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Stem cell therapy for inflammatory bowel disease
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CHAPTER 4

Mesenchymal stromal cell function is not affected by drugs used in the treatment of inflammatory bowel disease

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ABSTRACT

Background and aim

Mesenchymal stromal cells (MSCs) have both multilineage differentiation capacity and immunosuppressive properties. Promising results of MSC administration have been obtained in experimental colitis. Clinical application of MSCs for the treatment of inflammatory bowel diseases (IBD) is currently under investigation in phase I-III trials in patients with past or concurrent immunomodulating therapy. However, little is known about MSC interactions with these immunosuppressive drugs. To address this issue we studied the combined effect of MSCs and IBD drugs in in vitro functionality assays.

Methods

The effects of azathioprine, methotrexate, 6-mercaptopurine, and anti-TNF-α on MSC phenotype, survival, differentiation capacity, and immunosuppressive capacity were studied.

Results

MSC exposed to physiologically relevant concentrations of IBD drugs displayed a normal morphology and fulfilled phenotypic and functional criteria for MSCs. Differentiation into adipocyte and osteocyte lineages was not affected and cells exhibited normal survival after exposure to the various drugs. MSC suppression of peripheral blood mononuclear cell (PBMC) proliferation in vitro was not hampered by IBD drugs. In fact, in the presence of 6-mercaptopurine and anti-TNF-α antibodies, the inhibitory effect of this drug alone was enhanced, suggesting an additive effect of pharmacotherapy and MSC treatment.

Conclusions

This study demonstrates that in vitro, MSC phenotype and function are not affected by therapeutic concentrations of drugs commonly used in the treatment of IBD. These findings are important for the potential clinical use of MSCs in combination with immunomodulating drugs and anti-TNF-α therapy.

INTRODUCTION

Currently, mesenchymal stromal cell (MSC) administration is being evaluated as a novel treatment modality for Crohn's disease.[1] MSCs are pluripotent, non-hematopoietic cells easily isolated from various tissues. In the absence of a single specific marker, MSCs are characterized using a specific panel of markers. Isolated and expanded MSCs express surface molecules CD105, CD73, and CD90 but do not express CD14 and hematopoietic surface molecules (CD45 and CD34). Furthermore, human leukocyte antigen (HLA)-DR is not expressed unless cells are stimulated with interferon gamma (IFN-γ).[2] Under appropriate conditions, MSCs can differentiate in vitro into different cell types such as adipocytes, osteoblasts, and chondroblasts.

Clinical trials with MSCs in patients with Crohn's disease focus on either active luminal disease,[1] for which MSCs can be injected intravenously, or fistulizing disease, for which MSCs are injected locally into the fistula tracts.[3, 4] MSCs are isolated from bone marrow or adipose tissue either from the patient (autologous) or from a healthy donor (allogeneic). In general, patients with Crohn's disease included in the current trials are relatively refractory to various immunomodulatory drugs, which may have altered their MSCs prior to harvesting (in an autologous setting), or may change the function of administered MSCs. Thus far, trials attempt to deal with this by stopping anti-TNF-α treatment at least 8 weeks before the application of MSCs and restricting other concomitant drugs to a stable dosage regimen of immunomodulators, antibiotics or, in the case of fistulizing Crohn's disease, cyclosporine.[1, 5] However, scientific data providing rationale for (dis)continuation of drugs during MSC treatment have not been available yet.

Previous studies showed that immunosuppressive drugs can be harmful to hematopoietic stem cells or endothelial progenitor cell proliferation and significantly affect their functional capacities.[6-9] Likewise, immunosuppressive agents might have an effect on MSC function and could, therefore, change the outcome of MSC therapy and affect safety. Also, allogeneic MSCs are exposed to drugs taken by the patient after local or intravenous administration. Furthermore, administered MSCs could interfere with concomitant medical therapy. To optimize treatment efficacy and safety, it is important to assess the effects of drugs routinely used in patients with IBD on the function of MSCs, and vice versa. Therefore, in this study, we examined the effect of azathioprine, methorexate, 6-mercaptopurine and anti-TNF-α (infliximab) on bone marrow-derived MSCs, focusing on MSC phenotype, viability and function. Subsequently, we studied the effect of MSCs on the immunosuppressive properties of these drugs.

MATERIALS AND METHODS

MSC isolation and expansion

Donors underwent routine donor control examination and screening tests, according to the standard procedures required for bone marrow donors. MSCs from three different donors were expanded according to a common protocol devised by the European Group for Blood and Bone Marrow Transplantation (EBMT) developmental committee, as previously

described.[10] Bone marrow was harvested by aspiration from the iliac crest from patients under local anesthesia in the outpatient clinic. Bone marrow mononuclear cells were isolated by Ficoll density gradient (density 1.077 g/cm^3) centrifugation. Washed cells were resuspended in Dulbecco's modified Eagle's-low glucose medium (Invitrogen, Paisley, UK) supplemented with penicillin and streptomycin (Lonza, Verviers, Belgium) and 10% fetal calf serum (FCS, HyClone, Logan, UT), without any additional growth factors (hereafter referred to as standard culture medium). Mononuclear cells were plated at a density of 160 000 cells per cm^2. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 in 175 cm^2 flasks (Greiner Bio-One, Frickenhausen, Germany). When the cultures reached near confluence (>80%), the cells were detached by treatment with trypsin/ EDTA (Lonza, Verviers, Belgium) and replated at a density of 4000 cells per cm^2. Cells were used from passage 3 to passage 6.

IBD drugs

Azathioprine (Sigma-Aldrich, St. Louis, MO), methotrexate (Pharmachemie BV, Haarlem, the Netherlands), and 6-mercaptopurine (Sigma-Aldrich) were used in relevant physiologic concentrations of 1μM, 100 μg/mL, and 4μM respectively. Infliximab (Schering-Plough, Houten, the Netherlands) was prepared according to the manufacturer's recommendations. In all experiments, azathioprine and 6-mercaptopurine were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich), and cells treated with DMSO alone were used as control.

The concentrations[11-16] used in our experiments are concentrations commonly used in in vitro experiments and are therapeutic concentrations that are also obtained in vivo.

Flow cytometric analysis

MSC immunophenotype was analyzed using flow cytometry. MSCs were seeded in 25cm^2 flasks and were incubated with IBD drugs added to standard culture medium for 7 days in total, medium was changed on day 3. Cells were harvested and immunophenotyping of cultured MSCs was performed using flow cytometry for the following markers: CD105, CD73, CD90, CD45, CD34, CD14, CD19 and HLA-DR (BD Biosciences, Franklin Lakes, NJ). Samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences) and data were analyzed with FlowJo software (version 7.2.5., Treestar, San Carlos, CA).

Cell viability by MTT assay

MSC were seeded in a 96-well plate at 5000 cells/well and incubated with IBD drugs in standard culture medium for 48 hours or 7 days. To determine viable cell numbers the MTT assay was used. At the end of the experiment, medium was replaced with 100 μL fresh medium, and 25 μL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) in PBS was added and incubated for 4 hours at 37°C. DMSO (100μL) was then added and the intensity of color was determined at 550 nm using a microplate reader (VersaMax, Molecular Devices, Sunnyvale, CA).

In vitro differentiation

MSCs were plated at 5000 cells/cm^2 in 24-well culture plates and kept in standard culture medium until 80-90% confluency was reached. For differentiation, MSCs were cultured in differentiation medium in the presence of the indicated IBD drug. Control MSCs were grown in non-conditioned medium. For osteogenic differentiation, cells were grown in standard medium supplemented with 50 μg/mL ascorbic acid, 10 mM β-glycerolphosphate, and 10^{-8}M dexamethasone and were stained with Fast Blue for alkaline phosphatase activity and Alizarin Red for mineralization. For adipogenic differentiation, cultures were stimulated with complete medium supplemented with 0.5 mM 3-isobutyl-1-methylxanthine, 100 μM indomethacin, 5 μg/ml insulin and 10^{-6} M dexamethasone. Lipid droplets were revealed by staining with Oil Red O. All chemicals were from Sigma-Aldrich (St. Louis, MO). Cells were kept at 37°C, 5% CO_2 for up to 21 days and the media were changed twice weekly.

MSC/peripheral blood mononuclear cell (PBMC) proliferation assay

On day 1, cultured MSCs were plated at increasing numbers in flat bottom 96 well plates (Costar) and allowed to attach overnight. PBMCs were isolated from buffy coats, activated with αCD3/αCD28 coated beads (Invitrogen) (1 bead/5 cells) and seeded in Iscove's modified Dulbecco's medium (Sigma-Aldrich) containing indicated IBD drug, 5% human serum (Sanquin, Leiden, the Netherlands), 5% FCS and 100 IU/mL IL-2 (LUMC Pharmacy, Leiden, the Netherlands) at 100 000 per well on day 2. Proliferation was measured by ^{3}H-thymidine incorporation on day 7.

Statistical Analysis

Data were analyzed using SPSS (version 16.0, SPSS Inc., Chicago, IL) and GraphPad (Graphpad software Inc., La Jolla, CA). Results are representative of at least three independent experiments and show means ± standard error of the mean unless otherwise indicated. *P* values were calculated using the analysis of variance (ANOVA) and Student's t-test with values less than 0.05 considered statistically significant.

Ethical Considerations

MSCs were obtained from donors in previous studies.[10,17,18] Donors were informed about and consented to the possibility of the use of their MSCs for preclinical studies/analysis. The Medical Ethical Committee of the Leiden University Medical Center (LUMC) approved the use of these MSCs for preclinical studies.

RESULTS

MSC phenotype and viability are not affected by drugs commonly used in IBD

For lack of a specific marker identifying MSCs, this cell type is commonly identified using a panel of markers as described before.[2] In short, MSCs are characterized by expression of CD105, CD90, CD73, and the absence of hematopoietic markers. To determine whether MSCs retained their phenotype after exposure to IBD drugs, cells were grown in culture medium in the presence of these drugs. Exposure to either azathioprine, methotrexate, 6-mercaptopurines or anti-TNF-α antibodies for seven days did not alter MSC morphology and phenotype. These MSCs showed normal expression of CD73, CD90, and CD105 and absence of CD45,

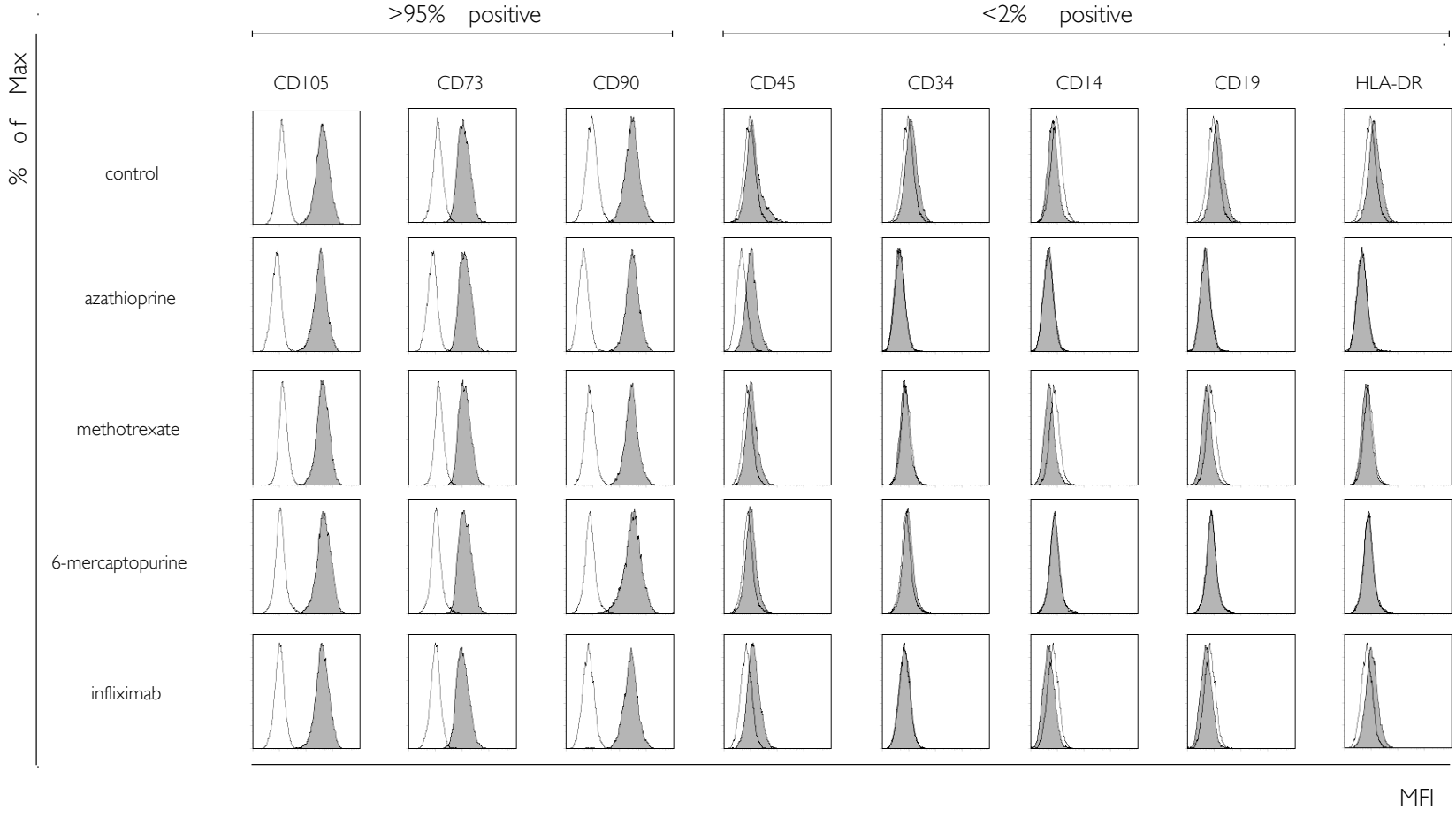

Figure 1. Effect of IBD drugs on MSC phenotype. Surface markers on control (upper panels) and IBD drug-treated MSCs (lower panels) were analyzed by flow cytometry. Markers are shown (solid histogram) compared to isotype control (line).

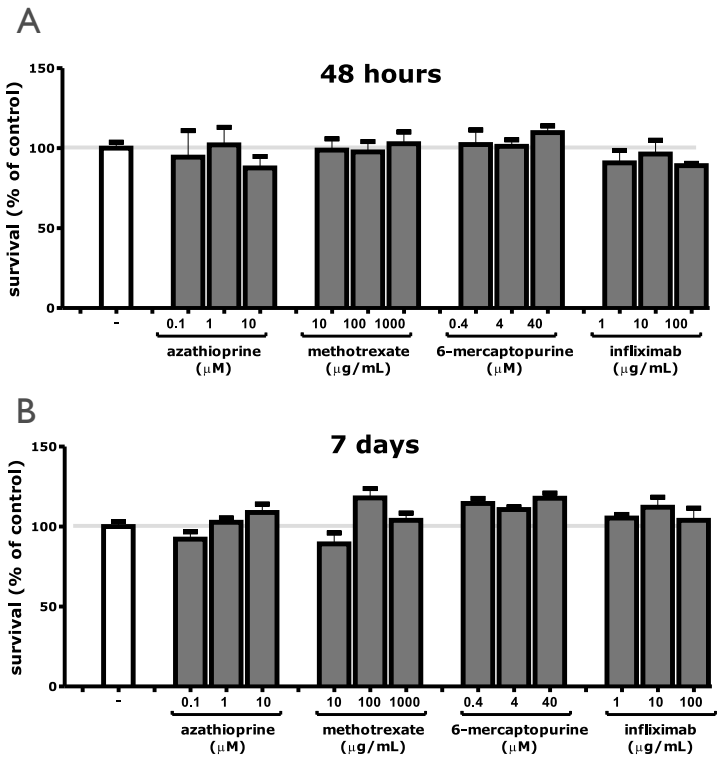

Figure 2. Cell viability of MSCs exposed to IBD drugs for 48 hours (A) and 7 days (B) as measured by MTT assay and expressed as a percentage of untreated control.

CD34, CD14, and CD19 (Figure 1). Furthermore, MSCs did not express HLA-DR, indicating they were not activated by the drugs.[19] Additionally, incubation with IBD drugs for 48 hours (Figure 2A) as well as 7 days (Figure 2B) had no effect on the viability of the MSCs.

MSCs maintain their multilineage differentiation capacity in the presence of IBD drugs

Minimal criteria for defining MSCs also include their adherence to plastic and the capacity to differentiate into various lineages in vitro, including adipocytes and osteocytes. To test the effects of IBD drugs on these functions, MSCs were cultured under various differentiation conditions in the presence of IBD drugs. MSC adherence to plastic was maintained and unaffected by any of the drugs. Also the spindle-shaped fibroblastic morphology was not altered (Figure 3A). To determine the effect of IBD drugs on MSC differentiation capacity, MSCs were concomitantly cultured under adipogenic and osteogenic conditions. MSCs cultured under these conditions maintained their capability to differentiate into adipocytes (Figure 3B) and osteoblasts (Figure 3C) in the presence of IBD drugs, and therefore fulfill the international MSC criteria.

IBD drugs combined with MSCs retain immunosuppressive properties on activated PBMCs

MSCs have the capacity to inhibit T-cell proliferation, as previously shown in various studies.[20-22] We tested the immunosuppressive capacity of the MSCs at different ratios to PBMCs stimulated with αCD3/αCD28 coated beads. In this setting, PBMC proliferation is inhibited by MSCs in a dose-

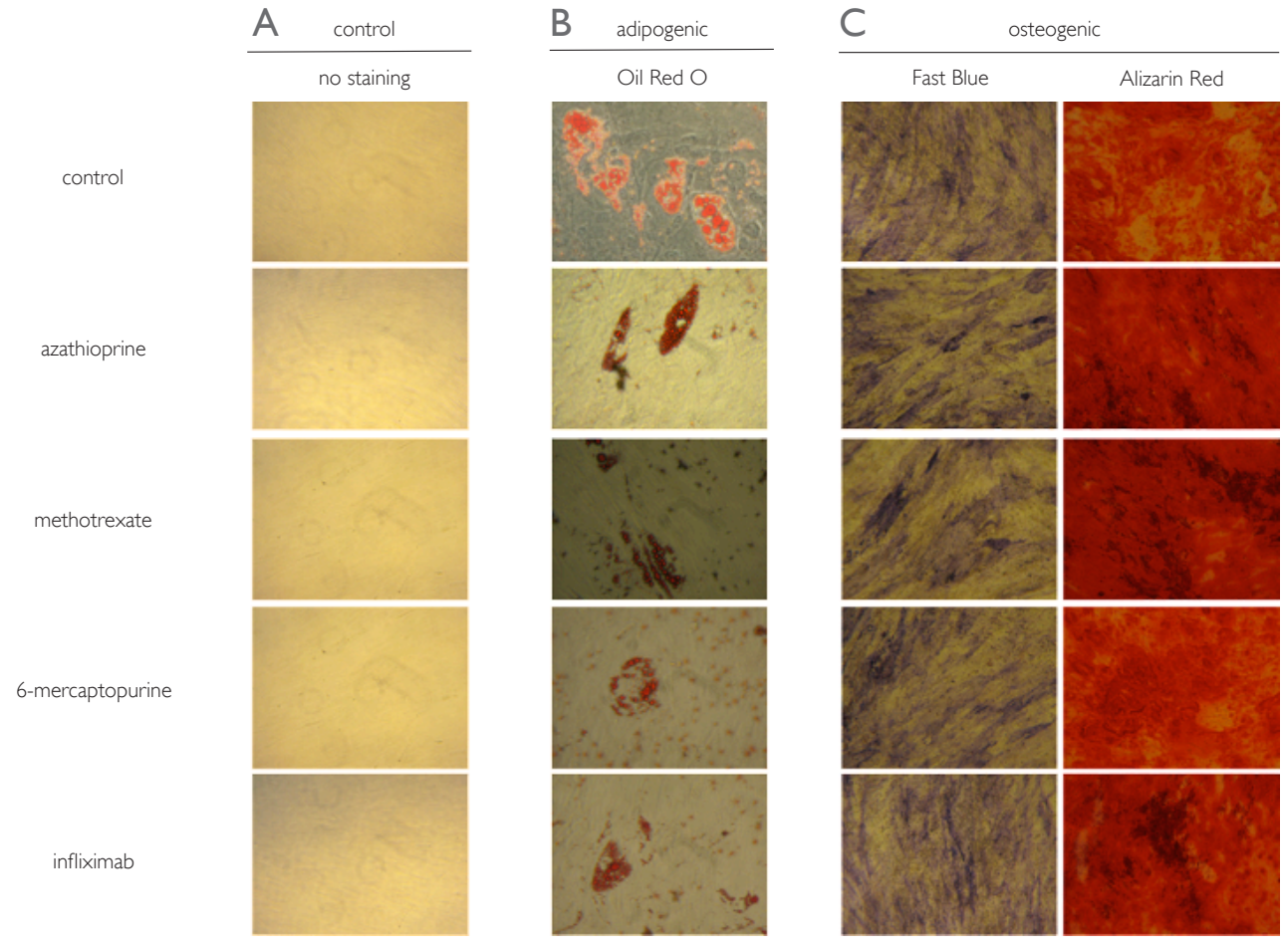

Figure 3. Differentiation capacity of MSCs into adipocytes and osteoblasts cultured in the presence of IBD drugs. A Control culture-expanded MSC morphology in the presence of IBD drug (no staining). B Lipid droplets in the cell cytoplasm of adipocytes were stained with Oil Red O. Magnifications 32x. C Osteogenic differentiation was detected with Fast Blue to show alkaline phosphatase activity and Alizarin Red to detect mineralization. Magnifications 10x.

dependent manner (Figure 4A). Next, we examined if MSCs would have a positive or negative effect on the immunosuppressive properties of azathioprine, methotrexate, and 6-mercaptopurine in vitro. We found that MSCs did not antagonize the effect of the immunomodulating drugs on PBMC proliferation. Azathioprine and methotrexate alone inhibit PBMC proliferation, as has been described previously.[23, 24] This effect was enhanced when MSCs were present in the culture, although this did not reach statistical significance (Figure 4B). Presence of MSCs in co-cultures containing 6-mercaptopurine did show a significant additive effect, with a decrease in PBMC proliferation of 53.5% (no MSCs 82.4 ± SD 7.1 vs 1000 MSCs 28.9 ± SD 6.0, *P*<.001).

It has been described that MSCs need to be stimulated by pro-inflammatory cytokines, e.g. IFN-γ and TNF-α, to exert their immunosuppressive effect.[25,26] By adding anti-TNF-α agents to PBMC cultures, TNF-α activity is neutralized,[27] thereby possibly affecting MSC function. Therefore, we analyzed the effect of increasing concentrations of the anti-TNF-α agent infliximab on MSC/activated PBMC co-cultures. In

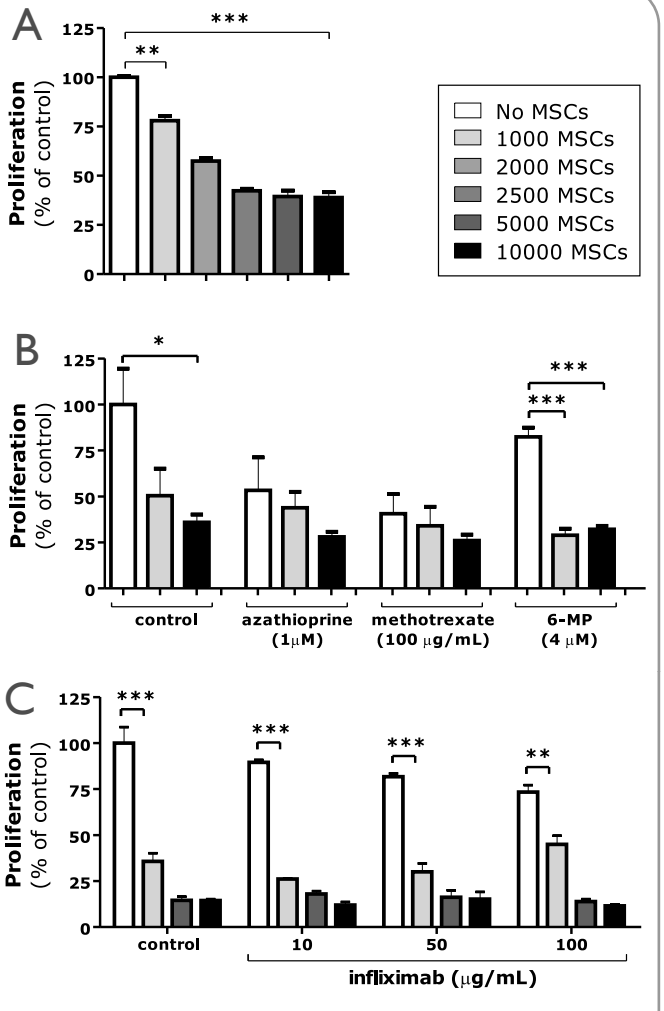

Figure 4. MSC and IBD drug interactions on PBMC proliferation. A MSCs inhibit the proliferation of activated PBMCs in a dose-dependent fashion. PBMCs (100 000 cells) were stimulated with αCD3/αCD28 beads and cocultured with different numbers of MSCs. B Effect of IBD drugs on the proliferation of PBMCs (100 000 cells) stimulated with αCD3/αCD28 beads in the presence of different numbers of MSCs (0, 1000 and 10 000). C Effect of increased concentrations of infliximab on the proliferation of PBMCs (100 000 cells) stimulated with αCD3/αCD28 beads in the presence of indicated number of MSCs (0, 1000, 5000 and 10 000). Proliferation was measured by 3H-thymidine uptake in counts per minute. Data are expressed as a percentage relative to PBMC proliferation in control medium (set to 100%).and presented as means ± SEM,*P<0.05, **P<0.01, ***P<.001.

this setting, inhibition of PBMC proliferation by MSCs persists also in the presence of high concentrations (50 and 100 μg/mL) of infliximab (Figure 4C), indicating that neutralization of TNF-α alone is insufficient to antagonize MSC function.

DISCUSSION

Currently, MSC administration is being evaluated as a potential new treatment for Crohn's disease. Studies in experimental colitis have generated promising results,[28-30] and phase I studies have not shown any major safety and feasibility concerns.[1, 3] At the moment, various phase II trials are undertaken attempting to optimize MSC treatment with regard to dosage, timing, and route of administration. However, the effect of concomitant use of various IBD drugs is unknown and raises questions on which patients to include in the various trials. Patient MSCs might be affected by past drug usage but also allogeneic MSCs are exposed to immunomodulating drugs after administration. Therefore, we first aimed to study the effects of commonly used IBD drugs on MSC phenotype and function. Subsequently, the effect of MSCs on the immunosuppressive properties of these drugs was studied.

This study demonstrates that MSCs exposed to IBD drugs fulfill the minimal set of standard criteria introduced by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy,[2] indicating that these drugs do not substantially change MSC morphology, phenotype, and differentiation capacities. Furthermore, MSC viability was not affected by any of the compounds examined.

MSCs inhibit PBMC proliferation in a dose-dependent manner.[21] Previously, it was demonstrated that bone marrow derived MSC have a synergistic effect with the calcineurin inhibitor cyclosporin on the activation of cytotoxic T cells.[31-33] Conversely, MSCs derived from heart tissue decreased the immunosuppressive effect of tacrolimus and rapamycin, raising some concern regarding the simultaneous use of immunomodulating drugs and MSC treatment.[34] Although cyclosporin and tacrolimus are used in the controlling IBD, they are still of limited value as randomized controlled studies are lacking.[35, 36] This chapter mainly focused on immunomodulating drugs frequently used in the treatment of IBD and we found that, in vitro, bone marrow-derived MSCs do not hamper the inhibitory effect of these drugs. Furthermore, we show that neutralization of TNF-α using the anti-TNF-α agent infliximab does not antagonize the inhibitory effect of MSCs. These findings indicate that in the setting of IBD, concomitant drug usage may not present a problem for MSC therapy and, conversely, that MSC administration does not appear to hamper the effects of other drugs. These observations may expand the scope for MSC therapy in IBD, as patients unable or unwilling to stop their therapeutic regimen, who are now excluded from the various studies, may yet become candidates in future trials.

The present study demonstrates that therapeutic concentrations of immunosuppressive drugs do not affect MSC phenotype and function. These findings are important for the potential clinical use of MSCs in combination with immunosuppressants and could further optimize current treatment developmental programs.

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