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ARTICLE

Bone marrow mesenchymal stromal cells support regeneration of intestinal damage in a colitis mouse model, independent of their CXCR4 expression

Burcu Pervin[1,2] | **Merve Gizer**[1,3] | **Mehmet Emin Şeker**[1,2] | **Özgür Doğuş Erol**[1,2] | **Sema Nur Gür**[1,2] | **Ece Gizem Polat**[1,2] | **Bahar Değirmenci**[4] | **Petek Korkusuz**[3,5] | **Fatima Aerts-Kaya**[1,2,6]

[1]Department of Stem Cell Sciences, Hacettepe University Graduate School of Health Sciences, Ankara, Turkey

[2]Hacettepe University Center for Stem Cell Research and Development (PediSTEM), Ankara, Turkey

[3]Micro-Electro-Mechanic Systems (MEMS) Center, Middle East Technical University, Ankara, Turkey

[4]Department of Molecular Biology and Genetics, Bilkent University, Ankara, Turkey

[5]Department of Histology and Embryology, Hacettepe University Faculty of Medicine, Ankara, Turkey

[6]Hacettepe University Experimental Animals Application and Research Center (HÜDHAM), Ankara, Turkey

Correspondence
Fatima Aerts-Kaya, Department of Stem Cell Sciences, Center for Stem Cell Research and Development, Hacettepe University, Sıhhiye 06100, Ankara, Turkey.
Email: fatimaaerts@yahoo.com; fatima.aerts@hacettepe.edu.tr

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Abstract

Inflammatory bowel disease (IBD) is characterized by a chronically dysregulated immune response in the gastrointestinal tract. Bone marrow multipotent mesenchymal stromal cells have an important immunomodulatory function and support regeneration of inflamed tissue by secretion of soluble factors as well as through direct local differentiation. CXCR4 is the receptor for CXCL12 (SDF-1, stromal-derived factor-1) and has been shown to be the main chemokine receptor, required for homing of MSCs. Increased expression of CXCL12 by inflamed intestinal tissue causes constitutive inflammation by attracting lymphocytes but can also be used to direct MSCs to sites of injury/inflammation. Trypsin is typically used to dissociate MSCs into single-cell suspensions but has also been shown to digest surface CXCR4. Here, we assessed the regenerative effects of CXCR4^{high} and CXCR4^{low} MSCs in an immune-deficient mouse model of DSS-induced colitis. We found that transplantation of MSCs resulted in clinical improvement and histological recovery of intestinal epithelium. In contrary to our expectations, the levels of CXCR4 on transplanted MSCs did not affect their regenerative supporting potential, indicating that paracrine effects of MSCs may be largely responsible for their regenerative/protective effects.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

Expression of the chemokine receptor CXCR4, known as the main regulator of directed stem cell migration, is largely lost after incubation with trypsin.

WHAT QUESTION DID THIS STUDY ADDRESS?

Here, we wanted to assess the effectiveness of transplantation of CXCR4^{low} and CXCR4^{high} MSCs in the repair of intestinal damage, induced by DSS.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

We found that despite low expression of CXCR4, MSCs were still capable of significant repair, indicating that the effects of MSC transplantation are at least partially mediated by paracrine factors.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

Clinical use of MSC-derived exosomes or its secretome may be sufficient to alleviate intestinal damage in inflammatory diseases, including inflammatory bowel disease.

INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are two separate conditions that together make up the clinical picture of inflammatory bowel diseases (IBD). IBD is mainly characterized by a chronically dysregulated immune response in the gastrointestinal tract. Microbiological, genetic, and environmental factors (like antibiotics and smoking) are all involved in the development of IBD.[1] Whereas CD affects the gastrointestinal tract transmurally, UC has been shown to mainly affect the colonic mucosa.[2] Treatment options for IBD typically include life-long medication, consisting of aminosalicylates in combination with anti-inflammatory medication, steroids, anti-TNFα agents, and/or immune suppressive drugs.[3] However, a fraction of the patients remains irresponsive to treatment or develops resistance against treatment in time.[2,4] Therefore, alternative strategies, such as use of (gut-specific) anti-integrins (vedolizumab, etrolizumab), anti-interleukins which target the production of inflammatory interleukins by activated T and NK cells (ustekinumab, risankizumab), use of small molecules, fecal transplantation, and stem cell transplantation are now being explored.[5] Especially stem cell therapy, which may consist of hematopoietic stem cell (HSC) transplantation (to reset the immune system) or local or intravenous infusions of MSCs (to suppress the overactivated immune system and participate in and support intestinal regeneration) have gained wide interest and are currently being assessed in a number of clinical trials.[6,7] Although local injections with MSCs have shown great promise in terms of suppression of clinical disease activity, results obtained after intravenous injections of MSCs have been puzzling, with some trials reporting clinical remission,[6,7] whereas others reported no effect or even clinical aggravation of symptoms.[8,9] Although the source (allogeneic/autologous), dose and infusion route of the MSCs all may affect the outcome of the stem cell treatment, we believe other factors need to be taken in account as well.

We and others have previously shown that human BM-MSCs express CXCR4 on their surface.[10,11] CXCR4 is the receptor for CXCL12 (SDF-1, stromal-derived factor-1) and has been shown to be the main chemokine receptor, required for homing of MSCs to the bone marrow or sites of injury/inflammation.[12,13] Increased expression of CXCL12 by intestinal epithelial cells (IECs) from patients with IBD has been shown to support constitutive inflammation by attracting activated T cells to the intestinal mucosa.[14,15] However, this increased expression of CXCL12 in the inflamed gut can be used to our advantage to direct migration of MSCs toward sites of inflammation. Previously, increased expression of CXCR4 by human endometrial regenerative cells through preincubation of cells with CXCL12 has been shown to alleviate experimentally induced colitis by supporting enhanced migration of the stem cells to the gut.[16]

Clinical application of MSCs requires considerable stem cell expansion in culture vessels. In addition, preparation of infusible cell concentrates requires the production of single-cell suspensions via collection of the adherent cells by enzymatic dissociation solutions, such as trypsin with or without the addition of EDTA.[17] However, we have previously shown that the use of trypsin, which is standardly used for collection of culture expanded human BM-MSCs for both research and clinical purposes, is correlated with a significant decrease in surface expression of CXCR4.[11] Although in vivo use of MSCs has shown a favorable safety profile, large discrepancies in the expected/predicted effects, based on in vitro data,[18,19] suggest that these differences in MSC potency may be related to the fact that the stem cells may not (or not in sufficient numbers) reach their target organ/tissue.

Therefore, we hypothesized that the use of trypsin may cut the CXCL12 binding domain of CXCR4, effectively rendering the MSCs blind and unable to migrate to areas of injury in response to a CXCL12 gradient. To test this hypothesis, we wanted to assess whether MSCs collected in the presence or in the absence of trypsin were able to migrate to damaged tissues and provide immunomodulatory

and/or regenerative effects, alleviating dextran sodium sulfate (DSS)-induced colitis in a mouse model of IBD.

METHODS

Animals

All mouse experiments were performed at the Hacettepe University Experimental Animals Application and Research Center after approval by the Hacettepe University Animal Experiments Ethical Committee (2021/06–19). And 8–12-week old healthy male donor Balb/c mice and immune-deficient female Balb/c-Rag2$^{-/-}$ (RAG2) mice[20] were bred and housed at the Hacettepe University Experimental Animals Research Laboratory in IVC cages. All animals were allowed access to irradiated chow ad libitum.

BM-MSC isolation

Femurs and tibias from Balb/c donor mice ($n=6$) were rinsed with PBS once and collected cells were run through a 40 μm cell strainer. Cells were counted with Turk's solution and plated in T25 flasks at a density of 4×10^5 cells/cm^2 in complete medium (CM), consisting of DMEM-LG (Gibco, Cat. No. 31885-023) supplemented with 20% fetal bovine serum (Gibco, Cat. No. 10270-106), 1% penicillin/streptomycin (Cegrogen, Cat. No. P0100-790) and 2 mM L-Glutamine (Sigma, Cat. No. G3126). Media were changed every 3–4 days.

Characterization of BM-MSCs

BM-MSCs were stained with fluorescent antibodies against CD29, CD44, and CD90.2 (BioLegend) for 15 min in the dark in the presence of PBN (PBS, 0.5% bovine serum albumin and 0.05% NaN_3) with 2% mouse serum and analyzed with a BDAccuri flow cytometer (Becton Dickinson) using the BD CSampler Analysis software 1.0 for Mac (Becton Dickinson). BM-MSCs were differentiated into adipogenic and osteogenic direction using the mouse mesenchymal stem cell functional ID kit (R&D systems, cat no SC010), according to the manufacturer's instructions. Media were changed every 3–4 days and after 21 days adipogenic cultures were stained with Oil Red O (Sigma, Cat. No. O-0625) and osteogenic cultures were stained with Alizarin Red S (Applichem, Cat. No. A2306). For semi-quantitative analysis of differentiation, ORO dye and calcium were extracted and assessed, as described before.[21] For immune fluorescent staining, cultures were fixed and permeabilized, followed by blocking with 10% normal donkey serum (Abcam, Cat. No. ab7475) in PBS. Adipogenic and osteogenic cultures were stained with anti-mFABP4 (R&D, Cat. No. 967799) and anti-mOsteopontin (R&D, Cat. No. 967802), respectively, followed by staining with a secondary donkey anti-goat antibody-NL-557 (R&D, Cat. No. NL001). Nuclei were counterstained with DAPI (Sigma, Cat. No. 8417).

Collection of CXCR4^{high} and CXCR4^{low} BM-MSCs

CXCR4^{high} BM-MSCs were collected using a non-enzymatic cell dissociation solution (NE-CDS, Biological Industries, Cat. No. BI03-071-1B) and run through a 40 μm cell strainer. CXCR4^{low} BM-MSCs were collected using standard trypsinization. Briefly, cells were incubated for 10 min at 37°C in the presence of 0.25% trypsin/2 mM EDTA. All cells were collected and counted with 0.4% Trypan Blue. CXCR4 expression levels were assessed using rat-anti-mouse CD184 (BioLegend, Cat. No. 146508). For assessment of CXCR4 re-expression kinetics, BM-MSCs ($n=5$) were trypsinized, washed once with PBS, spun down and resuspended in CM in 15 mL tubes. Cells were incubated at 37°C for 15, 30, 60, 120, and 480 min. After this incubation, cells were spun down and fixated in ice cold 4% paraformaldehyde and stained with anti-CD184. Re-expression of CXCR4 was analyzed using a BDAccuri or an Agilent Novocyte flow cytometer.

Induction of colitis and transplantation

RAG2 mice were exposed to 3% 40 kDa dextran sodium sulfate (DSS, Sigma, Cat. No. 42867) in their drinking water for five consecutive days. Control animals were provided with normal drinking water. DSS-induced colitis was assessed using the disease activity index (DAI), which uses a grading system (0–4) to assess weight loss, consistency of the stool, and presence of blood in the stool, where DAI 0 equals healthy, DAI 6 moderate colitis and DAI 12 severe colitis.[22] After 5 days of DSS treatment and confirmation of at least a grade DAI 6 colitis, animals were transplanted intraperitoneally (i.p.) with 1.5×10^6 CXCR4^{high} (pos, $n=3$) BM-MSCs or CXCR4^{low} (neg, $n=3$) BM-MSCs. Control mice did not receive any cells ($n=3$). In order to trace the cells after transplantation, CXCR4^{high} BM-MSCs were stained with red Fluorescent Cell Linker (Sigma, PKH26), whereas CXCR4^{low} BM-MSCs were stained with the green Fluorescent Cell Linker (Sigma, PKH67).

Assessment of intestinal permeability before and after transplantation

To assess intestinal permeability in response to DSS treatment before and after transplantation, animals were fed 15 mg/mouse FITC-Dextran (Sigma, Cat. No. FD4-1G) through oral gavage. Four hours later, serum was collected from the animals and fluorescence was detected using black 96-well plates and a SpectraMax i3x microplate reader at 528 nm.

Histological assessment of intestinal damage

Seven days after transplantation, ascending, transverse, and descending colons were carefully isolated to keep the mucosal microstructure intact. Untreated mice were used as negative control, mice receiving DSS but no BM-MSCs served as positive control. All samples were fixed in formaldehyde, dehydrated in a graded series of ethanol, and cleared in xylene. Hematoxylin/eosin (HE) stained sections (3 µm) were evaluated using a bright-field microscope (Leica, Germany) with a digital camera (DFC7000T, Leica) and images were analyzed for inflammatory cell infiltrate, epithelial changes, and mucosal architecture (Table S1) with LASX software (LASX, Leica) using a modified protocol.[23]

Tracking of transplanted BM-MSCs by confocal microscopy

Untreated negative control mice, DSS-treated positive control mice (not-transplanted) and DSS-treated mice transplanted with intraperitoneally infused PKH26 (fluorescent red)-labeled CXCR4^{high} and PKH67 (fluorescent green)-labeled CXCR4^{low} MSCs were sacrificed and samples from the ascending, transverse, and descending colons were snap frozen in liquid Nitrogen and assessed using confocal microscopy (LSM 980, Zeiss, Germany) to track the intestinal homing on serial frozen sections by producing 5–6 Z-stacks per sample. Intestinal mucosal morphology, including vascularity and cellularity of the stroma, was assessed for the presence of fluorescent cells. Nuclei were stained with DAPI (blue).

Prediction of trypsin cleavage sites

Trypsin is known to cleave peptide bonds between arginine (R) or lysine (K) residues and the adjacent amino acid, except when the amino acids are near a proline (P) residue on the carboxyl side of the cleavage site.[24] Furthermore, the cleavage rate is decelerated when they are in the vicinity of acidic amino acids, such as aspartate (D), glutamate (E) or cystine (C). Using these data, trypsin cleavage sites for human and murine CXCR4 were predicted manually, as well as by using the Expasy-Peptide cutter free online tool (https://web.expasy.org/peptide_cutter).

Statistical analysis

Statistical analyses were performed using the Excel spreadsheet program. For comparison of two groups Student's *t*-test was used, for comparison of more than two groups ANOVA was used. The Shapiro–Wilk test was used to assess the normality of distribution of histomorphometric data. Multiple comparison and pair-wise comparison of histological data were performed using the 2-way ANOVA and Tukey test, respectively on GraphPad Prism version 9.0 software (GraphPad, USA). Data were considered statistically significant at a p-value <0.05.

RESULTS

Trypsin abolishes murine BM-MSC CXCR4 expression

Healthy Balb/c mice BM-MSCs stained highly positive for CD29 (94.3 ± 4.1%), and to a lesser extent positive for CD44 (73.8 ± 16.4%) and CD90.2 (19.2 ± 15.8%). Murine BM-MSCs displayed the capacity for differentiation, similar to human BM-MSCs, as apparent by positive staining for ORO and mFABP4 for adipogenic and ARS and mOsteopontin staining for osteogenic lineage (Figure 1).

When healthy donor BM-MSCs were collected using different types of dissociation methods, we observed wide differences in cellular viability, with mechanical methods (such as pipetting and scraping) resulting in the highest levels of cell death, and enzymatic collection methods, including trypsin, resulting in the highest cell viability (Figure S1). However, as expected, the use of 0.25% trypsin/EDTA removed most of the CXCR4 surface expression by murine BM-MSCs (CXCR4^{low} cells), whereas non-enzymatic cell dissociation solution preserved most of the surface expressed CXCR4 (CXCR4^{high} cells), as shown in Figure 2. Transplantation assays were therefore performed with CXCR4^{low} and CXCR4^{high} cell fractions after correction for viability.

In order to assess the kinetics of re-expression of CXCR4 on the surface of BM-MSCs, cells were trypsinized and CXCR4 expression was followed in time (Figure S2). We found that when incubated in presence of CM, CXCR4 expression rapidly increased, but even after 480 min, levels were still lower (<55%) than before trypsinization or after digestion with non-enzymatic solutions (>95%).

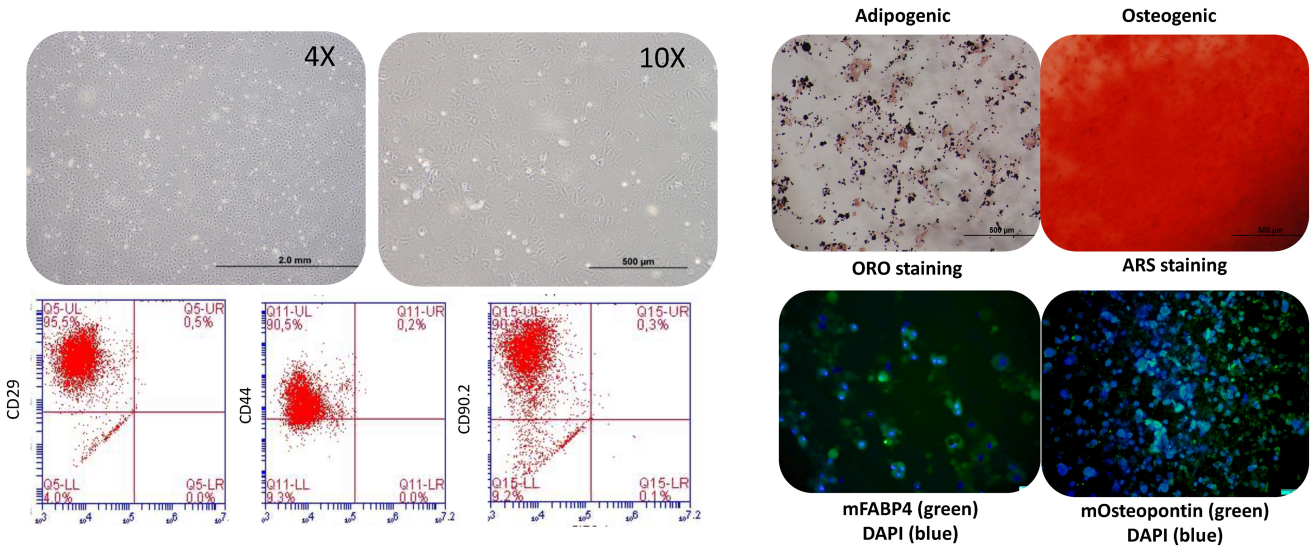

FIGURE 1 Characterization of murine BM-MSCs. Balb/c BM-MSCs showed spindle-shaped morphology (upper left), expression of CD29, CD44, and CD90.2 (lower left) and differentiation toward adipogenic and osteogenic lineage, as apparent by positive staining for ORO and ARS (upper right) and widespread presence of mFABP4 and mOsteopontin expressing cells (lower right) for adipogenic and osteogenic differentiation, respectively.

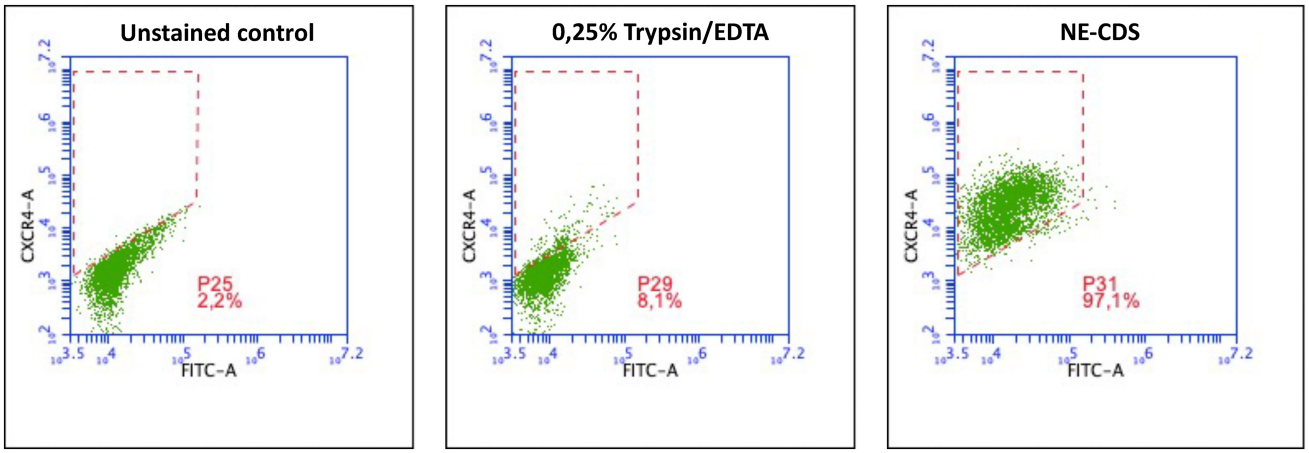

FIGURE 2 Non-enzymatic cell dissociation solution preserves CXCR4 expression by BM-MSCs. Healthy donor Balb/c BM-MSCs were collected with 0.25% trypsin/EDTA or non-enzymatic cell dissociation solution (NE-CDS). Y-axis: CXCR4. Representative dot plots.

Trypsin cleaves CXCL12 and anti-CD184 binding sites

When the known amino acid sequences of human CXCR4 (uniprot P61073) and murine CXCR4 (uniprot P70658) were blasted for similarity (blast.ncbi.nlm.nih.gov), we found a protein similarity of 89.64%. Based on the working mechanism of trypsin,[24,25] we predicted the most important effects on function of CXCR4 by trypsin is by cleavage of Lys25 and Lys38 in humans and Lys 27 and Lys40 in mice, thus preventing binding to both the anti-CD184 antibody and interaction with CXCL12 (Figure S3). Using the Expasy – Peptide cutter software we found that trypsin is predicted to cleave the CXCR4 protein at 30 different positions in human CXCR4 and 31 distinct positions in mouse CXCR4 (Figure S4). However, since both activation of signaling by CXCL12 and binding of the anti-CD184 antibody takes place at the N-terminal extracellular part of CXCR4, cleavage at these sites is predicted to affect the function of the chemokine receptor most.

BM-MSC transplantation alleviates clinical severity of DSS-induced colitis and supports intestinal regeneration

After 5 days of DSS administration, all mice displayed colitis-like symptoms and colitis severity was determined to be moderate (DAI score 7.0 ± 1.7) before transplantation. Mice were transplanted with 1.5×10^6

CXCR4^{low} or CXCR4^{high} BM-MSCs and monitored daily. Transplantation of BM-MSCs of both treatment groups resulted in relief of clinical signs of colitis, including a decrease in body weight loss, rectal bleeding, and diarrhea. However, the CXCR4^{high} group appeared to be more effective in lowering the clinical DAI score in comparison to the CXCR4^{low} group, with a DAI of 0.3 ± 1.5 ($p < 0.05$) and 2.3 ± 0.6 ($p < 0.005$), respectively at day 7 after transplantation.

Upon sacrifice, the effects of BM-MSC transplantation were assessed macroscopically by determining differences in total colon length. DSS treatment resulted in significant shortening of the colon, measured from cecum to anus in comparison to colons of untreated control mice ($p < 0.05$). Transplantation of both CXCR4^{low} and CXCR4^{high} BM-MSCs resulted in the normalization of colon length to pretreatment levels (Figure 3a,b). DSS treatment in mice induced increased colon permeability in comparison to

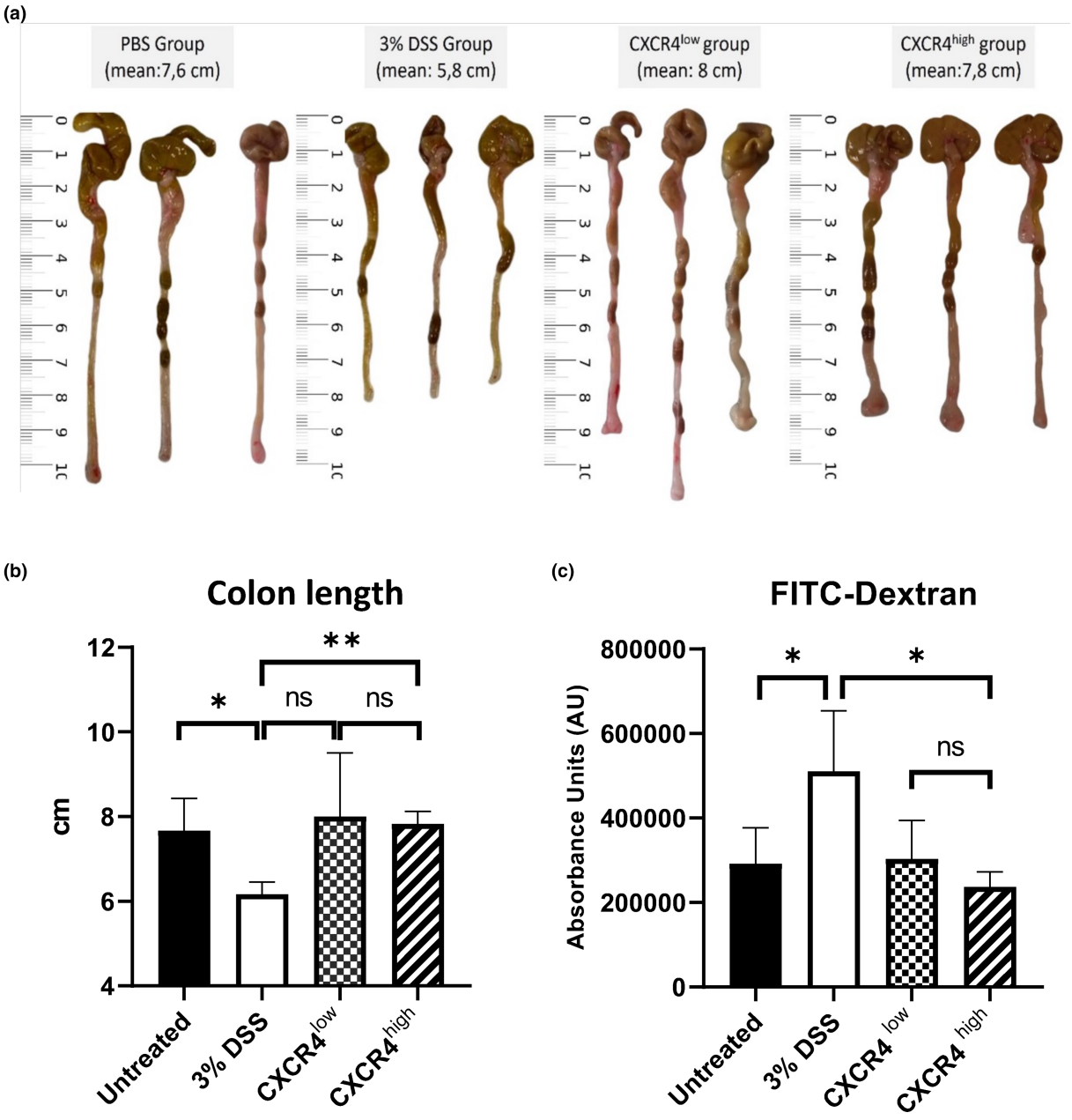

FIGURE 3 BM-MSC transplantation after DSS-induced intestinal damage improves colon length and permeability. Mice were treated with 3% DSS to induce intestinal damage and transplanted with CXCR4^{low} or CXCR4^{high} BM-MSCs. Control mice received normal drinking water (untreated). On day 7, mice were first given FITC-Dextran p.o. and 4 h later sacrificed and colons removed. (a) Colon lengths were measured in all mice from cecum to anus ($n = 3$ per group). (b) Average colon length in untreated, DSS-treated mice, and after transplantation with CXCR4^{low} and CXCR4^{high} BM-MSCs. (c) FITC-Dextran levels in untreated mice, after DSS, and after treatment with CXCR4^{low} and CXCR4^{high} BM-MSCs. Data are shown as average + standard deviation. *$p < 0.05$; **$p < 0.005$.

untreated mice ($p < 0.05$). To evaluate the protective effects of BM-MSC transplantation on colon permeability, mice were treated with oral FITC-Dextran and levels were measured in serum 4 h later. Transplantation of DSS-treated mice with either CXCR4^{high} and CXCR4^{low} BM-MSCs resulted in normalization of FITC-Dextran levels (Figure 3c). However, these data were only found to be significant for the group treated with CXCR4^{high} BM-MSCs ($p < 0.05$).

Thus, the effects of BM-MSC transplantation on intestinal permeability, combined with the morphological appearance of the colons indicate a strong regenerative or protective effect on the colon, even though no significant differences were found between the effects of the CXCR4^{high} and CXCR4^{low} groups ($p = 0.30$).

Levels of CXCR4 expression do not affect histological regeneration of intestinal epithelium

To assess the effects of DSS treatment and BM-MSC transplantation on microscopical changes in the intestinal architecture, H/E-stained sections from the ascending, transverse, and descending colons from treated, untreated, and transplanted mice were scored, according to Table S1. Transplantation of both CXCR4^{low} BM-MSCs and CXCR4^{high} BM-MSCs significantly reduced signs of inflammatory cell infiltration ($p < 0.05$), epithelial damage ($p < 0.0001$) and mucosal architectural disruption ($p < 0.01$) of colon sections in mice with DSS-induced colitis, when compared with the non-transplanted DSS positive controls (Figure 4). In general, histological recovery parameters were similar throughout the independently assessed anatomical areas of the ascending, transverse, and descending colon. Although mice treated with CXCR4^{high} BM-MSCs showed *grosso modo* the best recovery, quantifiable differences between histological regeneration of the full-length colons of CXCR4^{high} and CXCR4^{low} BM-MSC transplantation groups were negligible. These data indicate that although CXCR4 expression may render certain advantages in terms of homing, the overall regenerative and protective effects of BM-MSC transplantation on DSS-induced colitis in mice were largely independent of the presence or absence of CXCR4.

DSS treatment resulted in multifocal mucosal infiltration and patchy surface epithelial erosion with Goblet cell loss in all groups, and could be observed both before and after transplantation with either CXCR4^{high} and CXCR4^{low} BM-MSCs (Figure 5). Mucosal (involvement of

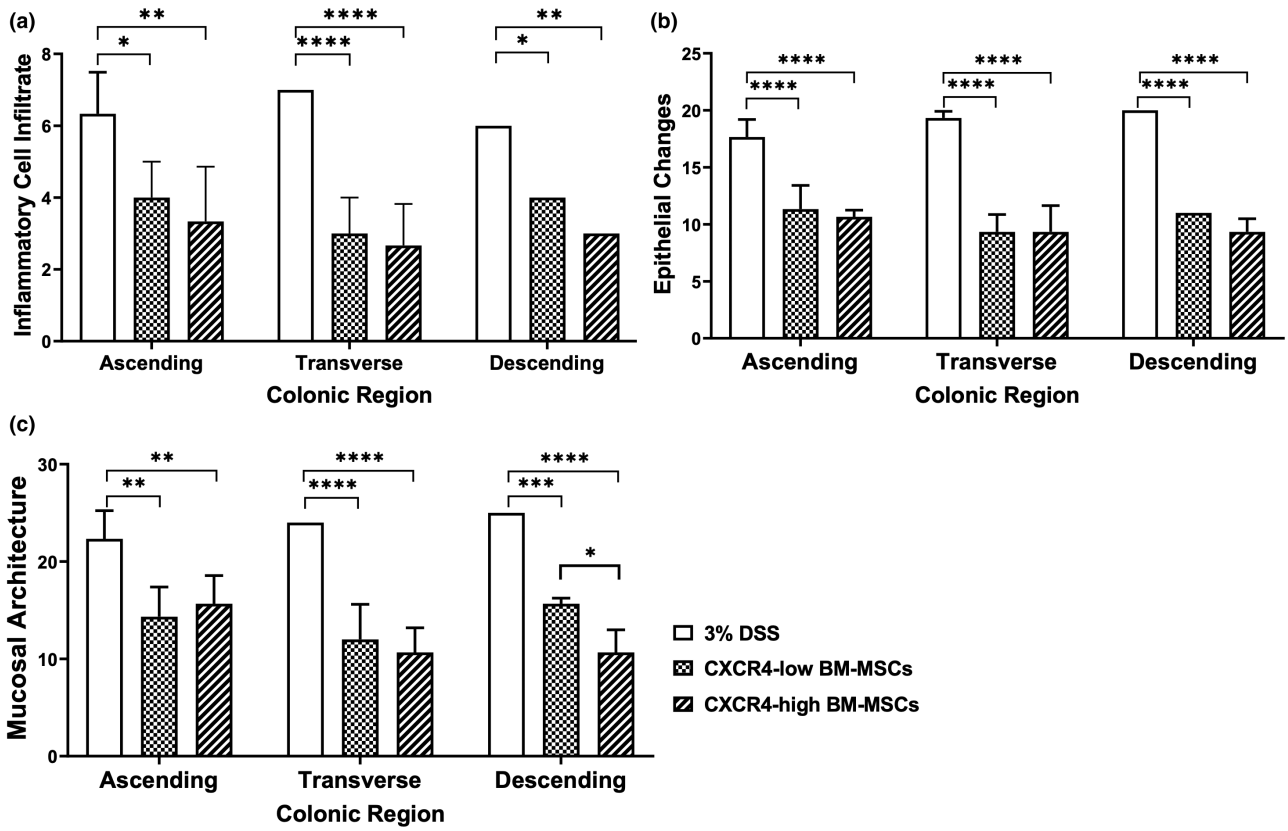

FIGURE 4 BM-MSC transplantation after DSS exposure improves colonic damage. DSS-treated mice were transplanted with CXCR4^{low} and CXCR4^{high} BM-MSCs and histological changes were scored and compared with the DSS-treated, non-transplanted positive controls. (a) inflammatory cell infiltrate; (b) epithelial changes; and (c) mucosal architecture scores. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

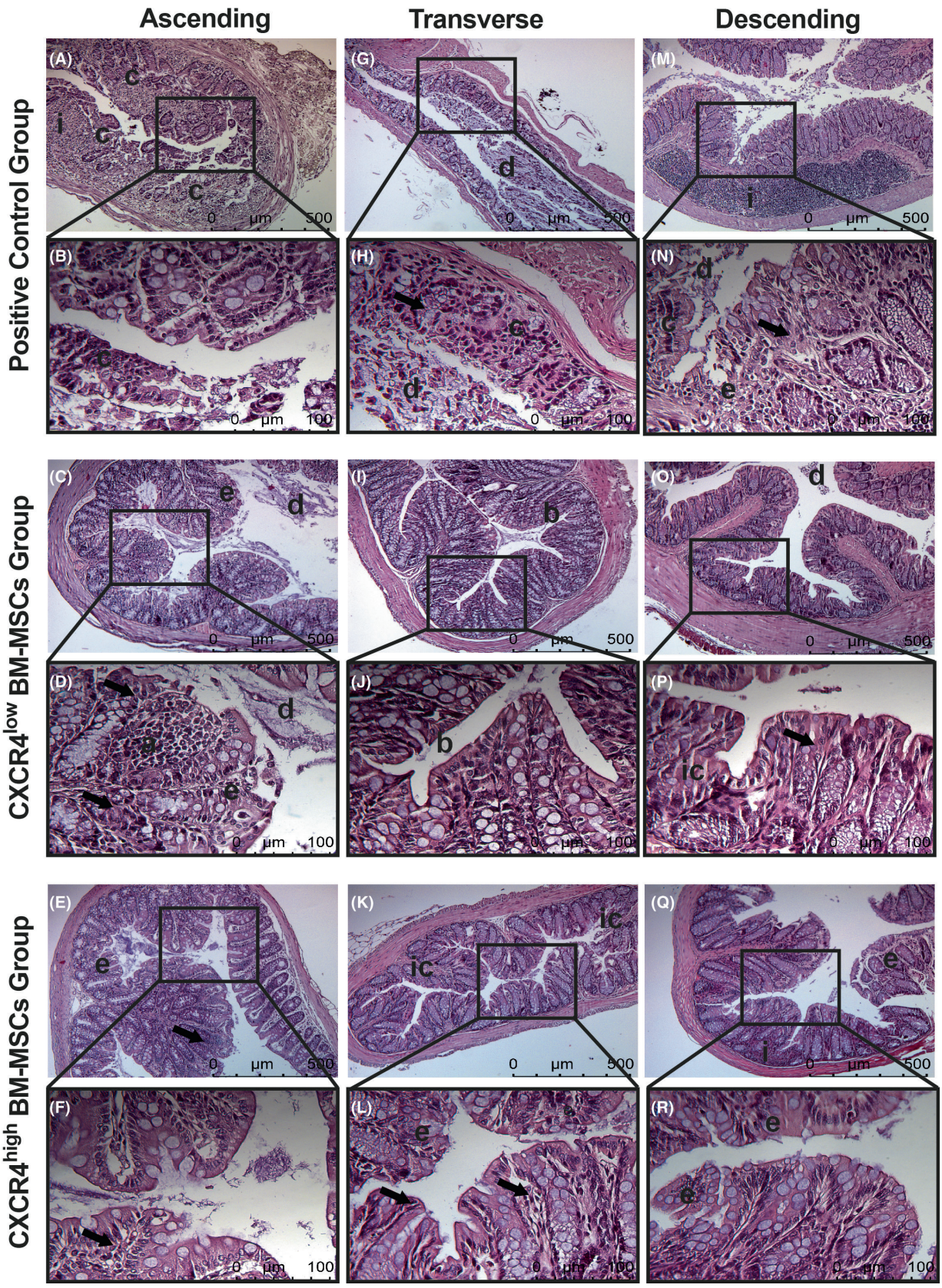

FIGURE 5 BM-MSC transplantation after DSS exposure improves colonic damage. The upper row of each panel represents low magnification, the lower rows high magnification micrographs. H/E staining reveals variable levels of Goblet cell loss (*black arrows*), crypt abscesses (*a*), bifurcations (*b*), crypt loss (*c*), debris (*d*), epithelial erosion (*e*), cellular infiltration (*i*), irregular crypts (*ic*) in mucosa and/or submucosa. Note mucosal epithelial recovery in both CXCR4^{low} BM-MSC and CXCR4^{high} BM-MSC- treated groups.

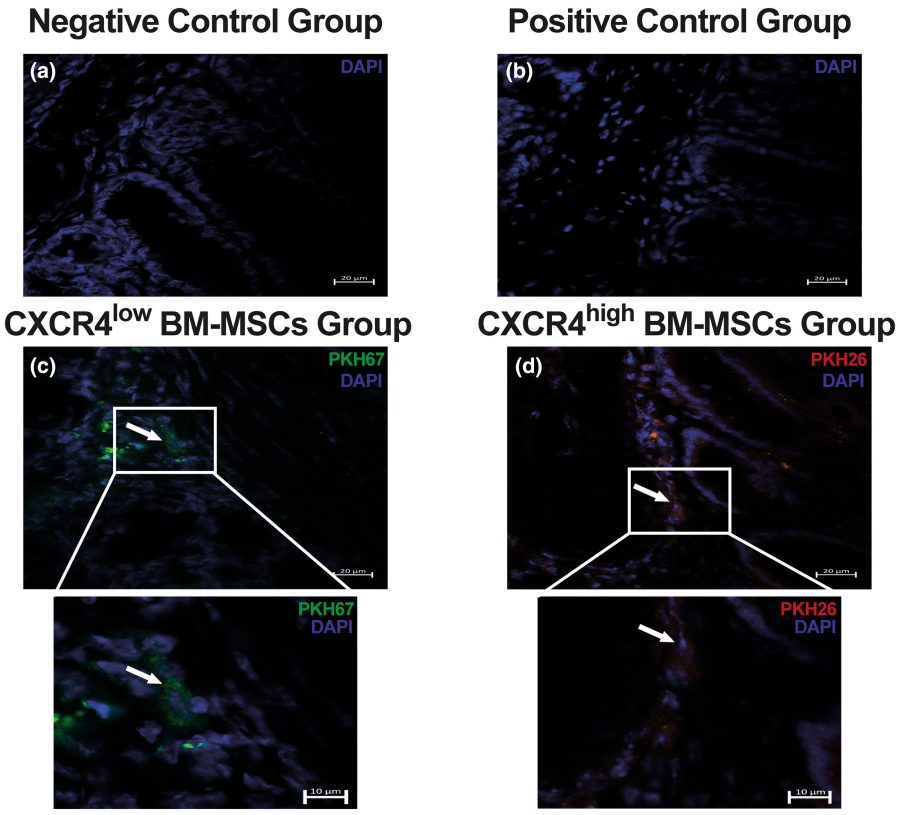

FIGURE 6 Tracking of fluorescently labeled CXCR4high and CXCR4low BM-MSCs after transplantation. Transplanted (d) CXCR4^{high} (red) and (c) CXCR4^{low} (green) BM-MSCs are found in small numbers in intestinal mucosal and submucosal layers. No labeling was detected in (a, b) control groups. Scale bar indicates 20 and 10 μm (inset). Nuclei were counterstained with DAPI (blue). The white arrows indicate the presence of PKH67 (green) CXCR4^{low} and PKH26 (red) CXCR4^{high} BM-MSCs.

lamina propria) and submucosal infiltration, was noted in the DSS-treated positive control samples (Figure 5M). Cryptic loss, abscess formation and focal ulceration induced by DSS resolved after BM-MSC transplantation, and were replaced by epithelized bifurcated and irregular crypts, which indicate epithelial recovery and mucosal regeneration.

In order to assess the homing capacities of the CXCR4^{high} and CXCR4^{low} BM-MSCs, we stained these cells with a red and green fluorescent dye, respectively, and assessed the presence of red and green stained BM-MSCs in frozen sections using confocal microscopy. Although we were only able to trace few fluorescent cells in either group, numbers of CXCR4^{high} BM-MSCs in the mucosal and submucosal layers of the inflamed colons were considerably higher than that of the CXCR4^{low} BM-MSC group (Figure 6).

DISCUSSION

The chemokine receptor CXCR4, is composed of 352 amino acids and belongs to the G-protein-coupled receptor (GPCR) family. It consists of an extracellular N-terminal (NT) end, three extracellular loops (ECLs), seven transmembrane (TM) helices and an intracellular C-terminus that is responsible for the activation of downstream signaling.[26] Studies using mutational, structural (NMR), functional, and phylogenetic analyses have shown that the amino acid residues 2–9, Glu14 and/or Glu15, and Tyr 21 at the NT end of the CXCR4 molecule, as well as the first and second ECL are the primary domains responsible for binding to CXCL12.[26–30] In a previous study using human bone marrow derived MSCs, we found that expression of the chemokine receptor CXCR4, which is the main regulator of directed stem cell migration, was largely lost after incubation with trypsin and other similar enzymatic solutions used for the dissociation of MSCs. Although we showed that the use of non-enzymatic solutions or physical collection, that is, rigorous pipetting or scraping, prevented loss of CXCR4 expression, the latter methods were associated with increased cell death.[11] Therefore, trypsinization remains the most commonly used method to collect adherent cells, both in basic research and for clinical use.

Trypsin itself has been shown to cleave the peptide bonds between arginine (R) or lysine (K) and the adjacent amino acid. However, also in the presence of a

proline (P) residue on the carboxyl side of the cleavage site, cleavage will not occur.[24] Furthermore, the rate of cleavage has been shown to decelerate when the lysine and arginine residues are located in the vicinity of acidic amino acids, such as aspartate (D) or glutamate (E), or cystine (C) residues.[25] Based on these data, and the high level of homology (91%–93%) between human and murine CXCR4,[28,31] we believe that trypsin most likely cleaves both human and murine CXCR4 molecule at the Lys38 residue, at the end of the NT domain of CXCR4 (Figure S2). Cleavage at this site would prevent interaction with/binding to CXCL12 and could therefore severely affect homing and cellular migration to injured tissues in response to a CXCL12 gradient.

Here, we first assessed the effects of trypsin (and other enzymatic and non-enzymatic dissociation methods) on expression of CXCR4 by murine BM-MSCs. We measured CXCR4 expression on murine BM-MSCs using the anti-mouse CD184 antibody (clone L276F12). This particular anti-CD184 antibody clone has been previously shown to block murine CXCR4 signaling in vivo.[31] Studies assessing binding of antibodies to the extracellular domains of CXCR4 using mutagenesis have shown that different antibodies may recognize different areas, including the NT end, ECL1, 2 or 3.[32] However, our data suggest that L276F12 most likely[31] competes with CXCL12 for its binding site on the NT end of CXCR4, which would also explain why use of trypsin abolishes binding of this antibody to CXCR4.

We then proceeded to investigate whether and how CXCR4 expression by BM-MSCs affects intestinal regeneration in a mouse model with DSS-induced colitis by transplantation of $CXCR4^{high}$ and $CXCR4^{low}$ BM-MSCs and we assessed the regenerative/supportive/protective effects of these cells on large intestine function, morphology, and histology. Dextran sulfate sodium has been used by many groups to induce colitis, but the effects may vary widely depending on the dose (1%–5%), the duration of exposure (3–7 days) and the molecular weight of the DSS used (30–40 kDa), as well as on mouse strain (e.g. Balb/c vs. C57Bl/6), age (newborn-adult) and weight.[33,34] The DSS murine colitis model is often preferred because of its easy use, reproducibility, and reliability of inducible colitis and it can be used for modeling of acute, chronic, and relapsing models of intestinal inflammation by adapting dosing schedules and retracting/reintroducing DSS treatment. In addition, since in contrast to humans, mice T and B cells are not required for development of colitis, immune-deficient mouse models, such as $RAG2^{-/-}$ mice can be used to specifically study the effects of the innate immune system in the development of intestinal inflammation.[34] In our optimized $RAG2^{-/-}$ model, we used 3% 40 kDa DSS for 5 days, after which we used the DAI to score the induced colitis. This treatment protocol typically results in induction of moderate colitis, which was confirmed clinically (DAI > 6), as apparent by weight loss and changes in stool consistency, and also by the presence of increased intestinal permeability.

We hypothesized that lack or low expression of CXCR4 by BM-MSCs would result in decreased migration to the inflamed intestine and result in measurable decreased regenerative effects. BM-MSCs have been used previously in a variety of animal colitis models for their immunomodulatory and anti-inflammatory properties.[35,36] In these models, in which colitis was induced by TNBS or DSS, regression in the course of the disease was observed after BM-MSC infusion and transplanted Balb/c mice displayed a lower DAI in terms of loose stool, visible fecal blood, and macroscopic inflammation than control mice. Additionally, body weight and colon length increased in these models after transplantation with BM-MSCs.[35,36] In our study, we found that although mice transplanted with $CXCR4^{high}$ BM-MSCs performed clinically better, transplantation of both $CXCR4^{high}$ and $CXCR4^{low}$ BM-MSCs resulted in an overall similar decrease in intestinal permeability, normalization of colon length and an improvement in colon epithelial recovery. These data indicate that in $RAG2^{-/-}$ mice exposure to 3% DSS, followed by infusion of 1.5×10^6 BM-MSCs i.p., transplantation of BM-MSCs results in significant improvement of intestinal damage, independent from the level of CXCR4 cell surface expression.

Numerous studies have shown that chemokines are critical for cell migration during systemic inflammation, but also for homeostasis and immune regulation.[37,38] A variety of chemokines is released by immune cells infiltrating the IBD lesions, including macrophages and neutrophils, which serve key roles in further development and progression of IBD.[39] Upregulated levels of chemokine ligands CCL2, CCL4, CCL7, and CXCL10 have been demonstrated in IBD tissues and have been correlated with immune infiltration and disease severity.[15,40] Most importantly, CXCL12 expression by IECs has been shown to be significantly increased in IBD and through continued chemotaxis of lymphocytes to the intestine and infiltration of the lamina propria (LP) has been shown to add to the pathophysiology of IBD by causing constitutive inflammation of the intestinal mucosa.[14] CXCL12 binds to two different chemokine receptors, that is, CXCR4 and CXCR7.[41] Many studies have shown that the CXCR4 chemokine receptor plays an important role in the migration of MSCs to damaged tissues, including the intestinal mucosa of IBD.[10,12,13,15] In contrast, CXCR7 expression by MSCs has been found to be responsible for adhesion and survival of MSCs,[42] as well as the regulation of

differentiation and modulation of immune modulatory capacities of MSCs.[43] To enhance the homing and therapeutic efficacy of BM-MSCs to inflamed tissue, cells have been genetically modified to overexpress CXCR4 using lentiviral vectors.[44–47] Furthermore, induction of CXCR4 expression with IL-1β has been shown to promote regeneration after DSS-induced colitis by enhancing homing of BM-MSCs to damaged intestinal mucosa, in comparison to unstimulated BM-MSCs.[48]

We have now shown that the use of most enzymatic dissociation methods, including trypsin, may affect CXCR4 expression by both human[11] and murine BM-MSCs. However, the absence of CXCR4 expression on murine BM-MSCs did not affect the regenerative potential of the cells, even though homing may have been decreased. We therefore believe that in this model, it is highly likely that since cells were injected i.p., direct paracrine effects of the cells may have been sufficient to support intestinal regeneration in a model of moderate colitis, as has been shown before.[49] However, paracrine effects are unlikely to be the case when cells are infused systemically, when cells need to home to other tissues further away from the injection site or when the disease is more severe. Therefore, other methods to promote homing and improve suboptimal performance of MSCs after transplantation in vivo need to be developed.[18]

In addition, is has been previously shown that i.p. delivered MSCs can alleviate the symptoms of experimental colitis in mice through induction of regulatory B Cells.[50] Although this mechanism is unlikely to be involved in our immune-deficient mouse model, it underlines the fact that multiple mechanisms may be involved in the immune modulating and regenerative effects of MSCs. In fact, is has been shown that MSCs injected into the peritoneal space spread mostly to abdominal organs, including liver, spleen, and intestine, in contrast to intravenously injected cells, which have a preference for liver and lung tissue within the first 24 h after injection.[51] In addition, intraperitoneally infused MSCs have been shown to home to the inflamed colon and ameliorate experimental colitis and have been observed throughout the colon wall 72 h after i.p. inoculation, predominantly in the submucosa and muscular layer of inflamed areas.[52]

Here, we aimed to show that migration of BM-MSCs in the absence of CXCR4 expression to the intestine would be decreased, resulting in decreased therapeutic efficacy. However, we showed that despite low CXCR4 expression, BM-MSCs still showed regenerative effects on intestinal epithelium that resembles the therapeutic effects of CXCR4 expressing BM-MSCs. Since our methods for detecting homing of cells were not sufficiently sensitive to find back significant amounts of transplanted cells in the whole colon, we cannot be sure whether the transplanted cells in fact homed to the intestine and how homing (if at all) was affected by the absence of the CXCR4 receptor. However, our confocal imaging data suggest that at least a fraction of the i.p. infused $CXCR4^{high}$ BM-MSCs have homed to the inflamed intestine, where they were observed in the submucosal and mucosal areas of the inflamed colon.

In addition, it is possible that CXCR4 was upregulated again after infusion of the cells since the protein is known to be continuously produced. In fact, we have now shown that CXCR4 expression is re-acquired by the BM-MSCs after trypsinization. However, upregulation of CXCR4 takes considerable time and levels remain below baseline levels even after 480 min. In addition, in vivo these kinetics may not be exactly the same as in vitro. Infusion of BM-MSCs in the peritoneum results in spread toward different organs, including the intestine. Furthermore, the number of cells is limited and dilution throughout the intestine makes it difficult to trace these cells. Although we found traces of both $CXCR4^{high}$ and $CXCR4^{low}$ BM-MSCs in the inflamed colon, numbers of $CXCR4^{high}$ BM-MSCs appear to be higher (as was expected) although their regenerative effects were highly similar. Therefore, these data support the idea that the mechanism through which BM-MSCs exert their regenerative potential is not alone by direct homing to the colon, but also through paracrine mechanisms.[53]

Therefore, we conclude here that intraperitoneal infusion of $CXCR4^{high}$ and $CXCR4^{low}$ BM-MSCs in an immune-deficient mouse model of colitis results in *grosso modo* similar intestinal regeneration and that CXCR4 expression by BM-MSCs, although important for homing of cells to damaged or inflamed tissue, is not the only critical factor that determines therapeutic efficacy of transplanted BM-MSCs. In our model, i.p. administration may have had a relative advantage, since MSCs transplanted i.p. have been shown to preferentially remain local, with migration to nearby organs, such as the liver, spleen, and intestine. Therefore, the role of CXCR4 on migration may have been relatively less important in this setting than if the cells would have been infused intravenously. Nevertheless, considering all the data we believe that it is important for clinical use to sustain the highest possible CXCR4 expression in transplanted BM-MSCs, either through use of non-enzymatic agents or through preincubation of the cells for at least 30 min in serum-containing medium to allow re-acquisition of surface expression of CXCR4, to ensure maximal homing capacity in addition to their paracrine effects.

AUTHOR CONTRIBUTIONS

B.P., M.G., P.K., and F.A.K. wrote the manuscript; B.P., B.D.U., and F.A.K. designed the research; B.P., M.G.,

M.E.Ş., Ö.D.E., S.N.G., and E.G.P performed the research; B.P., M.G., B.H.U., P.K., and F.A.K. analyzed the data; B.P., M.E.Ş contributed analytical tools.

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ORCID

Burcu Pervin https://orcid.org/0000-0002-5866-0955
Merve Gizer https://orcid.org/0000-0003-1911-2363
Mehmet Emin Şeker https://orcid.org/0000-0002-8240-5938
Özgür Doğuş Erol https://orcid.org/0000-0001-9301-5401
Sema Nur Gür https://orcid.org/0009-0006-4268-9979
Ece Gizem Polat https://orcid.org/0000-0003-3106-4384
Bahar Değirmenci https://orcid.org/0000-0001-8312-1411
Petek Korkusuz https://orcid.org/0000-0002-7553-3915
Fatima Aerts-Kaya https://orcid.org/0000-0002-9583-8572

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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