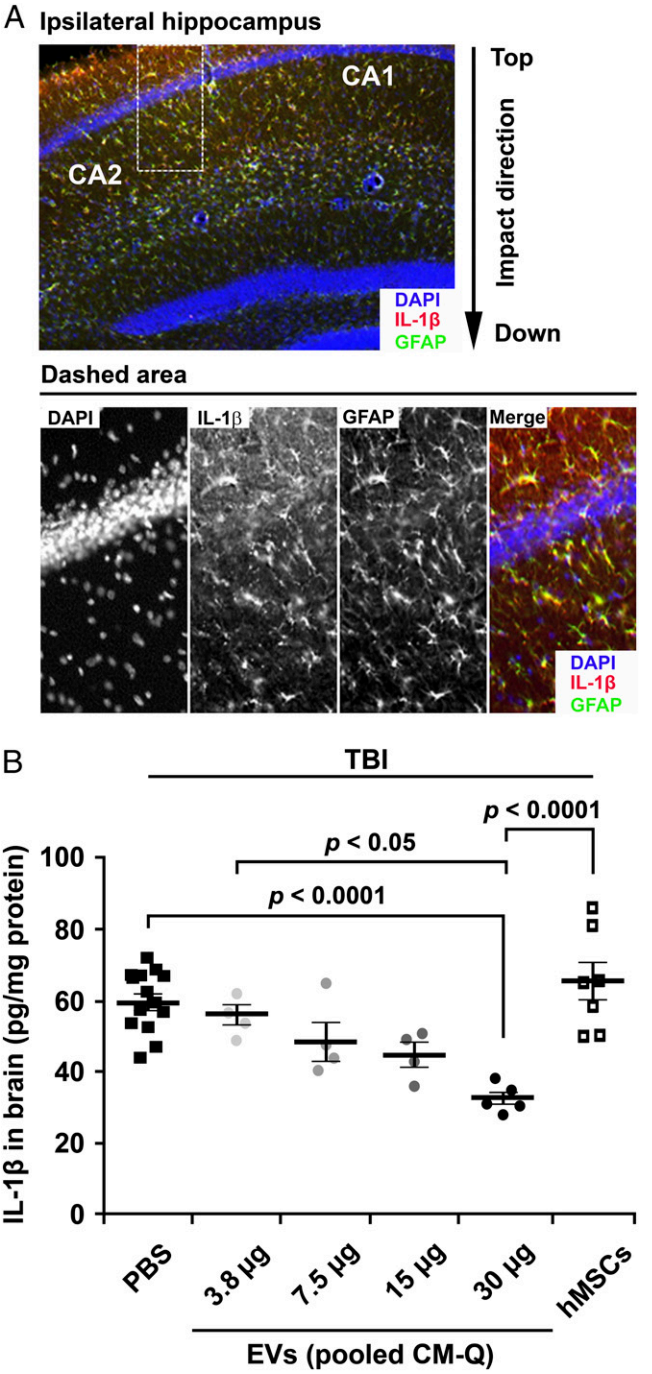

Fig. 4. Dose–response data for suppression of neuroinflammation by EVs after TBI. (*A*) Immunochemistry of brain sections demonstrated that TBI increased IL-1β in $GFAP^+$ astrocytes. Sections from brain recovered 12 h after TBI and sections from region indicated were stained for DAPI, IL-1β, and GFAP. (*B*) Dose-dependent decrease in IL-1β after i.v. administration of PBS or EVs. Amounts varied from 3.5 to 30 μg of protein or $1.8–15.3 \times 10^9$ EVs. PBS or EVs were administered 1 h after TBI, and assays were by ELISA on homogenates of ipsilateral brain isolated 12 h after TBI. The i.v. administration of 1 million MSCs cultured in CCM had little effect, apparently because they were not fully activated in 12 h to express TSG-6 by embolization of the lung (21).

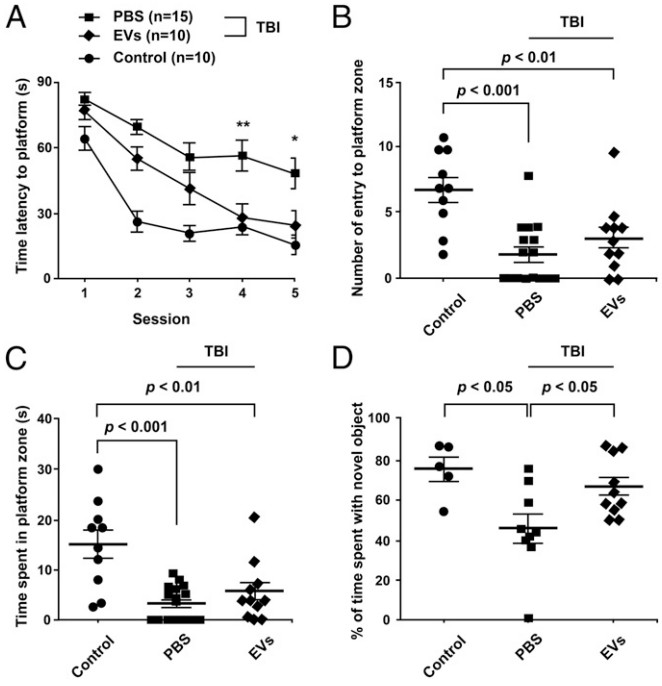

Fig. 5. Improved cognitive function after TBI and i.v. EVs. About 1 h after TBI, each mouse received i.v. PBS or 30 μg of protein (about 15.3×10^9 EVs) from the pooled peak from the anion exchange column (pooled CM-Q) (Fig. 3 *A*, *ii*). Behavior in the water maze was tested 28–33 d after TBI. The pattern separation test was performed 35 d after TBI. (*A*) In the water maze, treated mice with TBI learned to locate the hidden platform with the same latency as controls after four trials and better than TBI mice that received PBS. There was no significant effect of the therapy on the number of entries to platform zone (*B*) or time spent in the platform zone (*C*) in the probe test. (*D*) The treated mice performed better than TBI mice that received PBS in the pattern separation test.

ligands. Also, EVs can be engineered to contain novel cargos and cell-targeting ligands.

The experiments presented here addressed several issues that currently limit therapeutic applications of EVs. Two of the issues are the choice of the producing cells and the culture conditions under which the cells produce EVs. MSCs are attractive choices of cells for several reasons. They can be obtained from multiple tissues, including fat, umbilical cord, and synovial membranes (6–8). The cells from human bone marrow have been the most extensively studied and, like MSCs from other human tissues, can readily be expanded in culture (39). However, they are not tumorigenic (40). Also, they do not undergo spontaneous transformation during expansion in culture to generate malignant lines, as is observed with mouse MSCs (41) and as has been overlooked in multiple publications with murine MSCs (6, 40, 41). Therefore, they are unlikely to transfer tumorigenic factors in the EVs they produce, a risk inherent with immortal cell lines. Instead, the EVs produced by MSCs are likely to transfer therapeutic components, because MSCs have been shown to have beneficial effects in a large number of disease models (6, 7) and in a few clinical trials in patients (42–44). Moreover, the beneficial effects are largely explained by MSCs being activated by signals from injured cells to transfer a large number of therapeutic factors, including cytokines, chemokines, microRNAs, and mitochondria (6, 7, 45, 46). Also, MSCs tend to home to injured tissues, and some of the EVs they produce may retain this homing ability. In addition, MSCs are an attractive source of EVs because of an unusual property of the cells: They can survive for weeks in culture with medium containing no protein or growth factors but continue to produce EVs (47–50). During incubation of MSCs from bone marrow in α-MEM, the cultures underwent selection with survival of a subpopulation that expressed genes characteristic of embryonic cells (47). Pochampally and coworkers subsequently demonstrated that the MSCs incubated in α-MEM produced EVs, and they extensively characterized the EVs (48). They also demonstrated that the EVs supported tumor growth. Phinney et al. (49) demonstrated that EVs produced by human MSCs under different culture conditions partially rescued silica-induced fibrosis of lung in mice.

One limitation in using MSCs for producing EVs is the number of cells that can be obtained (29) because MSCs senesce after extensive expansion in culture (39, 40). Another limitation is that different preparations vary. The present protocol in part overcame these limitations by preselecting an initial preparation of bone marrow MSCs that could be expanded to provide up to 10^{15} cells from a small bone marrow aspirate of 2–4 mL (39). The 10^{15} MSCs are about 10^6 times the amount required for the experiments described here, but it is likely that larger samples of MSCs from bone marrow or other tissues will be required for clinical therapies. The preparation was also preselected by the criteria that the cells expressed high levels of the biomarker TSG-6 mRNA that was highly correlated with the efficacy of the MSCs in suppressing inflammation in three mouse models (34). As indicated here, the preselected cells expressed high levels of mRNA for CD63, an observation that may provide a potency marker for the efficiency of MSCs is producing EVs. To minimize the variations introduced by culture conditions, the cells were expanded at low density with a standardized protocol under which the cells retain most of their progenitor features (39). Also, to recover EVs, the cells were incubated in a chemically defined protein-free medium that was optimized for growth and production of recombinant proteins by CHO cells on a commercial scale (Invitrogen). Apparently, because it is protein-free, the medium induced stress on the MSCs, but it minimized the cell death seen with culturing MSCs under other conditions used to produce EVs (28, 49). The differences in culture conditions may explain why the medium harvested as a $2{,}600 \times g$ supernatant did not contain the large vesicles isolated by Phinney et al. (49).

The experiments were also designed to address two further issues: scalable protocols and quantitative assays for efficacy. To provide a scalable protocol, we used a chromatographic column to isolate the EVs. Use of the column provided a 500-fold concentration of the EVs and a protocol that can more readily be scaled up than protocols than use high-speed centrifugation and related techniques (29). We addressed the need for an efficacy assay with a protocol in which EVs were i.v. infused after TBI in mice and IL-1β levels in brain were measured by ELISA 12 h later. The EVs produced by human MSCs were effective in the WT mice, an observation consistent with the expression by human MSCs of undetectable levels of MHC class II and very low levels of MHC class I (6, 7). Also, it is consistent with the observation here that the EVs were HLA-a, -b, and -c negative. In addition, the observation that human EVs were effective is consistent with previous reports that i.v. administrations of human MSCs produced therapeutic effects in immune competent mice (6), including a model for TBI (36). Therefore, immune reactions to single administrations of human EVs in mouse models are unlikely to produce complicating immune reactions; models requiring repeated administrations may need to be examined more carefully.

Subsequently, we were able to demonstrate that infusion of the effective dose of the isolated EVs after TBI rescued pattern separation and spatial learning impairments 1 mo later. Therefore, the results suggested that, by modulating the initial inflammation produced by the TBI, the EVs interrupted the self-perpetuating cycle of tissue destruction and inflammation that largely explains the chronic effects of TBI (2–5).

At the same time, the results did not resolve several important issues. The $CD63^+CD81^+$ EVs seemed to account for most of the EVs secreted by the cells, but the data did not exclude the possibility that a small fraction of the EVs (less than 10% or

20%) were CD63-negative. The results are similar to the results reported by Vallabhaneni et al. (48), but the data are not directly comparable because of differences in the conditions used to produce MSCs and in many of the assays used. A second unresolved issue is the molecular mechanism whereby the i.v.-infused EVs reduced inflammation and rescue cognitive impairments in the TBI model. The EVs isolated here contained TSG-6, and previous results indicated that some of—but not necessarily all of—the antiinflammation effects of MSCs were explainable by the cells being activated to express TSG-6 (21, 22). However, the amount of TSG-6 in the effective dose of EVs was less than 1/10,000 the amount of recombinant TSG-6 required to suppress inflammation in several animal models. Therefore, the efficacy of the EVs observed here may well be explained by their containing many components other than TSG-6. Unfortunately, the technologies to define the active components of EVs and their effects of EVs on target cells are still challenging (26–29). For example, sequencing of the microRNAs and other RNAs in EVs provides data on millions of potential targets for the microRNAs (48), but it is difficult to identify those with significant effects on their target cells (48, 50).

Materials and Methods

Details are presented in *SI Materials and Methods* on all methods, including culture conditions, chromatographic isolation of EVs, PCR and ELISA assays, nanoparticle tracking analysis, controlled cortical impact injury, behavioral studies, and statistical tests. hMSCs were from the Center for Distribution (medicine.tamhsc.edu/irm/msc-distribution.html) and all animal protocols were approved by the Texas A&M Animal Care and Use Committee. Human MSCs were obtained from normal, healthy donors with informed consent under Scott & White and Texas A&M Institutional Review Boards approved procedures.

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