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

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1College of Health and Biomedicine, Victoria University, Melbourne, Australia; 2Department of Anatomy and Neuroscience, Monash University, Melbourne, Australia; 3Australian Regenerative Medicine Institute, Monash University, Melbourne, Australia; and 4Department of Physiology, Melbourne University, Melbourne, Australia

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Robinson AM, Sakkal S, Park A, Jovanovska V, Payne N, Carbone SE, Miller S, Bornstein JC, Bernard C, Boyd R, Nurgali K. Mesenchymal stem cells and conditioned medium avert enteric neuropathy and colon dysfunction in guinea pig TNBS-induced colitis. Am J Physiol Gastrointest Liver Physiol 307: G1115–G1129, 2014. First published October 9, 2014; doi:10.1152/ajpgi.00174.2014.—Damage to the enteric nervous system (ENS) associated with intestinal inflammation may underlie persistent alterations to gut functions, suggesting that enteric neurons are viable targets for novel therapies. Mesenchymal stem cells (MSCs) offer therapeutic benefits for attenuation of neurodegenerative diseases by homing to areas of inflammation and exhibiting neuroprotective, anti-inflammatory, and immunomodulatory properties. In culture, MSCs release soluble bioactive factors promoting neuronal survival and suppressing inflammation suggesting that MSC-conditioned medium (CM) provides essential factors to repair damaged tissues. We investigated whether MSC and CM treatments administered by enema attenuate 2,4,6-trinitrobenzene-sulfonic acid (TNBS)-induced enteric neuropathy and motility dysfunction in the guinea pig colon. Guinea pigs were randomly assigned to experimental groups and received a single application of TNBS (30 mg/kg) followed by 1 × 106 human bone marrow-derived MSCs, 300 μL unconditioned medium 3 h later. After 7 days, the effect of these treatments on enteric neurons was assessed by histological, immunohistochemical, and motility analyses. MSC and CM treatments prevented inflammation-associated weight loss and gross morphological damage in the colon; decreased the quantity of immune infiltrate in the colonic wall (P < 0.01) and at the level of the myenteric ganglia (P < 0.001); prevented loss of myenteric neurons (P < 0.05) and damage to nerve processes, changes in ChAT, and nNOS immunoreactivity (P < 0.05); and alleviated inflammation-induced colonic dysmotility (contraction speed; P < 0.001, contractions/min; P < 0.05). These results provide strong evidence that both MSC and CM treatments can effectively prevent damage to the ENS and alleviate gut dysfunction caused by TNBS-induced colitis. Inflammatory bowel disease; intestinal inflammation; mesenchymal stem cells; enteric neurons; colon motility

INFLAMMATORY BOWEL DISEASE (IBD) includes two major pathological conditions, Crohn’s disease and ulcerative colitis, characterized by chronic relapsing inflammation within the gastrointestinal (GI) tract. The symptoms experienced by IBD patients are associated with detrimental effects on the enteric nervous system (ENS), which innervates the GI tract and controls its functions, including gut motility, intestinal barrier function, secretion, visceral sensation, and blood flow (10, 27, 54). It is now recognized that persistent GI inflammation and immune activation affects inessential structural changes to enteric neurons, such as neuronal death and nerve fiber degeneration (10, 19, 54, 67), and that ENS functional changes, including hyperexcitability of enteric neurons and alterations in neurotransmission, can persist long after the resolution of acute intestinal inflammation (42, 46, 51, 52). Changes to neurons at the level of the myenteric ganglia have been associated with altered intestinal motility (19, 58). Immune cell invasion to the level of the myenteric and submucosal plexuses (plexitis) can be predictive of IBD recurrence (25, 73). Thus changes in the ENS may be prognostic of disease progression, play a role in IBD recurrence, and underlie gut dysfunction. This suggests that protection of enteric neurons may decrease disease severity.

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...
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-PerCP/Cy5.5, 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 were counted.

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 stunning and exsanguination (51, 52). Segments of the distal colon were collected for histology, immunohistochemistry (IHC), and motility 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 5 mm 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

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 allogenic rejection (64), hinder T cell proliferation (7), initiate T cell apoptosis (57), and inhibit dendritic cell maturation (2).

MSCs demonstrate neuroprotective properties via antiproliferative, 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).

Clinical trials have demonstrated administration of MSCs as a safe and feasible treatment option for complex perianal fistulas (4, 28). 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).

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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).

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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 (BII). 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 (BIII) but significant adipogenic differentiation of MSCs cultured in adipogenesis media (BIV). 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 (DI), as well as confirming cells within colonies to display typical MSC phenotype (DII). Overall 19% colony-forming efficiency was observed. Scale bars = 500 μm (B), 1 cm (D), 1 mm (DII), 100 μm (DIII). 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).
was significantly lower 1–3 days after TNBS administration compared with density of nerve fibers was determined by measuring preparation with Image J software (NIH). In cross sections, the neurons was measured by tracing neuronal profiles of 40 neurons per control (n = 4), sham (n = 5), MSC (n = 5), and CM-treated animals (n = 5, *; †P < 0.05 for all).

Imaging. Confocal microscopy was performed with a Nikon Eclipse Ti confocal laser scanning system (Japan). Fluorophores were visualized by a 488-nm excitation filter for Alexa 488 or FITC and a 559-nm excitation filter for Alexa 594 or rhodamine red. Z-series images were acquired at a nominal thickness of 0.5 μm. The total number of myenteric neurons immunoreactive (IR) for Hu, nNOS, and ChAT, as well as CD45-IR cells were counted within four randomly captured images (total area size 1 mm²) per preparation at ×60 magnification. The total soma area (μm²) of individual nNOS-IR neurons was measured by tracing neuronal profiles of 40 neurons per preparation with Image J software (NIH). In cross sections, the density of nerve fibers was determined by measuring β-tubulin (III)-IR per 1-mm² area with Image J software at ×60 magnification. Gross morphological damage in H&E-stained colon sections and goblet cell mucin in Alcian blue-stained sections were visualized by using a BX53 Olympus microscope and images captured with CellSense software.

Colonic motility. Colonic motility experiments were undertaken by applying methods previously employed (31). Guinea pig colon was placed in organ bath chambers superﬁxed with Krebs solution maintained at 37°C, cannulated at both ends, and positioned horizontally. The oral cannula was attached to a Krebs solution reservoir that was maintained at 37°C, cannulated at both ends, and positioned horizontally. 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-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-labeled MSCs and using anti-GFP antibodies. 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).

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

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
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<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>
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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>
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<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>
</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>
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<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>
</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>
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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).

![Figure 4](http://ajpgi.physiology.org/)

**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). 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-II; arrows). Colon sections from MSC- (A-IV) and CM-treated (A-V) animals revealed restoration of epithelial cells and mucosal repair 7 days after induction of colitis. Indications of mucosal damage and disruption of epithelial cells were present in UCM-treated guinea pigs (A-VI). Less numerous Alcian blue-positive cells in colon sections from control and sham-treated guinea pigs (B-I). Plentiful Alcian blue-positive cells in colon sections from control and sham-treated guinea pigs (B-II). 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-I). Examination of sections from guinea pigs 7 days after TNBS administration showed epithelial cell loss and glandular disruption (n = 5, Fig. 4A-II). Colonic architecture in MSC-treated (n = 5, Fig. 4A-I) and CM-treated (n = 5, Fig. 4A-II) 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-I–E-I). Infiltration of leukocytes into the colon wall (arrows) was observed in TNBS-administered animals (B-I). Treatment with MSCs (C-I) and CM (D-I) alleviated the number of immune cells present in the distal colon 7 days posttreatment, whereas treatment with UCM (E-I) 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-II–E-II). 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) 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).

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, and 6A). CD45-IR cells in colon sections from MSC and CM-treated animals were significantly sparser than in TNBS-administered animals (P < 0.001; Figs. 6, C–D) and 6A).

Inflammation and immune cell 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%) guinea pigs (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 < 0.005 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
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. Scale bars = 50 μm (A–E). ***P < 0.001.

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 ± 1.8) and UCM-treated (51.6 ± 1.8) guinea pigs compared with sham (26.7 ± 1.3) animals (P < 0.001 for all; Figs. 8, AII–EII and 9B). Consequently, the proportion of nNOS-IR neurons to the total number of neurons within 1-mm² 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 ± 0.3), MSC (28.0 ± 1.0), and CM-treated (30.3 ± 1.5) animals (P < 0.001 for all; Figs. 8, AII–EII 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 μm², P < 0.001, Fig. 8BII, inset) and UCM-treated animals (882.6 ± 35 μm², P < 0.001, Fig. 8EII, inset) compared with sham-treated animals (406.1 ± 53 μm², Fig. 8AII, inset). MSC and CM treatments prevented TNBS-induced nNOS-IR neuronal cell body hypertrophy in colon preparations (439.4 ± 136 and 350.3 ± 45 μm², P < 0.001, Fig. 8, CII–DII, 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, AIII–EIII, 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-mm² area of the distal colon (Figs. 10, AIII–EIII, 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-versus-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.](http://ajpgi.physiology.org/fig9.png)
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-
Neurons to the total number of neurons (per 1-mm² area; and CM treatments prevented a decrease in the number of ChAT-IR myenteric after induction of TNBS-colitis compared with sham-treated animals. MSC preparations from TNBS-administered and UCM-treated guinea pigs 7 days administration of MSCs or CM into the colon by enema ically involves epithelial cell obliteration and gross morphological damage to the intestinal wall (21). In our study, a single actioned CM that provide positive effects need to be further defined. CM observed in our study can provide a potential avenue for its mechanisms (6, 48, 61, 65). Therefore, the positive effects of trophic, and growth factors promoting endogenous repair

**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**

![Fig. 11. Quantitative analysis of total number of ChAT-IR neurons and the proportion of ChAT-IR neurons to total number of neurons in the 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.**
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 distribution 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


