



Monocytes Stimulated with Conditioned Medium from Calcitriol-Treated Mesenchymal Stem Cells Exhibit Inhibition of Inflammatory Responses in Experimental Ulcerative Colitis

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Received February 13, 2025; Accepted April 27, 2025; Online Published September 30, 2026

Abstract

Introduction: Mesenchymal stem cells (MSCs) are widely used to treat autoimmune and inflammatory diseases by modulating immune system activity. Several studies have shown that the administration of micronutrients such as calcitriol, which has active receptors on MSCs, can enhance the production of immunomodulatory substances. Conditioned medium (CM) derived from MSCs can induce monocytes and macrophages to initiate various anti-inflammatory pathways. This experimental study aimed to evaluate the effects of monocytes stimulated with CM from calcitriol-treated MSCs in a mouse model of ulcerative colitis (UC).

Materials and Methods: Male BALB/c mice were divided into the following groups: healthy controls (negative control), untreated UC (positive control), UC treated with untreated monocytes, UC treated with calcitriol-CM-stimulated monocytes, and UC treated with mesalazine. Following UC induction and the respective therapies, the animals were euthanized for tissue sampling. Levels of nitric oxide (NO), myeloperoxidase (MPO), and the pro-inflammatory cytokines interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF- α), as well as intestinal tissue lesions, were assessed using biochemical and histological analyses.

Results: Our results revealed that both mesalazine and treated monocytes mitigated intestinal tissue lesions by significantly reducing MPO and NO levels and suppressing the production of the pro-inflammatory cytokines IL-1, IL-6, and TNF- α ($p < 0.05$).

Conclusions: Monocytes stimulated with CM from calcitriol-treated MSCs exhibit anti-inflammatory properties and promote beneficial tissue regeneration in UC. This approach may be used in conjunction with standard pharmacological treatments for UC management.

Keywords: Ulcerative Colitis, MSCs-derived Conditioned Medium, Calcitriol, Monocyte, Mesalazine

Citation: Ghorashi M, Shayegh J, Esmaeili Gouvarchinghaleh H. Monocytes Stimulated with Conditioned Medium from Calcitriol-Treated Mesenchymal Stem Cells Exhibit Inhibition of Inflammatory Responses in Experimental Ulcerative Colitis. J Appl Biotechnol Rep. 2026;13(3): 2159-2167. doi:10.30491/jabr.2025.506447.1845

Introduction

Ulcerative colitis (UC) has a higher incidence rate compared to other digestive disorders. It is categorized as an autoimmune disease with an inflammatory basis. In affected individuals, the mucosal layer, and occasionally the submucosa, is subject to inflammation.¹ Various classifications have been established based on the extent of the disease, including ulcerative proctitis (limited to the rectum), distal colitis or proctosigmoiditis (extending to the sigmoid region), left-sided colitis (extending to the splenic flexure), and pancolitis (involving the cecum).² The initial treatment plan is typically determined by the severity and clinical indications of the disease, with the approach largely influenced by the early assessment of inflammatory extent.³ Colonoscopy remains a reliable and frequently employed method for evaluating UC severity.

Patients with mild to moderate symptoms are often prescribed aminosalicylates, which are associated with both dose-dependent and dose-independent side effects. Dose-dependent adverse effects include nausea, vomiting, anorexia, headache, alopecia, back pain, and impaired folate absorption, whereas dose-independent effects may include skin rashes, fever, hemolytic anemia, agranulocytosis, pancreatitis, hepatitis, reversible male infertility, colitis, fibrosing alveolitis, pericarditis, and myocarditis.⁴ Consequently, researchers are continuously seeking novel therapeutic strategies and potent agents with minimal adverse effects.

Mesenchymal stem cells (MSCs) have been investigated for their immunomodulatory potential in treating inflammatory conditions associated with dysregulated immune responses, such as type 1 diabetes, rheumatoid arthritis, and multiple

sclerosis.⁵ These cells exert immunosuppressive effects through the expression of various inhibitory molecules and anti-inflammatory cytokines, including PDL1, TGF- β , IL-10, and galectins. They also produce metabolites such as nitric oxide and IDO, and contain matrix metalloproteinases. MSCs are employed in the treatment of numerous conditions, including graft-versus-host disease, tissue repair, lung injuries, skin healing, myocardial infarction, kidney disorders, diabetes, and certain neurological conditions.^{6,7} MSCs can suppress immune responses both *in vivo* and *in vitro*, partly through the activation of Treg cells, which indirectly inhibit immune reactions. Additionally, they can modulate various immune cell types, including T cells, B cells, and natural killer cells.⁸ Following appropriate stimulation, MSCs inhibit T lymphocyte proliferation. *In vitro*, these cells impede the differentiation of monocytes into dendritic cells (DCs).⁹ The suppression of co-stimulatory molecules by MSCs hinders the differentiation, antigen presentation, and maturation of dendritic cells.¹⁰ Overall, the unique properties of MSCs render them promising candidates for cell therapy in autoimmune diseases, inflammatory disorders, and tissue injuries. By activating macrophages via an alternative pathway, reducing inflammation, and modulating immune responses, MSCs may facilitate tissue repair and enhance disease resistance.¹¹

Co-culturing monocytes with MSC supernatant can prevent their differentiation into antigen-presenting cells and promote the production of the anti-inflammatory cytokine IL-10, owing to the presence of IL-10 and IL-6 in the supernatant.^{12,13} The management of autoimmune and inflammatory diseases may also be supported by certain vitamins. Calcitriol is particularly significant in this context; it is synthesized in the skin upon sunlight exposure and can also be obtained through diet. It plays a crucial role in bone metabolism, regulates cell division and proliferation, and modulates immune responses.¹⁴ Numerous studies suggest that calcitriol can alleviate symptoms of autoimmune diseases such as multiple sclerosis by regulating immune responses, an effect mediated by the vitamin D receptor expressed on various human tissues and cells, including those of the immune system.¹⁵ Moreover, multiple studies have demonstrated that calcitriol can augment the secretion of immunomodulatory factors by MSCs, as these cells express the calcitriol receptor on their surface.¹⁶

In light of the aforementioned evidence, the use of MSC-conditioned medium to shift monocytes toward an anti-inflammatory phenotype, as well as the treatment of MSCs with calcitriol to enhance their immunomodulatory capacity, has been previously explored. In this hybrid therapy study, we utilized calcitriol as a stimulatory agent to enhance the ability of MSCs to induce an anti-inflammatory monocyte phenotype and investigated its therapeutic anti-inflammatory effects in UC. Accordingly, following UC induction and the

respective therapies, the animals were euthanized for tissue sampling. Levels of nitric oxide (NO), myeloperoxidase (MPO), and the pro-inflammatory cytokines interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF- α), as well as intestinal tissue lesions, were assessed using biochemical and histological analyses.

Materials and Methods

Animals

A total of sixty male BALB/c mice (6–8 weeks old, weighing 20–30 g) were obtained from the Pasteur Institute of Iran, Tehran. The animals were housed under controlled conditions at 25 °C with a 12-hour light/dark cycle and had free access to food and water. All procedures were performed in accordance with ethical guidelines (ethical code: IR.IAU.TABRIZ.REC.1401.329). Of these, 40 mice were used to establish the UC model, 10 served as the negative control group for *in vivo* experiments, and 10 were euthanized with ketamine/xylazine for the collection of bone marrow-derived MSCs.

Colitis Induction and Experimental Groups

An acute colitis model was induced in 40 male BALB/c mice following a 36-hour fast, as described previously.¹⁷ Under light sedation, a catheter was inserted into the colon, and 100 μ l of a 4% acetic acid solution was administered via enema. The mice were held in a vertical position for one minute to prevent leakage. They were then divided into five groups of ten mice each: a healthy group (negative control), a colitis group without treatment (positive control), and three treatment groups (monocyte, treated (T)-monocyte, and mesalazine). The treatment groups received intravenous cell therapy with 1×10^5 cells twice, at one-week intervals. One week after the final transplantation, the mice were euthanized, and colon tissues were harvested for analysis.

Isolation and Proliferation of MSCs

MSCs were isolated from the bone marrow of the mice's tibias and femurs using a previously established protocol.¹⁸ Under deep anesthesia, the bone marrow was extracted, washed in phosphate-buffered saline (PBS), and cultured in DMEM medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) at 37 °C in a humidified incubator with 5% CO₂. After four days, the culture media were collected, and the adherent cells were trypsinized, counted, and subcultured at a 1:3 ratio upon reaching 80% confluence. This passaging process was continued for 21 days, with fresh media added every three days. The cell suspension from the third passage was prepared for administration.¹⁸

Treatment of MSCs with Calcitriol

Approximately 5×10^6 MSCs were treated with 400 nM

calcitriol (Sigma, USA) and incubated for 24 hours. Following this, the medium was aspirated, the cells were washed with PBS, and the MSCs were incubated in serum-free DMEM (without FBS) for an additional 24 hours. The supernatants were collected, centrifuged to remove cellular debris, pooled from individual mice, filtered, and stored at -80°C . Negative control media were also prepared under identical conditions using serum-free DMEM.^{18,19}

Isolation of Monocytes and Treatment with Calcitriol-Treated MSC-Derived Conditioned Medium

Monocytes were isolated from deeply anesthetized mice via cardiac puncture to collect heparinized blood samples, which were then layered on a Ficoll-Hypaque gradient in RPMI-1640 medium (Gibco, USA). After centrifugation at $400\times g$ for 30 minutes, the mononuclear cell layer was collected, suspended in DMEM, and incubated in six-well plates at 37°C for two hours. Adherent monocytes were retained, while non-adherent lymphocytes were washed away with PBS. The adherent cells were then cultured in DMEM supplemented with 10% FBS and 50% conditioned media or negative control media.¹⁸

Macroscopic and Microscopic Disease Activity Index

Daily evaluations were performed to assess stool consistency and the presence of blood. The Disease Activity Index (DAI) was calculated by aggregating the scores for each parameter (Table 1). Colon tissue samples were collected and fixed in 10% formalin. Each sample was subsequently embedded in paraffin blocks, sectioned, and stained with hematoxylin and eosin (H&E) for microscopic examination. Two pathologists, blinded to the study groups, assessed the samples and assigned scores based on the following criteria: a score of 0 for intact epithelium with no leukocyte infiltration or hemorrhage; a score of 2 for focal hemorrhage; a score of 3 for up to 50% epithelial disruption with widespread leukocyte infiltration and hemorrhage; and a score of 4 for more than 50% epithelial disruption with extensive leukocyte infiltration and hemorrhage.²⁰

Table 1. Scoring criteria for the assessment of Disease Activity Index (DAI) based on stool consistency and fecal bleeding in mice

Score	Stool consistency	Blood in stool
0	Normal	Normal (no blood)
1	Soft	Red
2	Very Soft	Dark Red
3	Diarrhea	Black (melena)

Myeloperoxidase Assay

Distal colonic tissues were dissected, washed with PBS, homogenized in ice-cold physiological saline, and centrifuged at $10,000\times g$ for 10 min at 4°C . MPO activity in the colonic homogenate was measured as a marker of neutrophil

infiltration. The homogenate was mixed with a TMB solution and incubated at 37°C for 15 min. The reaction was terminated by adding 2 M H_2SO_4 , and the absorbance was recorded at 450 nm using a microplate reader. The assay employed horseradish peroxidase (HRP) standards at specified concentrations. MPO activity was calculated from the HRP standard curve and expressed in milliunits per milliliter (mU/ml).²⁰

Nitric Oxide Assay

Nitric oxide (NO) levels in colonic tissues were quantified using the Griess reagent, a standard colorimetric method. Aliquots of 50 μl of tissue homogenate were mixed with 50 μl of Griess reagent and incubated at 25°C in the dark for 10 min. Absorbance was then measured at 540 nm using a microplate reader, and nitrite concentrations were determined from a standard curve.²⁰

Cytokine Assay

The levels of TNF- α , IL-1 β , and IL-6 in colon samples were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit, strictly following the manufacturer's instructions (ZellBio GmbH, Germany).¹⁷

Statistical Analysis

Statistical analyses were performed using SPSS software (version 23.0). The Kruskal–Wallis test was used for non-parametric comparisons of disease severity scores, followed by the Mann–Whitney U test with Bonferroni correction. For continuous variables, one-way ANOVA followed by Dunnett's post hoc test was employed after confirming normal distribution using the Kolmogorov–Smirnov test. Results are expressed as mean $\pm$ standard deviation (SD), and statistical significance was set at $p < 0.05$.

Results

Disease Activity Index

Stool consistency and the presence of fecal blood were monitored daily and scored on a scale of 0 to 3 throughout the study period, from day 0 until the end of the experiment (Table 1). Intergroup comparisons revealed that both the T-monocyte and mesalazine treatment groups exhibited significantly lower Disease Activity Index (DAI) scores compared to the untreated positive control group (Figure 1). In contrast, treatment with unstimulated monocytes did not significantly reduce the DAI.

MPO Assay

MPO levels were significantly lower in all treatment groups compared to the positive control group (Figure 2, $p < 0.05$). Specifically, the T-monocyte-treated group exhibited a marked reduction in MPO levels relative to the positive control ($p < 0.0001$), whereas no significant difference was

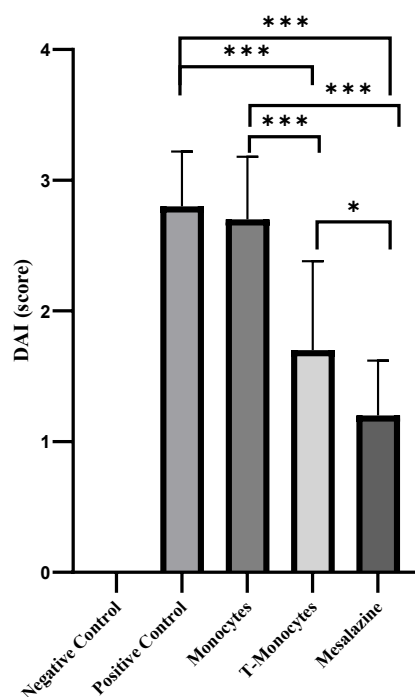


Figure 1. Assessment of Disease Activity Index (DAI) Across the Experimental Groups. The DAI serves as a quantitative measure of colitis severity in the murine model. Treatment with T-monocytes and mesalazine significantly reduced clinical scores compared to the untreated positive control group, indicating comparable therapeutic efficacy (* $p < 0.05$; *** $p < 0.001$).

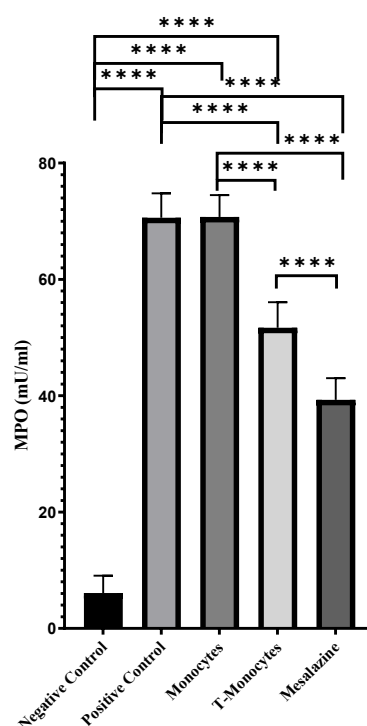


Figure 2. Myeloperoxidase (MPO) Activity Across the Experimental Groups. Both T-monocyte and mesalazine treatments significantly reduced MPO activity compared to the positive control (untreated colitis) group (**** $p < 0.0001$).

observed between the monocyte-treated group and the positive control ($p > 0.05$). Moreover, as expected, MPO levels in the mesalazine group were significantly lower than those in all other experimental groups ($p < 0.0001$).

NO Assay

NO levels were significantly lower in all treated groups compared to the positive control group (Figure 3, $p < 0.05$). Among the treatment groups, the T-monocyte-treated group

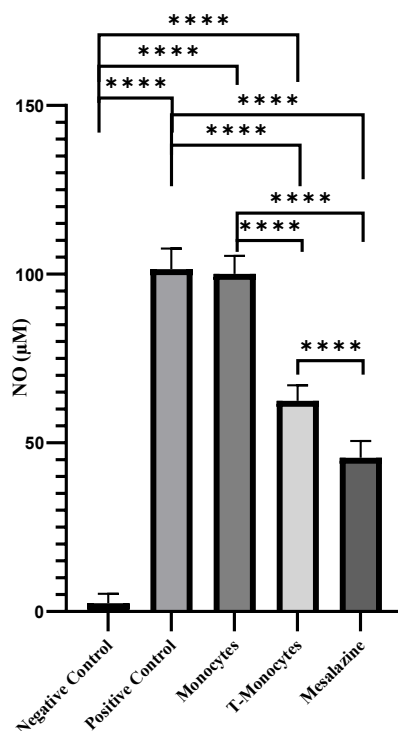
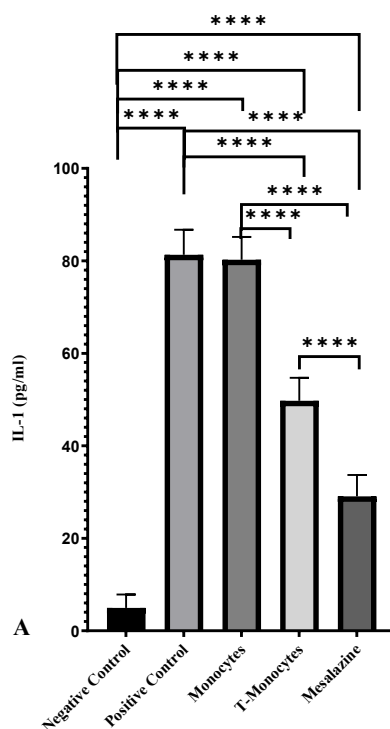


Figure 3. Nitric oxide (NO) Levels Across the Experimental Groups. Both T-monocyte and mesalazine treatments significantly reduced NO levels compared to the positive control (untreated colitis) group (**** $p < 0.0001$).

showed a marked reduction in NO levels relative to the positive control ($p < 0.0001$), whereas no significant difference was observed between the monocyte-treated group and the positive control ($p > 0.05$). As expected, the mesalazine group exhibited significantly lower NO levels than all other experimental groups ($p < 0.0001$).

IL-1, IL-6, and TNF- α Assay

Cytokine analysis revealed that disease induction significantly elevated the levels of inflammatory cytokines in the positive control group ($p < 0.05$). In contrast, treatment with either mesalazine or T-monocytes significantly reduced these cytokines levels compared to the positive control group ($p < 0.0001$), indicating effective suppression of the inflammatory response.



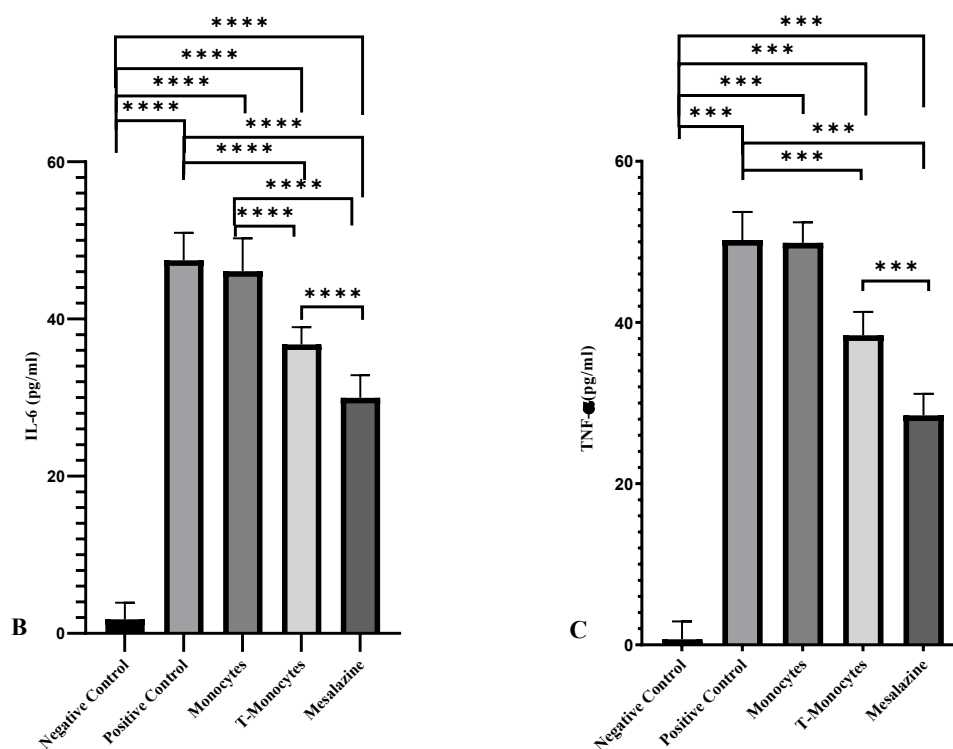


Figure 4. Levels of IL-1 (A), IL-6 (B), and TNF- α (C) Across the Experimental Groups. Both T-monocyte and mesalazine treatments significantly reduced pro-inflammatory cytokine levels compared to the positive control (untreated colitis) group (** $p < 0.001$), (**** $p < 0.0001$).

Microscopic Evaluation of Lesions

Histological examination of colonic tissue sections provided a quantitative assessment of the extent of tissue injury (Figure 5). The highest histological damage score was observed in the positive control (untreated colitis) group, which served as a reference for disease severity. In contrast,

treatment with either T-monocytes or mesalazine significantly reduced the degree of inflammation and tissue damage, as evidenced by diminished inflammatory cell infiltration into the colonic mucosa. Compared to the positive control group, both treated groups demonstrated substantially improved histological outcomes.

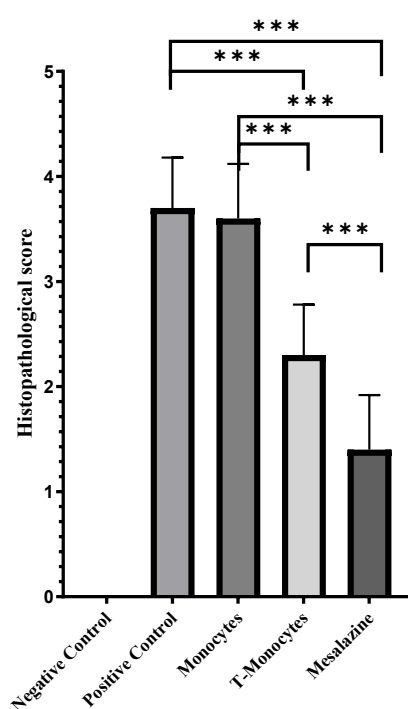


Figure 5. Histological Assessment of Lesions Across the Experimental Groups. Both T-monocyte and mesalazine treatments significantly reduced tissue inflammation compared to the positive control (untreated colitis) group (** $p < 0.001$).

Discussion

Ulcerative colitis (UC) and Crohn's disease (CD) are the two primary forms of inflammatory bowel disease (IBD), characterized by persistent and chronic inflammation of the digestive tract. The primary goal of most IBD therapeutic strategies is to alleviate clinical symptoms and prevent recurrence. However, the majority of currently available pharmaceutical and nutritional therapies are primarily symptomatic and supportive, lacking the capacity to achieve complete remission.²¹ This underscores the urgent need for novel therapeutic approaches with minimal adverse effects and potent anti-inflammatory properties.²² In recent years, researchers have increasingly investigated the use of cell-based therapies for immune- and inflammation-mediated disorders.^{18,23} In the present study, we evaluated the clinical and biochemical outcomes in an experimental UC model using monocytes stimulated with conditioned medium derived from calcitriol-treated MSCs. The rationale for this approach and the criteria for cell selection are discussed below.

The primary objective in the clinical management of UC is the repair of the damaged colonic mucosa. MSCs possess considerable therapeutic potential in regenerative medicine, owing to their capacity for proliferation, multipotent differentiation, and secretion of a wide array of bioactive molecules.²⁴ MSCs can be isolated from various tissues, including bone marrow, adipose tissue, umbilical cord, and muscle. They exhibit low immunogenicity and do not provoke host immune responses prior to terminal differentiation, a property attributed to the limited expression of HLA class I molecules, the absence of HLA class II, and low levels of co-stimulatory molecules. Their anti-inflammatory and tissue-reparative functions are mediated through both direct cell–cell interactions and paracrine signaling.^{24,25} Numerous studies have highlighted the role of MSC-derived mediators in immune modulation. For instance, MSC-secreted proteins can directly regulate CD4⁺ T cell activity, and HLA-G5 has been shown to induce Treg cell development.²⁶ Given their ability to promote tissue repair, enhance angiogenesis, and prevent fibrosis, MSCs represent a promising cell-based therapeutic option for IBD. Furthermore, their capacity to modulate inflammation is particularly relevant, as mucosal healing in IBD is closely linked to the regulation of inflammatory processes within the gastrointestinal tract.²⁷

MSCs are believed to influence immune cell function through the secretion of soluble factors, including anti-inflammatory cytokines such as IL-10, TGF- β , and IL-6, as well as metabolites like prostaglandin E2 and inhibitory enzymes such as matrix metalloproteinases.²⁸ It has been demonstrated that activation of MSC surface receptors with appropriate ligands under optimal conditions can enhance their therapeutic efficacy. Calcitriol (1,25-dihydroxyvitamin D₃), a derivative of the steroid hormone family, participates

in the regulation of gene expression.²⁹ Vitamin D is essential for bone metabolism, calcium homeostasis, and immune regulation. Its active metabolite, calcitriol, can modulate immune responses by binding to the vitamin D receptor (VDR) in the cell nucleus.³⁰ The VDR is expressed on various immune cells, including macrophages, dendritic cells, lymphocytes, and natural killer cells. Calcitriol can be obtained from two environmental sources: dietary intake and cutaneous synthesis following exposure to ultraviolet light. Populations with higher dietary vitamin D intake exhibit a lower prevalence of autoimmune diseases, suggesting a link between vitamin D₃ and immune modulation.³¹ Studies have shown that calcitriol can enhance antioxidant enzyme activity and reduce oxidative damage in both systemic and intestinal tissues of UC patients.³² Furthermore, calcitriol has been shown to augment the immunomodulatory properties of MSCs, as demonstrated by co-culture experiments with neutrophils.¹⁹ Additionally, combining MSCs with calcitriol can promote macrophage survival and stimulate the production of immunomodulatory factors.³³

MSCs can inhibit the differentiation of monocytes into immunogenic antigen-presenting cells and promote their differentiation toward a phenotype that produces the anti-inflammatory cytokine IL-10.¹³ Monocytes treated with MSC supernatant have been shown to attenuate asthma symptoms, reduce tissue damage, and decrease the production of inflammatory cytokines.¹⁸ In a rat model of experimental colitis, MSCs contributed to tissue repair by differentiating into colonic interstitial cells and supplying VEGF and TGF- β to the injured area.³⁴ In a murine model of experimental colitis, systemic administration of bone marrow-derived MSCs (BM-MSCs) reduced the levels of the pro-inflammatory cytokines IL-1 and TNF- α .³⁵ Furthermore, intravenous administration of MSCs proved more effective than intraperitoneal delivery in attenuating colitis severity, as evidenced by reduced weight loss, diarrhea, and inflammation, accompanied by downregulation of TNF- α and IL-6.³⁶ BM-MSC transplantation has also been reported to reduce oxidative stress and restore intestinal mucosal permeability in mice with chemically induced colitis.³⁷

Yang et al. (2015) demonstrated that extracellular vesicles derived from BM-MSCs (BMSC-EVs) effectively ameliorated experimental UC by reducing colonic inflammation, oxidative stress, and apoptosis. Intravenous administration of BMSC-EVs was associated with decreased DAI scores and reduced colonic tissue damage, along with downregulation of NF- κ B p65, TNF- α , iNOS, COX-2, and IL-1, while IL-10 expression was upregulated. Moreover, BMSC-EVs suppressed oxidative stress by decreasing MPO and MDA activity and enhancing SOD and GSH activity.³⁸ Similarly, Pouya et al. (2018) found that MSCs with immunomodulatory properties reduced inflammatory responses in a mouse model of acute colitis, with improvements in mucosal damage and colonic

inflammation, accompanied by increased Treg cell populations and elevated IL-10 and TGF- β levels, alongside decreased IL-17 levels.³⁹ Heydari et al. (2021) demonstrated that exosomes derived from adipose-derived MSCs reduced inflammatory cytokine levels and modulated Treg cell populations to alleviate DSS-induced acute colitis, with treated mice showing reduced colon shortening, weight loss, hemorrhage, and colonic damage, as well as elevated IL-4, IL-10, and TGF- β levels in lymph nodes and spleen.⁴⁰

Conclusion

Monocytes stimulated with conditioned medium from calcitriol-treated MSCs exhibit substantial anti-inflammatory properties and enhance tissue regenerative characteristics in the context of UC. This novel approach suggests that the interplay between monocytes and MSCs can improve therapeutic outcomes by reducing inflammation and promoting healing in affected tissues. Our findings indicate that calcitriol-treated MSC-CM can potentially modulate the immune response, leading to decreased production of inflammatory cytokines and fostering a microenvironment conducive to tissue repair. This strategy may serve as a complementary therapy alongside standard pharmacological treatments for UC, offering a dual mechanism that addresses both the inflammatory and regenerative aspects of the disease. Incorporating this method into existing treatment protocols may enhance overall therapeutic efficacy, providing patients with improved healing and symptom relief. Further research is warranted to fully elucidate the therapeutic potential of this approach and its clinical implications in the management of ulcerative colitis.

Authors' Contributions

MGH, JSh, and HEGG developed the theoretical formalism, performed the analytic calculations, and performed the numerical simulations. JSh supervised the project. All authors contributed to the final version of the manuscript.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

Acknowledgment

The authors would like to thank the Central Laboratory of Baqiyatallah University of Medical Sciences, for all their support and guidance during carrying out this study.

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