Mesenchymal stem cells and conditioned medium avert enteric neuropathy and colon dysfunction in guinea pig TNBS-induced colitis

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In recent years, mesenchymal stem cells (MSCs) have generated much interest in the field of regenerative medicine because of their differentiation potential and ability to advance tissue repair via the release of biologically active molecules.
(18). MSCs play a major role in tissue regeneration, repair, and homeostasis with capacity for self-renewal and differentiation into cells of mesodermal lineage such as bone, cartilage, muscle, and fat (4, 20), as well as cells of neuronal and glial lineage when cultured under particular experimental conditions (3, 88). These multipotent cells can be derived from many adult tissues, including bone marrow (BM), adipose tissue, umbilical cord, and placenta (71). Most commonly derived from the BM, MSCs only represent a minute fraction (0.001–0.01%) of the total population of BM nucleated cells; however, their adherent nature enables them to be isolated and rapidly expanded for a number of passages under appropriate cell culturing conditions (4, 28).

Numerous studies, under an array of pathological conditions, have established the capacity of MSCs to migrate to sites of tissue damage and inflammation where they participate in mechanisms of regeneration, regardless of the tissue (36, 44, 55, 74, 83). MSCs act in a paracrine manner to release biologically active molecules, such as cytokines, growth factors, and neuroregulatory molecules, with anti-inflammatory, antiapoptotic, and neuroprotective properties (48, 56, 69). Subsequently, MSCs have been recognized to have immunomodulatory properties, including the capacity to evade allogeneic rejection (64), hinder T cell proliferation (7), initiate T cell apoptosis (57), and inhibit dendritic cell maturation (2).

MSCs demonstrate neuroprotective properties via antiapoptotic, anti-inflammatory, and antiapoptotic influences on neurons within the central nervous system (CNS) (35, 56, 69, 72, 77). These neuroprotective and immunomodulatory MSC qualities imply a therapeutic potential of these cells in attaining neural repair and protection. Furthermore, soluble factors secreted by MSCs into the culture medium may provide all the vital elements for tissue repair (61, 65).

MSCs have been proposed for the treatment of IBD (38, 78). Clinical trials have demonstrated administration of MSCs as a safe and feasible treatment option for complex perianal fistulas associated with Crohn’s disease (17, 23). In experimental models of colitis, MSCs administered intraperitoneally (IP), intravenously (IV), or directly into the colonic wall have proven effective for tissue repair (13, 33, 79). However, the potential of MSCs to alter enteric neural dysfunction resulting from inflammation is unknown.

Despite advances in the prospective of MSCs for the treatment of IBD and numerous nervous system disorders, no studies have evaluated the capacity of MSCs and conditioned medium (CM) to attenuate enteric neuropathy associated with IBD. Considerable evidence implicating persistent ENS damage in the generation of IBD symptoms together with the demonstrated therapeutic abilities of MSCs in nervous system disorders indicates that the study of MSCs for the treatment of enteric neuropathy associated with IBD is warranted. Accordingly, we have investigated the therapeutic potential of enema-applied MSCs and CM for the treatment of enteric neuropathy in the guinea pig model of 2,4,6-trinitrobenzene-sulfonic acid (TNBS)-induced colitis. Human BM-derived MSCs have been most studied and can uniquely evade immune rejection when administered to other species (64, 81); therefore, they were used in this study.

MATERIALS AND METHODS

Animals. Male and female Hartley guinea pigs (140–280 g) (n = 34) were obtained from South Australian Health and Medical Research Institute and housed in a temperature-controlled environment with 12-h day-night cycles. Animals had free access to food and water. All procedures used throughout this study were conducted according to the Australian National Health and Medical Research Council guidelines and approved by the Victoria University Animal Experimentation Ethics Committee.

MSC culture. Human BM-MSC cell lines BM-7025, BM-7081, and BM-5077 (Tulane University) were cultured in accordance with MSC culturing procedures previously described (56). Briefly, 60 cells/cm² were cultured in 20 ml minimum essential medium, 16.5% MSC qualified fetal bovine serum (FBS), 100 U/ml penicillin/streptomycin, and 100× GlutaMAX at 37°C. The medium was replaced every 48–72 h for 10–14 days. When MSCs were 70–75% confluent, cells were lifted by the enzymatic action of TrypLE Select (GIBCO, Life technologies). CM was collected following a minimum of 48 h incubation with MSCs. Unconditioned medium (UCM) was not exposed to MSCs.

MSC characterization. Phenotypic analysis of passage 4 MSCs by flow cytometry was performed (56). MSCs were labeled with anti-human CD34-phycocerythrin, CD45-PerCPCy5.5, CD29-Alexa Fluor 488, CD44-brilliant violet 421, CD73-brilliant violet 421, and CD90-Alexa Fluor 647 antibodies (Biolegend). Peripheral blood mononuclear cells were employed as controls. Osteogenic and adipogenic differentiation potential of MSCs was revealed by 1% alizarin red S or 0.3% Oil Red O labeling as previously described (56). Oil Red O was eluted with 100% isopropanol and quantified colorimetrically (500 nm) on a Bio-Rad plate reader. MSC colony-forming potential was assessed by crystal violet staining methods described previously (30). Briefly, MSCs were seeded (10 cells/cm²) for 14 days and stained with 5% crystal violet for 10 min. After washing, colonies > 2 mm were counted.

Induction of colitis and treatment with MSCs, CM, and UCM. TNBS (30 mg/kg) was administered into the guinea pig distal colon under isoflurane anesthesia as described previously (43). Sham-treated guinea pigs underwent the same procedure without TNBS administration. Guinea pigs in MSC-treated, CM-treated, and UCM-treated groups were additionally anesthetized with isoflurane 3 h after TNBS administration, at the peak of acute intestinal inflammation (59), and received either 1 × 10⁶ MSCs in 300 μl UCM, or 300 μl CM, or 300 μl UCM at the site of inflammation by enema. After application of MSC-based therapies, guinea pigs were held at an inverted angle to ensure treatments did not leak from the rectum. Animals were weighed and monitored 7 days following treatment then culled via stunning and exsanguination (51, 52). Segments of the distal colon were collected for histology, immunohistochemistry (IHC), and mobility analyses.

Immunohistochemistry and histology. IHC was performed in whole-mount longitudinal muscle-myenteric plexus (LMMP) preparations and cross sections of the colon as described previously (53). After 1 h incubation in 10% normal donkey serum (Merck Millipore) at room temperature, whole-mount preparations were labeled with primary antibodies: goat anti-neuronal nitric oxide synthase (nNOS) (1:500) (Novus Biologicals), goat anti-choline acetyltransferase (ChAT) (1:500) (Merck Millipore), mouse anti-CD45 (1: 200), mouse anti-Hu (1:500) and chicken anti-PGP9.5 (1:500) (Abcam) followed by secondary donkey antibodies: anti-mouse Alexa Fluor 594 (1:200), anti-goat FITC 488 (1:200), anti-mouse FITC 488 (1:200), and anti-chicken Alexa Fluor 594 (1:200) (Jackson Immunoresearch Laboratories) and mounted in fluorescent medium (DAKO). For cross sections, tissues were frozen and sections of 20-μm thickness were cut and labeled with rabbit anti-β-tubulin (III) (1:1,000) and mouse anti-CD45 (1:200) monoclonal antibodies (Abcam) followed by donkey anti-rabbit Alexa...
Fluor 594 (1:200) and donkey anti-mouse FITC 488 (1:200) antibodies (Jackson Immunoresearch Laboratories). Green fluorescent-labeled (GFP)-MSCs and FITC anti-human leukocyte antigen (HLA)-A,B,C antibody (1:50) (BioLegend) were used to detect presence of MSCs within the colonic wall. Tissues for histology were paraffin embedded, sectioned at 5 μm, deparaffinized, cleared, and rehydrated in graded ethanol concentrations for standard hematoxylin and eosin (H&E) and Alcian blue staining. Gross morphological damage was assessed by histological grading of four parameters: mucosal flattening (0 = normal, 3 = severe flattening), occurrence of hemorrhagic sites (0 = none, 3 = frequent sites), variation of the circular muscle (0 = normal, 3 = considerable thickening and disorganization of muscular layer), and depletion of goblet cells (0 = normal, 3 = severe depletion of goblet cells).

Fluor 594 (1:200) and donkey anti-mouse FITC 488 (1:200) antibodies (Jackson Immunoresearch Laboratories). Green fluorescent-labeled (GFP)-MSCs and FITC anti-human leukocyte antigen (HLA)-A,B,C antibody (1:50) (BioLegend) were used to detect presence of MSCs within the colonic wall. Tissues for histology were paraffin embedded, sectioned at 5 μm, deparaffinized, cleared, and rehydrated in graded ethanol concentrations for standard hematoxylin and eosin (H&E) and Alcian blue staining. Gross morphological damage was assessed by histological grading of four parameters: mucosal flattening (0 = normal, 3 = severe flattening), occurrence of hemorrhagic sites (0 = none, 3 = frequent sites), variation of the circular muscle (0 = normal, 3 = considerable thickening and disorganization of muscular layer), and depletion of goblet cells (0 = normal, 3 = severe depletion of goblet cells).

Fig. 1. Phenotypic and functional validation of mesenchymal stem cells (MSCs). Flow cytometric analysis showed MSCs to positively express CD29, CD44, CD73, and CD90, but not CD34 and CD45 (red) compared with isotope controls (blue) (A). After 14 days in culture, MSCs were subjected to an osteogenic assay and labeled with alizarin red. Osteogenic differentiation, absent for MSCs cultured in control media (B), was evident when MSCs were cultured in osteogenic differentiation media (B'). MSCs were also subjected to adipogenesis assay after 14–21 days. Labeling of MSCs with Oil Red O revealed a lack of adipogenesis in control media after 21 days (B'') but significant adipogenic differentiation of MSCs cultured in adipogenesis media (B'''). Quantification of adipogenesis plates for the uptake of Oil Red O at 21 days demonstrated a significant increase in MSCs cultured in adipogenesis media compared with control media (P < 0.05) (C). Crystal violet staining demonstrated MSC colony-forming capacity when subjected to a colony-forming unit fibroblast (CFU-F) assay (D). High-power imaging of CFU-F established formation of separate MSC colonies (D'), as well as confirming cells within colonies to display typical MSC phenotype (D''). Overall 19% colony-forming efficiency was observed. Scale bars = 500 μm (B), 1 cm (D'), 1 mm (D''), 100 μm (D'''). Data presented as means ± SD (C).

Fig. 2. MSC migration and homing within the colon. Fluorescent microscopy confirms transmural migration of green fluorescent protein (GFP)-labeled MSCs within the colon wall 7 days after treatment in animals with 2,4,6-trinitrobenzene-sulfonic acid (TNBS)-induced colitis (A). Migration and homing of MSCs through the layers of the colon at the site of TNBS-induced inflammation was further evidenced by labeling of human leukocyte antigen (HLA)-A,B,C-immunoreactive (IR) human cells within guinea pig colon (B). HLA-A,B,C-IR cells were absent in the colon from MSC-only-treated animals, demonstrating lack of MSC homing to noninflamed tissue (C). Scale bars = 50 μm (A), 100 μm (B and C).
recorded colonic contractile activity (30 min equilibration, 3 × 40 min videos). Spatiotemporal maps were produced (Scribble v2.0; MATLAB v2012a) and subsequently utilized to analyze colonic migrating motor complexes (CMMCs) defined as constrictions propagating over >50% of the colon segment.

Statistical analysis. Data were analyzed by one-way or two-way ANOVA with Tukey-Kramer post hoc test for multiple group comparisons. Statistical analyses were generated with GraphPad Prism v5.0. Data were presented as means ± SE, if not specified otherwise. Differences were considered statistically significant when P < 0.05.

RESULTS

Phenotypic and functional validation of MSCs. MSCs were characterized by flow cytometry, differentiation assays, and a colony-forming unit assay. At passage 4, flow cytometric analysis revealed that MSCs met the International Society for Cellular Therapy proposed criteria for defining multipotent MSCs. For a population of cells to qualify as MSCs, the minimal requirements of three criteria must be met: 1) ability to adhere to tissue culture plastic; 2) coexpression of certain CD antigens; and 3) capacity to differentiate into osteoblasts, adipocytes, and chondroblasts under standard in vitro differentiating conditions (22). Cells expressed MSC markers CD73 and CD90, as well as cell migration molecules CD29 and CD44, and lacked expression of CD34 and CD45 (Fig. 1A). MSCs readily differentiated into osteocytes and adipocytes after incubation in osteogenic and adipogenic differentiation media for 14–21 days as indicated by positive staining of calcium deposits with alizarin red S and lipid vacuoles with Oil Red O, respectively (Fig. 1B). Some spontaneous adipogenic differentiation was seen for MSCs cultured in control medium, as described by others (1, 87), but adipogenic differentiation was significantly greater for MSCs cultured in specific medium at 21 days (P < 0.05; Fig. 1C). MSCs were subjected to a colony-forming unit fibroblast (CFU-F) assay with crystal violet labeling. CFU-F assays established a 19% MSC colony-forming efficiency (Fig. 1D). A colony-forming efficiency between 15–20% is considered high for BM-MSCs and not indicative of loss of stemness (63).

MSC homing capacity. To ascertain the homing capacity of MSCs to the area of tissue damage, they were identified in colon cross sections via detection of GFP-labeled MSCs and using antisera for HLA-A,B,C protein specific for human cells. The successful engraftment of MSCs in regions of the colonic inflammation was evident by localization of GFP-MSCs in the lamina propria and at the level of myenteric ganglia 7 days after MSC treatment (Fig. 2A). To further substantiate MSC migration capacities, cross sections of the distal colon from TNBS+MSC administered (n = 3) and MSC-only (without TNBS) administered (n = 3) guinea pigs were labeled with anti-human HLA-

Table 1. Changes in guinea pig body weight (%) for 7 days following treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
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</thead>
<tbody>
<tr>
<td>Control (n = 4)</td>
<td>100.0</td>
<td>104.1±1.8</td>
<td>105.3±1.1</td>
<td>108.0±1.5</td>
<td>110.6±2.1</td>
<td>113.1±1.6</td>
<td>116.9±2.1</td>
<td>119.5±2.4</td>
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<tr>
<td>Sham (n = 5)</td>
<td>100.0</td>
<td>103.1±3.0</td>
<td>105.2±1.9</td>
<td>108.0±2.5</td>
<td>110.2±3.7</td>
<td>112.9±2.8</td>
<td>117.8±3.7</td>
<td>119.9±4.2</td>
</tr>
<tr>
<td>TNBS (n = 5)</td>
<td>100.0</td>
<td>97.2±1.5</td>
<td>97.7±2.0</td>
<td>99.6±0.2</td>
<td>105.2±2.1</td>
<td>111.2±0.9</td>
<td>111.3±2.2</td>
<td>113.7±2.7</td>
</tr>
<tr>
<td>TNBS + MSC (n = 5)</td>
<td>100.0</td>
<td>100.4±0.9</td>
<td>104.2±1.5</td>
<td>106.3±1.8</td>
<td>107.5±1.1</td>
<td>111.5±0.5</td>
<td>112.7±0.9</td>
<td>113.9±1.0</td>
</tr>
<tr>
<td>TNBS + CM (n = 5)</td>
<td>100.0</td>
<td>100.2±1.0</td>
<td>102.7±1.3</td>
<td>105.6±1.5</td>
<td>106.7±1.8</td>
<td>111.2±1.8</td>
<td>113.1±1.7</td>
<td>115.3±1.5</td>
</tr>
<tr>
<td>TNBS + UCM (n = 5)</td>
<td>100.0</td>
<td>98.7±1.9</td>
<td>100.0±3.3</td>
<td>102.7±4.5</td>
<td>106.3±3.5</td>
<td>107.8±3.8</td>
<td>107.7±4.2</td>
<td>109.5±5.0</td>
</tr>
</tbody>
</table>

TNBS, 2,4,6-trinitrobenzene-sulfonic acid; MSC, mesenchymal stem cell; CM, conditioned medium; UCM, unconditioned medium. *P < .05 significantly different from TNBS group, †P < .05 significantly different from TNBS + UCM group.
A,B,C antibody to detect major histocompatibility class I (MHC-I) antigens expressed by all human nucleated cells. Transmural migration and engraftment of human MSCs into the colon wall was evident in sections from TNBS+MSC-treated guinea pigs (n = 3, Fig. 2B). HLA-A,B,C-IR cells were absent in the colon from MSC-only-administered animals (n = 3) demonstrating that MSCs did not home to noninflamed tissues (Fig. 2C).

MSC and CM treatments prevent TNBS-induced weight loss. Guinea pig weight was monitored daily prior to and for 7 days following treatment to demonstrate the systemic influence of therapies tested (Fig. 3, Table 1). Control (n = 4) and sham-treated (n = 5) guinea pigs consistently gained weight. TNBS administration caused significant weight loss during the first 3 days compared with control and sham-treated animals (P < 0.05). Treatment with MSCs (n = 5) and CM (n = 5) applied by enema 3 h after TNBS administration prevented weight loss compared with animals administered with TNBS only (n = 5; P < 0.05 for both). Treatment with UCM (n = 5) did not prevent weight loss compared with control, sham, and MSC-treated guinea pigs (P < 0.05 for all).

Fig. 4. MSC and CM treatments prevent TNBS-induced gross morphological damage to the colon. Histological changes to the colonic architecture and mucin expression 7 days after TNBS application and treatment, as indicated by hematoxylin and eosin (H&E; A) and Alcian blue staining (B). Intact epithelium and an orderly arrangement of muscular layers, submucosa, and mucosal glands were observed in colon tissues from control and sham-treated animals (A-I, II). Disruption to the mucosal lining and obliteration of epithelial cells with some evidence of crypt cell abscesses were observed in colon tissues from TNBS-administered guinea pigs (A-III, IV). Indications of mucosal damage and disruption of epithelial cells were present in UCM-treated guinea pigs (A-V). Plentiful Alcian blue-positive cells in colon sections from control and sham-treated guinea pigs (B-I, II). Less numerous Alcian blue-positive cells were evident in TNBS-treated guinea pigs (B-III). Conversely, treatment with MSCs (B-IV) and CM (B-V) attenuated TNBS-induced Alcian blue-positive cell loss in the guinea pig colon. Scale bars = 50 μm (A), 20 μm (B).
MSC and CM treatments prevent TNBS-induced gross morphological damage to the colon. Gross morphological assessment of H&E and Alcian blue-stained colon sections enabled definition of changes to colonic architecture and presence of goblet cells 7 days after treatment (Fig. 4). Normal functional arrangements of goblet, crypt, and epithelial cells, as well as distinct colonic layers, were seen in H&E-stained sections from control (n = 4) and sham-treated animals (n = 5, Fig. 4A-II). Examination of sections from guinea pigs 7 days after TNBS administration showed epithelial cell loss and glandular disruption (n = 5, Fig. 4AIII). Colonic architecture in MSC-treated (n = 5, Fig. 4AIV) and CM-treated (n = 5, Fig. 4AV) animals was comparable to control and sham-treated animals. The colon from UCM-treated animals displayed morphological

Fig. 5. MSC and CM treatments reduce leukocyte numbers and prevent damage to nerve processes in the distal colon following TNBS-induced colitis. Neuronal processes innervating mucosal glands (arrows) in cross sections of the distal colon from sham-treated (A), TNBS-administered (B), MSC-treated (C), CM-treated (D), and UCM-treated (E) guinea pigs 7 days after treatment were labeled by neuron specific anti-β-tubulin (III) antibody (red) (n = 4/group). Damage to neuronal processes revealed in the colon of TNBS-administered animals was prevented by MSC and CM treatments. UCM treatment had no effect (A–E). The infiltration of leukocytes was determined by double-labeling with anti-CD45 antibody (green) (A’–E’). Infiltration of leukocytes into the colon wall (arrows) was observed in TNBS-administered animals (B’). Treatment with MSCs (C’) and CM (D’) alleviated the number of immune cells present in the distal colon 7 days posttreatment, whereas treatment with UCM (E’) had no effect on leukocyte numbers. Merged images denote colocalization of CD45-IR cells and β-tubulin (III) IR fibers within colon cross sections (A”–E”). Scale bars = 50 μm.
changes similar to untreated TNBS-administered animals (n = 5, Fig. 4A).

Compared with colon sections from control and sham-treated guinea pigs (n = 5, Fig. 4B–D), Alcian blue-stained colon sections from TNBS-only (n = 5, Fig. 4B) and UCM-treated (n = 5, Fig. 4B–V) animals revealed fewer goblet cells because there was a reduction of Alcian blue-positive cells within the mucosa. Treatment with MSCs and CM attenuated changes in goblet cell expression, so that there were large accumulations of Alcian blue-positive cells similar to control and sham-treated colon sections (n = 5, Fig. 4B–V).

Grading of histological parameters indicated colonic inflammation in sections from TNBS-administered and UCM-treated animals. Treatments with MSCs and CM decreased the scores for all histological parameters (mucosal flattening, hemorrhagic sites, muscular change, goblet cell depletion) from 2–3 to 0–1 compared with TNBS- and UCM-treated animals. Since no differences were observed between control and sham-treated animals, only sham-treated animals were used in further experiments.

**MSC and CM treatments reduce the TNBS-induced inflammatory response in the distal colon.** Quantitative analyses of CD45+ leukocytes in the colon were employed as a measure of the severity of inflammatory reaction and the efficacy of the treatment. Colon cross sections (n = 4/group) were double labeled with anti-CD45 antibody specific to leukocyte common antigen and anti-β-tubulin (III) antibody specific to neurons (Fig. 5). Significant increases in leukocyte numbers, indicating inflammatory infiltrate, were evident in TNBS and UCM-treated animals compared with shams (P < 0.01; Figs. 5A–B, 5E–H, and 6A). CD45-IR cells in colon sections from MSC and CM-treated animals were significantly sparser than in TNBS-administered animals (P < 0.01; Figs. 5, C–D) and 6A).

Inflammation and immune infiltration to the level of myenteric ganglia was assessed in whole-mount preparations (Fig. 7, A–E). Quantitative analysis of CD45-IR cells per 1-mm² area (n = 4/group) showed significantly lower numbers of immune cells in LMMP preparations of the colon from sham-treated guinea pigs compared with TNBS-administered and UCM-treated animals (P < 0.001; Fig. 7F). The number of leukocytes at the myenteric level was significantly reduced in MSC and CM-treated compared with TNBS-administered and UCM-treated animals (P < 0.001; Figs. 7, B–D).

**MSC and CM treatments prevent damage to nerve processes.** Neuronal cell bodies and processes innervating smooth muscles and mucosa were labeled by an antibody specific to neuronal microtubule protein β-tubulin (III) (n = 4/group; Fig. 5, A–E). Colon sections from sham-treated guinea pigs showed orderly arrangement of β-tubulin (III)-IR fibers within the mucosal gland cores, submucosal and muscular layers of the colonic wall (Fig. 5A). At 7 days after TNBS administration, β-tubulin (III)-IR fibers were fragmented, disorderly, and erratically distributed within the mucosa (Fig. 5B). The arrangement and distribution of β-tubulin (III)-IR fibers in colon sections from MSC-treated and CM-treated guinea pigs were comparable to those in sections from sham-treated animals (Fig. 5, C and D), suggesting that MSC and CM treatments prevented TNBS-induced axonal damage. Treatment with UCM did not attenuate the damage to nerve processes caused by inflammation (Fig. 5E). Quantification of β-tubulin (III)-IR fiber density correlated with observations in axon structure (Fig. 6B). The total area of β-tubulin (III)-IR area (n = 4/group) was significantly reduced in the colon segments from TNBS-administered (6.8 ± 0.3%) and UCM-treated (6.7 ± 0.6%) compared with sham-treated (10.5 ± 0.9%) animals (P < 0.001 for both). Treatment with MSCs and CM significantly prevented losses in nerve fiber density (9.7 ± 0.4%, 9.8 ± 0.7%, respectively) compared with TNBS-administered and UCM-treated animals (P < 0.05 for all, Fig. 6B).

**MSC and CM treatments protect against TNBS-induced neuronal loss.** Enteric neurons were identified by using the pan-neuronal marker Hu in whole-mount LMMP preparations of the distal colon (n = 4/group, Figs. 8 and 9). The number of Hu-IR myenteric neurons counted within 1-mm² area of the colon from TNBS only- and UCM-treated guinea pigs was reduced by 29 and 24%, respectively, compared with sham-treated animals (P < 0.001 for both; Figs. 8 and 9A). Colon tissues from MSC and CM-treated guinea pigs following

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Fig. 6. Quantitative analysis of the total number of CD45-IR cells and density of nerve fibers in cross sections of TNBS-inflamed colon. The total number of CD45-IR cells counted in cross sections of the distal colon per 1-mm² area (A) was significantly less in sham-treated compared with TNBS-administered animals 7 days after induction of colitis. Both MSC and CM treatments significantly suppressed the inflammation-induced immune infiltration of the colon compared with TNBS-administered animals. Colon sections from UCM-treated animals displayed significantly greater CD45-IR compared with sham, MSC-, and CM-treated guinea pigs. The density of nerve fibers measured as a percentage β-tubulin (III)-IR per 1-mm² area (B) was significantly reduced in the colon sections from TNBS-administered and UCM-treated animals compared with the colon sections from sham-treated guinea pigs. MSC and CM treatments preserved nerve fiber density 7 days following an inflammatory assay. *P < 0.05, **P < 0.01.
TNBS administration exhibited only 2 and 10% fewer Hu-IR cells than sham-treated animals, but significantly higher numbers of Hu-IR neurons compared with TNBS- and UCM-treated guinea pigs ($P < 0.05$ for all, Figs. 8, 9A).

**MSC and CM treatments prevent TNBS-induced changes in the number and proportion of nNOS-IR neurons.** The number of nNOS-IR myenteric neurons ($n = 4$/group) was significantly higher in the colon of TNBS-administered (52.8 ± 3.9) and UCM-treated (51.6 ± 2.1) guinea pigs compared with sham (37.6 ± 1.3), $P < 0.01$ for both), MSC (40.4 ± 1.1, $P < 0.05$ for both), and CM-treated (39.3 ± 2.7, $P < 0.05$ for both; Figs. 8, A$^I$–E$^I$ and 9B). Consequently, the proportion of nNOS-IR neurons to the total number of neurons within 1-mm$^2$ area of the colon was significantly greater in TNBS-administered (52.8 ± 1.8) and UCM-treated (46.7 ± 3.1) guinea pigs compared with sham (26.7 ± 0.5), MSC (28.0 ± 1.0), and CM-treated (30.3 ± 1.5) animals ($P < 0.001$ for all; Figs. 8, A$^{II}$–E$^{II}$ and 9C). An increase in the soma size of nNOS-IR myenteric neurons was observed in the colon from TNBS-administered animals (1,057.7 ± 239 μm$^2$, $P < 0.001$, Fig. 8B, inset) and UCM-treated animals (882.6 ± 35 μm$^2$, $P < 0.001$, Fig. 8E, inset) compared with sham-treated animals (406.1 ± 53 μm$^2$, Fig. 8A, inset). MSC and CM treatments prevented TNBS-induced nNOS-IR neuronal cell body hypertrophy in colon preparations (439.4 ± 136 and 350.3 ± 45 μm$^2$, $P < 0.001$, Fig. 8, C$^I$–D$^I$, insets).

**MSC and CM treatments prevent TNBS-induced changes in the number of ChAT-IR neurons.** The number of ChAT-IR myenteric neurons ($n = 4$/group) was significantly lower in the colon of TNBS-administered (48.5 ± 1.3) and UCM-treated (52.8 ± 1.8) guinea pigs compared with sham (77.5 ± 2.3), MSC (74.5 ± 2.1), and CM-treated (74.3 ± 2.3) animals ($P < 0.001$ for all; Figs. 10, A$^{II}$–E$^{II}$, and 11A). Changes in the total number of ChAT-IR neurons corresponded with losses in the total number of neurons and there were no significant differences in the proportion of ChAT-IR to total number of neurons within 1-mm$^2$ area of the distal colon (Figs. 10, A$^{II}$–E$^{II}$, and 11B).

**MSC and CM treatments prevent TNBS-induced changes in colonic motility.** CMMCs were assessed to determine the effectiveness of MSC and CM treatments to prevent TNBS-induced colon dysfunction (Fig. 12A). The speed of CMMC propagation, 7 days after TNBS administration (7.5 ± 0.8 mm/s, $n = 8$), was significantly greater than in sham-treated animals (4.1 ± 0.1 mm/s, $n = 5$, $P < 0.001$; Fig. 12B). The frequency of contractions was lower in the colon from TNBS group (1.6 ± 0.1 min$^{-1}$) than from sham-treated guinea pigs (2.3 ± 0.04 min$^{-1}$, $P < 0.01$; Fig. 12C). Treatment with MSC and CM ($n = 5$ for both) significantly attenuated the speed of CMMC propagation (3.8 ± 0.2 mm/s, 4.3 ± 0.5 mm/s; $P < 0.001$ for all) and increased the frequency of contractions (2.3 ± 0.07 min$^{-1}$, 2.2 ± 0.2 min$^{-1}$; $P < 0.05$ for all; Fig. 12, B–C). Treatment with UCM ($n = 7$) produced a decrease in the...
contraction speed (5.5 ± 0.3 mm/s; Fig. 12B) and an increase in the CMMC frequency (1.9 ± 0.1 min⁻¹; Fig. 12C) observed in the colon from TNBS-administered guinea pigs; however, this effect was not significant compared with the TNBS group, nor compared with sham, MSC, or CM-treated animals (P > 0.05).

**DISCUSSION**

This study is the first to examine the effects of MSC and CM treatments administered by enema on the enteric neurons in the guinea pig model of TNBS-induced colitis. Key findings revealed that local application of human BM-MSCs...
and CM 3 h after the induction of colitis preserves colonic architecture, reduces the quantity of immune infiltrate both transmurally and at the level of the myenteric ganglia, prevents neuronal loss and axonal damage, and alleviates inflammation-induced changes to GI motility 7 days after administration of treatment.

It is well recognized that MSCs possess remarkable immunomodulatory properties in regulating the inflammatory response via downregulation of proinflammatory cytokines and/or upregulation of anti-inflammatory factors. In both in vitro and in vivo studies, MSCs have been shown to suppress T1, macrophage, and natural killer cell activities; induce regulatory T cells (2, 82); and modulate dendritic cell maturation (34). Regardless of their tissue of origin, MSCs have displayed immunosuppressive and anti-inflammatory actions in animal models and clinical trials for a variety of autoimmune and inflammatory diseases, such as Crohn’s disease (17, 23), graft-vs.-host disease (86), systemic lupus erythematosus (76), and Type 1 diabetes (26).

Human MSCs express moderate levels of HLA MHC-I but lack expression of MHC-II, enabling human derived MSCs to escape immune rejection when administered to various species (81). Both in vitro and in vivo studies show that MSCs do not elicit a proliferative response from allogeneic lymphocytes, evading normal alloresponses and enabling successful transplantation into diverse allogeneic and xenogeneic locations (49, 62, 64). Human MSCs isolated from the BM are the most established in animal and clinical studies (41). Therefore, human BM-MSCs were utilized in this study.

In vitro culture of MSCs can influence several characteristics important to their in vivo efficiency (68). We conducted analyses of BM-MSCs to ensure that culture conditions did not affect expression of cell surface markers, cell growth, or their differentiation potential. Subsequently, our results have shown that MSCs used in this study have all characteristics of MSCs defined by the International Society for Cellular Therapy (22). The in vitro differentiation capacity of MSCs cannot be extrapolated to in vivo conditions. This study was performed at a time point of 7 days after the induction of colitis; therefore the capacity to see in vivo differentiation into particular cell lineages was restricted. Furthermore, it has also been reported that in vivo administered MSCs may not survive long enough for differentiation owing to their limited telomerase activity (12, 24) and that MSCs frequently exhibit therapeutic benefits without engraftment or differentiation (60).

Passage 4 MSCs were employed for in vivo experiments in this study because MSC secretion of anti-inflammatory cytokines, neurotrophic factors, and immunomodulating agents is most optimal at early passages, gradually declining with successive passages (16). Timing of administration can additionally impact MSC efficacy and homing capacity. MSC expression of surface chemokine receptors and adhesion molecules enables their homing and migration to sites of inflammation and injury (36). Injured tissues upregulate chemoattractants and release them into the circulation, stimulating MSCs to downregulate the adhesion molecules holding them at their niche (45). In our study, MSCs were administered 3 h after TNBS-induced inflammation, when the most significant damage to the mucosa occurs (59). Immunolabeling of MSCs with anti-HLA-A,B,C antibody, as well as use of GFP-labeled MSCs, confirmed their successful migration and engraftment within the inflamed colon wall, but not in the noninflamed colon. The migratory capacity of MSCs is one of their unique biological attributes. Hence, the significance of these cells migrating throughout the layers of the colon indicates their

Fig. 9. Quantitative analysis of the total number of neurons and subpopulation of nNOS-IR neurons in the myenteric ganglia. The total number of myenteric neurons labeled with anti-Hu antibody counted in whole-mount preparations per 1-mm² area (n = 4/group) (A) was significantly less in the distal colon of TNBS-administered and UCM-treated animals compared with sham-treated guinea pigs. MSC and CM treatments prevented neuronal loss compared with TNBS-administered animals. The total number of nNOS-IR neurons (B) was significantly greater in the colon from TNBS-administered compared with sham, MSC-, and CM-treated guinea pigs. The proportion of nNOS-IR neurons to total number of neurons (C) was significantly higher in the colon of TNBS-administered and UCM-treated guinea pigs compared with sham-treated, MSC-treated, and CM-treated animals. *P < 0.05, **P < 0.01, ***P < 0.001.

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expression of chemokine receptors and other molecules associated with MSC homing (36).

This study used the novel approach of MSC application by enema, which has not been previously reported. In contrast to prior studies, using systemic IP and IV injections of MSCs, application by enema is less invasive and avoids the initial uptake and accumulation of MSCs within filtering organs, such as the lungs, liver, or spleen associated with IV and IP delivery (36). Direct injection of MSCs into the colonic wall has been investigated (33), but this is highly invasive and not clinically feasible. Application of MSCs by enema is safe, convenient, and minimally invasive, provid-

Fig. 10. Effects of MSC and CM treatments on choline acetyltransferase (ChAT)-IR myenteric neurons. Whole-mount LMMP preparations of the distal colon from sham-treated (A), TNBS-administered (B), MSC-treated (C), CM-treated (D), and UCM-treated (E) guinea pigs 7 days after administration of treatment (n = 4/group). Confocal microscopy was used to visualize and capture Hu-IR (red) and ChAT-IR (green) myenteric neurons. Neuronal cell bodies were distinguished by HuC/HuD labeling (A–E). Cell bodies and processes of ChAT-IR subpopulation of neurons were labeled with anti-ChAT antibody (A′–E′). The merged images of Hu-IR and ChAT-IR neurons indicate the subpopulation of ChAT-IR neurons within the total population of myenteric neurons (A″–E″). Scale bars = 50 μm.
neurons to the total number of neurons (per 1-mm² area; and CM treatments prevented a decrease in the number of ChAT-IR myenteric ganglia following TNBS-induced colitis. The total number of ChAT-IR neurons (per 1-mm² area; n = 4/group) (A) was significantly less in the colon preparations from TNBS-administered and UCM-treated guinea pigs 7 days after induction of TNBS-colitis compared with sham-treated animals. MSC and CM treatments prevented a decrease in the number of ChAT-IR myenteric neurons caused by TNBS-induced inflammation. The proportion of ChAT-IR neurons to the total number of neurons (per 1-mm² area; n = 4/group) (B) was not significantly different between sham-treated, TNBS-administered, MSC-treated, CM-treated, or UCM-treated guinea pigs. ***p < 0.001.

Our results demonstrate that treatment with CM is as effective for alleviation of acute inflammation and enteric neuropathy as MSC treatment. MSCs act in a paracrine manner to secrete large amounts of anti-inflammatory, antiapoptotic, trophic, and growth factors promoting endogenous repair mechanisms (6, 48, 61, 65). Therefore, the positive effects of CM observed in our study can provide a potential avenue for its use in the treatment of intestinal inflammation. Specific factors within CM that provide positive effects need to be further defined.

Results of this study indicate that the systemic influences of both MSC and CM treatments applied by enema were actioned rapidly, significantly preventing TNBS-induced weight loss within the first 24 h after application and maintaining longstanding effect, at least for 7 days. TNBS-induced colitis typically involves epithelial cell obliteration and gross morphological damage to the intestinal wall (21). In our study, a single administration of MSCs or CM into the colon by enema restored inflammation-induced epithelial and goblet cell loss, as well as aiding repair of colonic architecture to control and sham-treated levels. Our results are consistent with previous studies involving IP and IV administration of MSCs, which reported reductions in gross morphological damage to the colon following MSC treatment (33, 79).

Administration of TNBS into the distal colon initiates inflammation through a cell-mediated immune response consistent with transmural T1-lymphocyte infiltration (29, 85). Invasion of immune cells into the level of myenteric ganglia is indicative of an acute inflammatory process, and previous studies have positively correlated the severity of inflammatory infiltrate with the severity of IBD recurrence (25, 50). Our results indicate the severity of colitis by infiltration of CD45-IR inflammatory cells through the colonic wall to the level of myenteric ganglia. MSCs produce an immunomodulatory effect by interacting with both innate and adaptive immune cells. This was confirmed in our study where leukocyte numbers were significantly reduced throughout the distal colon wall and specifically at the level of myenteric ganglia in tissues from MSC- and CM-treated animals compared with TNBS-administered animals. These results correlated with our findings that MSC and CM treatments prevented loss of myenteric neurons and damage to the structure and density of nerve fibers associated with colitis.

Acute inflammation induced by TNBS caused a 29% loss of myenteric neurons, as well as a significantly reduced nerve fiber density, in the distal colon compared with sham-treated animals; these data are consistent with previous studies (10, 43, 53). The results of this study demonstrated that after MSC and CM treatments, the number of myenteric neurons was similar to the levels in the colon from sham-treated animals. This can probably be attributed to prevention of neuronal loss rather than differentiation of MSCs into enteric neurons. Several studies have described the in vitro differentiation of MSCs into cells of the neuronal and glial lineage (3, 88), but this occurs only when MSCs are cultured under particular experimental conditions as discussed above. The neuroprotective effects of MSC and CM treatments found in our study correlate with previous reports in animal models of CNS disorders (37, 39), in which MSCs prevented axonal damage and promoted nerve fiber regeneration through the production of trophic factors. The loss of myenteric neurons in TNBS-administered animals correlated with colonic dysmotility, indicating disruption of the appropriate coordination and activation of motility reflex circuits. TNBS-induced disruption of motility persists at least 7 days after inflammation. Significant differences in velocity and frequency of CMMCs between TNBS-administered and sham-treated animals established in our study validate the functional consequences of inflammation-induced structural alterations to myenteric neurons. Application of MSCs and CM reestablished the velocity and frequency of CMMCs. Treatment with UCM had some effect on propagation speed and rate of contraction that was not significant compared with all groups. These changes are probably due to direct effects of culture medium on intestinal smooth muscles. It has been previously observed that visceral smooth muscles in culture medium display increases in number of contractions, as well as speed of contraction (14).

Inhibition of colonic motility observed in TNBS-administered animals correlated with changes in the subpopulations of
myenteric neurons immunoreactive to nNOS and ChAT. nNOS-producing neurons and ChAT-producing neurons are involved in the regulation of gut motility, and disturbances in nNOS/ChAT balance have been described in certain intestinal motility disorders (15). nNOS is characteristically expressed by inhibitory motor neurons and interneurons innervating the circular and longitudinal muscles of the GI tract. Our results demonstrated an increase in the total number and proportion of nNOS-IR neurons in the TNBS-inflamed colon, as well as alterations in the size of nNOS-IR neuronal cell bodies. The TNBS-induced increase in nNOS-IR neurons in the inflamed colon is consistent with previous findings (11). Furthermore, changes in the size and proportion of nNOS neurons have been associated with oxidative stress (5). MSC and CM treatment counteracted morphological changes and inflammation-induced increases in the quantity and proportion of nNOS-IR neurons. Cholinergic neurons are the major excitatory neurons of the ENS and include intrinsic sensory neurons, interneurons, and excitatory motor neurons (32). In this study, decrease in the total number of ChAT-IR neurons in the TNBS-inflamed colon corresponded with losses in the total number of neurons. MSC and CM treatments protected the ChAT-IR subpopulation of enteric neurons from inflammation-induced loss.

In conclusion, this study clearly indicates the therapeutic potential of enema-administered MSC and CM treatments for preventing enteric neuropathy and alleviating colonic dysfunction associated with TNBS-induced colitis. Our results demonstrated, for the first time, that BM-MSCs have the capacity to resolve insults to the ENS 7 days after the induction of acute intestinal inflammation. Furthermore, we have shown the equivalent proficiency of CM treatment for exhibiting neuroprotective effects, promoting enteric neuronal repair and functional recovery. Further research should include investigations to determine which specific factors released by MSCs contribute the most to neuroprotective and anti-inflammatory effects in intestinal inflammation, as well as the long-term effects of MSC and CM treatments.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

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