The use of unlicensed bone marrow-derived platelet lysate-expanded mesenchymal stromal cells in colitis : a pre-clinical study

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2019-02

Salmenkari , H , Laitinen , A , Forsgård , R A , Holappa , M , Linden , J , Pasanen , L , Korhonen , M , Korpela , R & Nystedt , J 2019 , ' The use of unlicensed bone marrow-derived platelet lysate-expanded mesenchymal stromal cells in colitis : a pre-clinical study ' , Cytotherapy , vol. 21 , no. 2 , pp. 175-188 . https://doi.org/10.1016/j.jcyt.2018.11.011

http://hdl.handle.net/10138/310822
https://doi.org/10.1016/j.jcyt.2018.11.011

Title page

Title: The utilization of unlicensed bone marrow-derived platelet lysate-expanded mesenchymal stromal cells in colitis: a preclinical study

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Key words: Mesenchymal stromal cells; MSCs; Cryopreservation; Licensing; Dextran sodium sulfate colitis; Renin-angiotensin system

Abstract

Background: Mesenchymal stromal cells (MSCs) are a promising candidate for treatment of inflammatory disorders, but their efficacy in human inflammatory bowel diseases (IBD) has been inconsistent. Comparing of the results from various preclinical and clinical IBD studies is also challenging due to a large variation in study designs.

Methods: In this comparative preclinical study, we compared two administration routes and investigated the safety and feasibility of both fresh and cryopreserved platelet-lysate expanded human bone marrow-derived MSCs without additional licensing in a dextran sodium sulfate (DSS) colitis mouse model both in the acute and regenerative phases of colitis. Body weight, macroscopic score for inflammation, and colonic IL-1β and TNFα concentrations were determined in both phases of colitis. Additionally, histopathology was assessed and *Il-1β* and *Agtr1a* mRNA levels and angiotensin-converting enzyme (ACE) protein levels were measured in the colon in the regenerative phase of colitis.

Results: Intravenously administered MSCs exhibited modest anti-inflammatory capacity in the acute phase of colitis by reducing IL-1β protein levels in the inflamed colon. There were no clear improvements in mice treated with fresh or cryopreserved unlicensed MSCs according to weight monitoring results, histopathology and macroscopic score results. Pro-inflammatory ACE protein expression and shedding were reduced by cryopreserved MSCs in the colon.

Conclusions: In conclusion, we observed a good safety profile for bone marrow-derived platelet-lysate expanded MSCs in a mouse preclinical colitis model, but the therapeutic effect of MSCs prepared without additional licensing, i.e. such as MSCs are administered in graft-versus-host-disease, was modest in the chosen *in vivo* model system and limited to biochemical improvements in cytokines without a clear benefit in histopathology or body weight development.

Background

Inflammatory bowel diseases (IBD) are multifactorial inflammatory diseases that present as inflammation of intestinal tissue, bloody diarrhea, and ulceration. The two main forms of IBD are ulcerative colitis and Crohn's disease. Ulcerative colitis mainly manifests as inflammation in the colon, whereas in Crohn's disease inflamed patches can be present throughout the gastrointestinal tract[1-3]. A common feature of IBD patients is an alternation between remission and an active disease state. The goal of current treatment regimens is to maintain disease remission or shorten active disease periods. Conventional treatments for IBD, such as anti-inflammatory medication (aminosalicylates and corticosteroids), immune suppressors (thiopurines and methotrexate), monoclonal anti-TNFα antibodies, and surgery may have significant side effects and often offer only temporary relief (for example due to drug resistance)[1, 4-6]. Furthermore, new treatment options are needed as a subset of patients responds poorly to these treatments[4-6]. Cell therapy with mesenchymal stromal cells (MSCs) is one interesting option.

MSCs are promising candidates for suppressing undesired immune reactivity and for promoting tissue healing and regeneration[7, 8]. MSCs secrete soluble factors, such as cytokines and growth factors, which could inhibit lymphocyte proliferation and promote immune cell differentiation to regulatory populations, but their immune-suppressive mechanisms *in vivo* are not completely resolved[9]. It has been thought that allogenic MSCs do not provoke an overt immune reaction from the host even when the host and donor are not HLA matched[7, 9], but a recent study presented convincingly a completely new immunomodulatory mechanism for MSCs based on apoptosis of MSCs and where an immune activation of the host cytotoxic T-cells against MSCs is critical for effective immune-suppression through macrophage polarization[10]. The immune-suppressive and anti-inflammatory properties have made these cells potential candidates in clinical applications for many diseases, including myocardial infarction, arthritis, and refractory graft-versus-host disease[7, 11, 12]. Several studies have also evaluated their efficacy in the treatment of refractory IBD. In preclinical studies, MSCs have alleviated the symptoms of dextran sodium sulfate (DSS)[13-16] and trinitrobenzene sulfonic acid[17, 18] -induced colitis, but results from clinical trials are inconsistent[19-22]. Fistulizing Crohn's disease can be very difficult to treat, but good outcomes (measured as fistula closure) were reported in trials using bone marrow (BM)-derived MSCs in local treatment of fistulas[23, 24]. Two phase III trials using adipose tissue-derived

MSCs in the treatment of perianal fistulas found MSC therapy effective[21, 25], but it is noteworthy that one of these trials only concluded the MSC therapy to be as effective as fibrin glue alone[25]. Although systemic infusions of MSCs have been well tolerated and feasible in phase I-II studies on luminal Crohn's disease[26-28], only one of the studies have demonstrated efficacy[28].

Functional properties of MSC may be affected by differences in the manufacturing strategy and culturing conditions[22]. Allogenic or autologous MSCs are most commonly derived from BM and cultured for several passages *ex vivo* to reach adequate numbers of cells for clinical use. In pre-clinical studies the cells can be easily licensed with various cytokines prior to administration to potentially improve their efficacy[15, 16, 29], but in clinical applications additional licensing would increase the level of manipulation of the cells. Most of the preclinical studies with animal colitis models have utilized either licensed human MSCs or syngeneic murine MSCs. To our knowledge all clinical trials have utilized native MSCs without additional licensing prior to administration. MSC preparations can either be fresh, meaning the cells are detached from the cell cultures just before administration to patients, or the cells can be cryopreserved and thawed bedside just before administration. A cryopreservation step in the manufacturing process brings important quality benefits as it enables a completion of all quality testing before batch release and administration to the patient. It also enables the administration of identical cell doses in repeated cell administration regimes. Cryopreservation is also the only feasible option for MSC banking strategies and is practical with regards to logistics. Some recent reports, however, suggest that cryopreserved MSCs may have impaired functional properties when compared with freshly harvested MSCs from continuous cultures[30-33]. On the contrary, some studies have shown that the efficacy of cryopreserved MSCs is comparable to fresh MSCs[34, 35]. These conflicting results warrant further studies to elucidate the impact of a cryopreservation step in manufacture of clinical-grade MSCs.

In this comparative preclinical study, we investigated the feasibility and safety of unlicensed platelet-lysate expanded human BM-derived MSCs in a DSS-induced murine experimental colitis model. We used a minimally expanded (cultured until passage 2) cryopreserved MSC product, which has been proven to be effective to some extent in the treatment of acute graft-versus-host-disease (GvHD) [36]. We compared this product to its fresh, unfrozen counterpart in the same passage. We chose not to stimulate the platelet-lysate expanded MSCs with any additional cytokines to be able to study the effectiveness of an unlicensed MSC product i,e, such as MSCs are currently prepared and administered for the treatment of steroid-resistant GvHD. First, we compared the administration routes using fresh MSCs (fresh-MSC) by injecting them either intravenously (IV) or intraperitoneally (IP) and compared the anti-inflammatory properties of MSCs during the acute phase of colitis. Second, we compared IV MSC treatments with either fresh or cryopreserved (cryo-MSC) cell preparations in the regenerative phase of colitis. We further investigated the anti-inflammatory and tissue healing-promoting effects of MSCs in the colon by measuring cytokine levels, angiotensin-converting enzyme (ACE) protein expression and shedding, and anti-inflammatory corticosterone production in colon preparations. This study further demonstrated the safety and feasibility of MSCs but provided evidence of only modest therapeutic effect in treatment of experimental colitis when utilizing unlicensed MSCs. The differences between fresh and cryopreserved MSCs remained unresolved. Interestingly, we observed evidence of MSC involvement in regulation of the intestinal renin-angiotensin system (RAS).

Methods

MSC expansion, characterization, and preparation before administration

Human bone marrow was harvested and MSCs were expanded as previously described[37]. Briefly, the MSCs were expanded in medium consisting of D-MEM low glucose (Life Technologies, Paisley, Scotland, UK) supplemented with 40 IU/ml heparin (Heparin LEO 5000 IE/KY/ml, Leo Pharma, Ballerup, Denmark), 10% platelet lysate (PL1 supplement as described previously by Laitinen et al.[37]), 100 U/ml penicillin, and 100 µg/ml streptomycin (Life Technologies, Grand Island, NY, USA). For fresh MSC preparations, cells in p2 were trypsinized with Tryple Select CTS™ (LifeTechnologies) and resuspended for the injections in 0.9% NaCl + 5% human serum albumin (HSA) (Albunorm 200 g/L, Octapharma, Lachen, Switzerland) (administration route study) or 0.9% NaCl + 3.6% HSA (fresh versus cryo study) solution at 5 x 10^6 cells/ml (fresh-MSC). For cryopreserved MSC preparations, the cells were frozen at p2 in HSA and 10% DMSO (CryoSure, WAK-Chemie Medical GmbH, Germany) at 7 x 10^6/ml. The cryopreserved cells were thawed in a 37°C water bath, centrifuged at 300 g for 5 min after a short rest at RT and finally resuspended in 0.9% NaCl + 3.6% HSA at 5 x 10^6 cells/ml (cryo-MSC). Comparative cell batches were used in the fresh versus cryo study and the fresh MSC preparations were prepared from interim frozen p1 cells and entered to subsequent culturing until p2 according to the administration schedule.

The MSCs were characterized for cell surface markers and immunosuppression and differentiation capacity as described previously[37] and were verified to have a typical MSC phenotype (with an HLA-DR+ phenotype as described by Laitinen et al.[37]), osteogenic and adipogenic differentiation capacity and evident T-cell immunosuppression capacity *in vitro* (Figure S1). Cell numbers and viability were determined using NucleoCounter NC-100™ (ChemoMetec, Allerod, Denmark). The viability of cryo-MSCs was >95% after thawing and >90% 1h after thawing (data not shown).

Animals

The animal experiments were approved by the national Animal Experiment Board in Finland (ESAVI/6314/04.10.03/2012 and ESAVI/114/04.10.07/2015) according to the Finnish Act on Animal Experimentation (62/2006). Male balb/c mice obtained from Scanbur AB (Sollentuna, Sweden) at 8 weeks of age were used for the study. The mice were housed in a 12-h light/dark cycle at 22°C ± 2°C and relative humidity of 55% ± 15%. The animals were given a 2018 Teklad Global 18% Protein Rodent Diet (Harlan Laboratories, Indianapolis, IN, USA) standard rodent food and experimental drinks *ad libitum*. The animals were weighed daily throughout the experiment.

Study design and induction of colitis

The study design is presented in detail in Figure 1.

Administration route study with a short follow-up in the acute phase of the colitis

To compare IV and IP MSC administration in the acute phase of colitis, colitis was induced in four groups (n=8 in each group) via administration of 3% DSS (DB001, TdB Consultancy Ab, Uppsala, Sweden) in the drinking water for 7 days (days 1-8). On days 3 and 5 of the experiment, 100 µl of fresh MSCs (0.5 x 10^6 MSCs in 0.9% NaCl + 5% HSA) or vehicle (VE) (0.9 % NaCl + 5% HSA) were injected either IV via the tail vein or IP under isoflurane-inhalation anesthesia (Colitis groups: VE IV, MSC IV, VE IP and MSC IP). The healthy control group had access to tap water throughout the experiment and did not receive MSCs or VE. On day 8, the mice were sacrificed by CO_2 and decapitation.

Fresh-MSC versus cryo-MSC study with one administration route and longer follow-up in the regenerative phase of colitis

To compare fresh and cryopreserved MSCs in the regenerative phase of colitis, colitis was induced in three groups (n=10 in each group) via administration of 3% DSS in water for 6 days (days 1-7), after which the mice received tap water for 7 days (days 7-14). On days 3 and 5 of the experiment, 100 µl of either fresh-MSC or cryo-MSCs (0.5 x 10^6 MSCs in 0.9 % NaCl + 3.6% HSA) or VE (0.9% NaCl + 3.6% HSA; DSS-control group), were injected IV via the tail vein of the mice in the colitis groups (DSS control, Fresh-MSC and Cryo-MSC) under isoflurane-inhalation anesthesia. The healthy control group had access to tap water throughout the experiment and received no MSCs or VE. On day 14, the animals were sacrificed by cardiac puncture in isoflurane anesthesia.

Macroscopic assessment of inflammation

Upon sacrifice, macroscopic inflammation was scored in the colons of all mice. Colons were excised, and their lengths were measured. The colons were opened longitudinally, and stool consistency was evaluated on a scale from 0-2 (0=normal, 1=loose, and 2=liquid). The colons were then cleared of intestinal content and weighed, after which colonic edema and presence of blood in the colonic mucosa were evaluated on a scale from 0-2 (where 0=none present and 2=clearly observable). All the scores were subsequently combined into a total macroscopic score (scale 0-6).

Preparation of tissue samples

To compare the histopathological changes in mice receiving fresh-MSCs or cryo-MSCs, pieces of distal colon were fixed in 10% neutral buffered formalin (Sigma Aldrich, St. Louis, MO, USA) for 24 hours and embedded in paraffin blocks. For both studies, tissue samples of mid colon were flash frozen in liquid nitrogen for immunochemical analyses. Pieces of proximal colon were incubated in pre-oxygenated Krebs buffer (119 mmol/l NaCl, 25 mmol/l $NaHCO_3$, 15 mmol/l KCl, 11 mmol/l glucose, 1.6 mmol/l $CaCl_2$, 1.2 mmol/l KH_2PO_4, 1.2 mmol/l $MgSO_4$) for 90 min after which the samples were centrifuged at 13 300 rpm for 3 min and the supernatant was collected for corticosterone and ACE measurements.

Microscopic assessment of inflammation

Colon slides were stained with hematoxylin and eosin (H&E) dye and evaluated for severity of inflammation. Inflammation activity, mucosal atrophy, and crypt regeneration in atrophied tissue were evaluated blinded on a grade of 0 to 5 by a trained pathologist. Inflammation activity and mucosal atrophy scores were combined into a histopathology score on a scale of 0 to 10.

Immunochemical analyses

Frozen colon pieces were homogenized in a Precellys 24 homogenator (Bertin Technologies, Montigny le Bretonneux, France) in 100 mM Tris – 120 mM NaCl, pH 8.3. The lysates were centrifuged for 20 min at 4°C and supernatant was stored at -80°C until use. IL-1β and TNFα were quantified using AlphaLisa (AL503 and AL504, Perkin Elmer, Waltham, MA, USA). ACE shedding and corticosterone were measured from the incubation supernatants and tissue ACE from lysed tissue samples using ACE DuoSet ELISA (#DY1513 R&D System, Minnesota, MO, USA) and Corticosterone EIA (#500655 Cayman Chemical, Michigan, MI, USA). Analyte concentrations were normalized to total protein concentration of the corresponding tissue piece (Pierce™ BCA Protein Assay Kit, Thermo Scientific).

Reverse transcriptase quantitative PCR

Colon RNA was extracted using NucleoSpin RNA (Macherey Nagel, Duren, Germany) and reverse-transcribed into cDNA using iScript™ cDNA Synthesis Kit (BioRad, Hercules, CS, USA). RT-qPCR was performed in a LightCycler® 480 with LightCycler® 480 SYBR Green I Master (Roche Diagnostics Corp., Indianapolis, IN, USA). All primers were ordered from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). Primer sequences were *β-Actin* F: 5’-CTGAATGGCCCAGGTCTGAG-3’, R: 5’-AAGTCAGTGTACAGGCCAGC-3’, *S18* F: 5’- AACGAACGAGACTCTGGCAT-3’, R: 5’-ACGCCACTTGTCCCTCTAAG-3’, *Il-1β* F: 5’- CTCCAGCCAAGCTTCCTTGT-3’, R: 5’-

TCATCACTGTCAAAAGGTGGCA-3’ and *Agtr1a* F: 5’- CTGCTCTCCCGGACTTAACA -3’, R: 5’-GCACTTGATCTGGTGATGGC-3’. n = 3 to 5 in each group in RT-qPCR experiments.

Statistical analysis

The gene expression data are presented in text as relative quantity in percent and as individual data points and geometric mean in the figures. All other data are presented in text and tables as mean ± SEM and in graphs as individual data points and mean. The differences between multiple groups were analyzed using one-way ANOVA with Tukey’s *post hoc* test or Kruskal-Wallis test where applicable. Non-parametric tests were conducted by adding noise to values below the detection limit of each assay. Statistical analyses were done and outliers removed in SPSS versions 22 and 23. P values lower than .05 were considered statistically significant.

Results

Macroscopic signs of inflammation were not improved by MSCs in acute phase of DSS colitis

We first compared IV-administered and IP-administered fresh MSCs during a seven-day DSS challenge (Figure 1A). MSC treatments had no obvious adverse effects on the general wellbeing of the animals. DSS induced significant weight loss (MSC IV group, $p = 0.002$; VE IV group, $p = 0.009$; MSC IP group, $p < 0.001$; VE IP group, $p = 0.001$) (Figure 2A) and colon shortening ($p < 0.001$ for all groups) (Table 1) in all colitis groups compared with healthy controls. Macroscopic scores (stool consistency, colonic edema, and mucosal blood) were significantly increased compared with those from healthy controls in all other colitis groups (MSC IV group, $p = 0.022$; MSC IP group, $p = 0.003$; VE IP group, $p = 0.002$) except in the VE IV group ($p = 0.093$), in which two mice had normal macroscopic findings (Table 1). Body weight, colon length, and macroscopic scores were similar in the IV-treated and IP-treated MSC groups and did not differ from their respective vehicle controls (Figure 2A, Table 1). There were no statistically significant differences in colon weight compared with body weight between any of the groups, although these values appeared to be lower in the MSC IV group than those from the other colitis groups (Figure 2B).

MSCs reduce the levels of colonic IL-1β in acute phase of DSS colitis

As there were no statistically significant differences in macroscopic signs of inflammation between IV and IP administration routes, we measured the concentrations of the pro-inflammatory cytokines IL-1β and TNFα in the colons of healthy, MSC IV, and VE IV mice. IL-1β was undetectable in samples from healthy control mice (Figure 2C). DSS caused an increase in IL-1β concentration (543 ± 246 pg/mg in the VE IV group, $p = 0.032$), which was reduced by MSC treatment (undetectable in the MSC IV group, $p = 0.032$). TNFα levels were increased in the VE IV group compared with healthy controls (55 ± 29 pg/mg and 1.8 ± 1.6 pg/mg, respectively, $p = 0.027$) (Figure 2D). There was a trend for lower TNFα concentrations in MSC-treated mice (9.1 ± 6.9 pg/mg) compared with the VE IV group, but the difference was not statistically significant ($p = 0.188$).

Severity of colitis not improved by MSCs in the regenerative phase of DSS colitis

We next compared the safety and therapeutic efficacy of fresh and cryopreserved IV-administered MSCs in a colitis model with a longer follow-up to simulate the regenerative phase of colitis (Figure 1B). Body weight in the colitis groups started to decline at day 4 of DSS administration as expected and was at its lowest on day 11 (DSS control and fresh-MSC groups) and on day 10 (cryo-MSC group) (Figure 3A). The change in body weight was similar in all colitis groups. Two mice in the DSS control group and one mouse in both the fresh-MSC and cryo-MSC groups were sacrificed due to excess weight loss before day 14 and excluded from all analyses (final n=9 in the fresh-MSC and cryo-MSC groups and n=8 in the DSS control group). The colon lengths were reduced (DSS control group: $p = 0.003$, fresh-MSC group: $p = 0.018$ and cryo-MSC group: $p = 0.014$) and colon weights increased ($p < 0.001$ for all groups) in all colitis groups compared with the healthy controls. There were no statistically significant differences between the colitis groups (Table 2). Macroscopic scores were increased (DSS control group: $p = 0.002$, fresh-MSC group: $p = 0.002$ and cryo-MSC group: $p = 0.005$ for) compared with the healthy controls (Table 2, Figure 3B) in the colons of all colitis groups without any statistical differences between the colitis groups. DSS induced marked crypt atrophy and inflammatory infiltration in all colitis groups (Figure 3B). Similarly, the histopathology scores (crypt atrophy and inflammatory infiltration) were increased in all colitis groups (DSS control group: $p = 0.006$, fresh-MSC group: $p = 0.004$ and cryo-MSC group: $p = 0.001$) compared with the healthy controls. There were no statistically significant differences between the colitis groups (Table 2). The regeneration scores were 2 ± 0.4 (DSS control group), 3.3 ± 0.3 (fresh-MSC group), and 2.4 ± 0.4 (cryo-MSC group) (Table 2). However, the differences in numeric grades were not statistically significant between the colitis groups.

Inflammation markers in mice receiving fresh MSCs in the regenerative phase of DSS colitis indicates no clear therapeutic effect of MSCs

To further study the anti-inflammatory effects of MSCs in DSS colitis, we measured the concentrations of the pro-inflammatory cytokines IL-1β and TNFα in colon tissue homogenates in the regenerative phase of the colitis (Figure 4). Consistent with the results from the acute phase of colitis in the administration route study, IL-1β (Figure 4A) and TNFα (Figure 4B) concentrations were not detectable in samples from healthy controls. DSS increased the concentrations of both IL-1β (453 ± 129 pg/mg, $p = 0.002$) and TNFα (8.5 ± 3.3 pg/mg, $p = 0.006$) compared with the healthy controls. Neither fresh nor cryopreserved MSCs reduced levels of IL-1β (244 ± 95 pg/mg and 389 ± 139 pg/mg, respectively) or TNFα (2.1 ± 1.1 pg/mg and 6.5 ± 1.7 pg/mg, respectively) compared with DSS controls. IL-1β and TNFα levels were significantly increased in the cryo-MSC group ($p = 0.011$ and $p = 0.001$, respectively) but not in the fresh-MSC group ($p = 0.97$ and $p = 0.371$, respectively) compared with healthy controls. We also measured colon *Il-1β* mRNA expression in the mice. DSS increased the expression of *Il-1β* by 254% in the DSS control ($p = 0.026$) and by 325% in the cryo-MSC ($p = 0.034$) groups but not in the fresh-MSC group (47% increase, $p > 0.999$) compared with healthy controls (Figure 4C). The *Il-1β* mRNA levels were not significantly decreased by either MSC treatments compared with DSS controls, and there were no statistically significant differences between fresh-MSC and cryo-MSC groups. We also measured the concentrations of the anti-inflammatory glucocorticoid hormone corticosterone in the incubation supernatants of the colon (Figure 4D). Corticosterone production was similar in all groups and there were no statistically significant differences between the groups. IL-6 was measured in colons, but it was below the detection limit of the assay in majority of samples (data not shown).

MSC treatment reduces intestinal tissue ACE and ACE shedding

To investigate whether MSC treatments modulate the intestinal RAS, we measured the amount of colonic ACE, ACE-ectodomain shedding, and *Angiotensin II receptor, type 1a* (*Agtr1a*) mRNA expression. The amount of ACE in colon tissue homogenates was similar in healthy (18.5 ± 1.3 ng/mg), DSS control (15.7 ± 1.1 ng/mg) and fresh-MSC (15.1 ± 1.2 ng/mg) groups (Figure 5A). Cryo-MSCs decreased the levels of ACE (11.2 ± 0.8 ng/mg) compared with healthy ($p < 0.001$) and DSS control ($p = 0.024$) groups, but the difference between cryo-MSC and fresh-MSC groups was not clear ($p = 0.05$). Consistent with these results, ACE shedding in colon (measured as released ACE protein in intestinal incubation supernatants) was similar in the healthy (4.23 ± 0.47 ng/mg) and DSS control (4.28 ± 0.43 ng/mg) groups (Figure 5B). ACE shedding was lower in the cryo-MSC group (1.63 ± 0.15 ng/mg) compared with DSS controls ($p = 0.002$) and healthy controls ($p < 0.001$). The reduction in ACE shedding by fresh-MSCs (2.88 ± 0.44 ng/mg) compared with healthy controls was not statistically significant ($p = 0.052$). DSS treatment decreased *Agtr1a* expression by 71% compared with healthy controls ($p = 0.018$, Figure 5C). In the fresh-MSC and cryo-MSC groups, *Agtr1a* expression was reduced by 62% and 58%, respectively, from the level of the healthy control group and the decrease was not statistically significant.

Discussion

MSCs have therapeutic potential in the treatment of various inflammatory conditions and in regenerative medicine. However, much uncertainty remains in manufacturing strategies and treatment protocols and even in the therapeutic effect of MSCs[15, 23, 30, 38, 39]. The results of preclinical studies are also conflicting, which might be due to variation in MSC source, culture methods, dosing schemes, and of course due to differences in the animal models used in different studies[40]. In addition, different administration routes might alter the biodistribution of MSCs and potentially their therapeutic efficacy[38, 39, 41]. The use of cryopreserved or fresh MSCs is also a subject of dispute. There are only a few studies comparing fresh and cryopreserved MSCs in *in vivo* animal models[33-35, 42] and to the best of our knowledge there are no comparisons between different MSC preparations in animal colitis models. Therefore, the aim of our current study was to investigate the safety profile and feasibility of a MSC product already in use for refractory graft-versus-host-disease[36], and to compare the inflammation-alleviating efficacy of cryopreserved and fresh MSCs administered either IV or IP in the DSS-colitis model. We specifically wanted to utilize unlicensed MSCs, since all clinical trials thus far have utilized only unlicensed, native MSCs.

Unstimulated murine MSCs have alleviated DSS-induced colitis in mice and rats when using large doses ranging from 1 x 10^6 to 5 x 10^6 cells per injection[13, 14, 39, 43]. However, unstimulated human MSCs have not been effective in xenogeneic colitis models, but promising therapeutic effects have been demonstrated with licensed MSCs in xenotransplantation models [15, 16]. Specifically, IL-1β- and IFNγ-stimulated human MSCs have reduced intestinal damage in DSS and trinitrobenzene sulfonic acid (TNBS) colitis models[15, 16]. In addition, in a study utilizing a radiation-induced intestinal injury model, IL-1β, TNFα, and nitric oxide were shown to induce secretion of anti-inflammatory mediators from MSCs as demonstrated by better survival and lesser degree of mucosal damage in MSC-conditioned medium-treated rats[29]. In our present study, two doses of 0.5 x 10^6 unlicensed human MSCs did not improve histopathology, body weight development, or macroscopic scores. It is possible that licensing or a higher cell amount is required to elicit the full therapeutic effect of human MSCs in a xenotransplantation model. However, licensing with cytokines increases the level of cell manipulation and could pose an additional risk in clinical applications. To our knowledge all published clinical results thus far, both with positive and negative outcome, have been received utilizing unlicensed MSCs. It is pivotal to investigate human cell products in animal models in order to develop human therapeutics, but the therapeutic outcome in animal xenotransplantation models should not be generalized to human diseases without reservations, especially in inflammatory models since it is possible that murine inflammatory cytokines simply has little or no reactivity toward human MSCs and therefore MSCs are not induced to become suppressive. The suitability of the DSS model in studying the efficacy and mechanism of action of MSCs with regards to human IBD has been criticized as the inflammation in DSS colitis is mediated mainly by innate immunity and not T-cells, which are important in IBD pathogenesis[40]. On the other hand, MSCs do have the ability to polarize macrophages (which are abundant in the colonic inflammatory infiltrates in DSS colitis) to anti-inflammatory M2 macrophages, which promote tissue repair and wound healing and ultimately T-cell polarization[7, 44]. Nonetheless, results from clinical studies in IBD using uninduced human bone-marrow derived MSCs indicate that they can be effective in treating IBD, as demonstrated by studies employing recurring systemic infusions[28] or local administration in fistulas[21, 23, 28].

MSCs have been administered in patients either by local injections (e.g. in fistulas) or IV by systemic infusions[23-28]. Goncalves et al.[38] reported that IV-administered MSCs had stronger anti-inflammatory effects than IP-injected MSCs, whereas in other studies IP-administered MSCs were more effective than IV administered MSCs[39, 41]. In our study, we found no statistically significant differences between the two administration routes. Nevertheless, IV administration appeared better with regards to colon weight in relation to body weight, which was lower in the MSC IV group (indicating less colonic edema). On the basis of several clinical studies, the safety profile of MSCs is deemed to be good[45, 46]. However, it is well established that IV-administered MSCs are prominently retained in the lung during the first pass before clearance to the circulation[41, 47, 48]. In our study, we report that both IV and IP MSC administration appear safe as no obvious adverse effects (e.g. infections or emboli) were observed at any stage of the study. As the IV administration route is clinically more feasible, we chose to continue the study using IV injections.

There are reports implying that cryopreservation impairs the immunosuppressive effects of MSCs *in vitro*[31, 32], but, cryopreserved MSCs have been explored in several clinical studies for graft-versus-host disease by us and others with partially encouraging results[36, 49-51]. While presenting a good safety profile, neither fresh nor cryopreserved MSCs improved the colitis in the regenerative phase as measured by weight change, and macroscopic or histopathology scores or by colonic pro-inflammatory cytokine and corticosterone levels. While MSCs prevented the DSS-induced upregulation of the pro-inflammatory cytokine IL-1β in the acute phase of colitis, indicating a mild anti-inflammatory effect of the MSCs, the reduction was diminished in the regenerative phase. The possible therapeutic differences between fresh and cryopreserved MSCs will remain unsolved in this study, since the overall minimal therapeutic effect might be confounding the comparison. It is also noteworthy that the loss and subsequent data exclusion of the animals with the most severe colitis, especially in the DSS control group (2 in DSS control group and 1 in each MSC group), might obscure the differences in the data set.

Recent findings in experimental models suggest that systemic and local RAS are involved in regulation of intestinal inflammation[52-56]. RAS is a critical regulator of blood pressure, but its components are also found throughout the intestine. The key enzyme of RAS, angiotensin converting-enzyme (ACE), cleaves angiotensin I to angiotensin II and it is prominently expressed in various cell types in the intestine[56]. In experimental models, activation of RAS promotes colitis[53] while RAS inhibition is protective against colitis[54, 55, 57-65]. Clinical studies have shown that angiotensin I and angiotensin II are elevated in the intestinal mucosa of

Crohn's disease patients with active inflammation[66] and that susceptibility to Crohn's disease is linked to polymorphisms in the ACE gene[67]. Despite accumulating evidence showing the beneficial effects of RAS modulation in experimental colitis, the functions of intestinal RAS remain incompletely understood in human health and disease.

In this study, we investigated how MSCs interact with intestinal RAS and we show that tissue ACE and ACE-ectodomain shedding in the colon are downregulated by cryopreserved MSCs. Based on our previous studies, ACE shedding is enhanced by inflammation in certain parts of the intestine eg. jejunum and mid to distal colon[68, 69]and unpublished observations]. In proximal colon, ACE shedding was not modulated by colitis itself but could be reduced by ACE-inhibiting agents[69]. ACE has been suggested to be secreted by intestinal crypt cells or cleaved by a specific sheddase (ADAM9)[70]. The specific purpose of ACE shedding in the intestine is not known, but since ACE is considered pro-inflammatory, it is possible that the reduction of ACE levels by MSCs might be a beneficial response to reduce signaling via the pro-inflammatory and pro-fibrotic ACE-Ang II-AGT1Ra axis. *Agtr1a* expression is induced in inflammatory and infectious conditions in the vasculature[71-73] and in the gastric mucosa[74]. Downregulation of the pro-inflammatory AGTR1a during inflammation and tissue healing might indicate a negative-feedback response to increased pro-inflammatory angiotensin II levels. We measured *Agtr1a* expression during the tissue regeneration process after the initial DSS insult had passed and found that *Agtr1a* expression was significantly downregulated in colons of DSS animals. There was a small but not statistically significant trend towards an increase in *Agtr1a* expression in the MSC-treated animals. Interestingly, in studies of experimental renal hypertension, MSCs normalized the upregulation of ACE and AGT1R protein and mRNA expression in damaged kidneys[75, 76]. Nevertheless, this study demonstrates that MSCs regulate intestinal RAS. Whether the anti-inflammatory properties of MSCs are partly facilitated via target tissue RAS should be elucidated in further studies.

Conclusions

We evaluated fresh and cryopreserved unlicensed platelet-lysate expanded human BM-derived MSCs in a preclinical mouse model during both the acute and the regenerative phases of DSS colitis. Macroscopic, microscopic and molecular parameters revealed no adverse effects of the MSCs, further strengthening the safety profile of systemically administered MSCs. In this xenotransplantation model, the therapeutic effect of unlicensed human MSCs, i.e. such as they are utilized in GvHD, was modest and limited to improvements in the levels of pro-inflammatory cytokines. Colonic IL-1β levels were reduced by MSCs during acute inflammation, but a beneficial effect was not as evident in the regenerative phase of DSS colitis. Taken together, this might indicate that the full anti-inflammatory capacity of MSCs is obscured in a severe colitis xenotransplantation model mediated mainly by the innate immune system, but results from a xenotransplantation model should not be extrapolated to efficacy in treatment of human IBD. Furthermore, we conclude that MSCs regulate intestinal RAS by reducing pro-inflammatory ACE protein expression and ectodomain shedding in the colon, which might implicate a novel beneficial mechanism of immunomodulation by MSCs. Additional studies using unlicensed versus licensed MSCs in the treatment of IBD are needed to verify the optimal manufacturing strategies for the best therapeutic effect of MSCs.

List of abbreviations

ACE: Angiotensin-converting enzyme **BM:** Bone marrow **DSS:** Dextran sodium sulfate **H&E:** Hematoxylin and eosin **GvHD:** Graft-versus-host disease **IBD:** Inflammatory bowel disease **IP:** Intraperitoneally **IV:** Intravenously **MSC:** Mesenchymal stromal cell **Cryo-MSC:** Cryopreserved MSCs **Fresh-MSC:** Freshly cultivated MSCs **RAS:** Renin-angiotensin system

Declarations

Ethics approval

The animal experiments were approved by the national Animal Experiment Board in Finland (ESAVI/6314/04.10.03/2012 and ESAVI/114/04.10.07/2015) according to the Finnish Act on Animal Experimentation (62/2006).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was partially funded by the Finnish Funding Agency for Technology and Innovation (Tekes) to the Salwe GID (Personalized Diagnostics and Care) program (Grant No. 3982/31/2013), Juhani Aho Medical Research Foundation, Finland, Finska Läkaresällskapet, Einar och Karin Stroems Stiftelse, Finland.

Authors' contributions

HS, AL, RK and JN designed the study. HS, AL, RF, MH, JL and LP performed the experiments and acquired data. HS, AL, RF, MH, JL and JN analyzed the data. HS, AL, RK and JN wrote the paper. MK, AL and JN provided study materials. Study supervision and administration by JN. All authors read and approved the final manuscript.

Acknowledgments

We are deeply thankful to Prof. emer. Heikki Vapaatalo for the helpful discussions and support during this project and we sincerely thank the technical personnel at the FRCBS Advanced Cell Therapy Centre for expert technical assistance. We are grateful to Niina Siiskonen at the Tissue preparation and histochemistry unit, Department of Anatomy, Faculty of Medicine, University of Helsinki for preparation of the H&E-stained tissue slides. We thank Prof. emer. Lauri Tarkkonen and Mikko Arvas for helpful discussions on statistical analyses.

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Figure legends

Figure 1. Study design presented as schematic diagrams of the two experiments in this article. A) Schematic of the study comparing the intravenous and intraperitoneal administration routes. B) Schematic of the study comparing fresh and cryopreserved MSCs in the regenerative phase of the colitis.

Figure 2. Body weight, colon weight and pro-inflammatory cytokine levels in the colon during the acute phase of colitis. A) Weight change of the animals from the beginning of DSS administration. B) Colon weight normalized to body weight of the animals. C) IL-1β and D) TNFα protein levels in the colons of healthy, DSS + VE IV and DSS + MSC IV mice.

Figure 3. Weight development and histopathology in the study comparing fresh and cryopreserved MSCs. A) Weight of the animals in colitis groups developed similarly. There were no statistically significant differences between colitis groups. The arrows indicate the days of MSC or vehicle injection. B) Histopathology in colon during the regenerative phase of colitis. H&E-stained sections of healthy control (top left), DSS control (top right), fresh-MSC (bottom left), and cryo-MSC (bottom right) group colons after 6 days of DSS administration and a 7-day recovery period. All DSS groups displayed inflammatory activity, mucosal atrophy, and crypt regeneration in H&E-stained tissue slides. Differences between colitis groups were minor and no statistically significant differences between colitis groups were observed. 20x objective magnifications.

Figure 4. Markers of inflammation in the colons of mice in the regenerative phase of colitis. A) IL-1β, B) TNFα and C) *Il-1β* mRNA expression were increased in DSS control and cryo-MSC groups but not in the fresh-MSC group compared with healthy controls. D) Corticosterone secreted by the colon during a 90-min *ex vivo* incubation.

Figure 5. ACE protein expression and shedding and *Atgr1a* mRNA expression in the colons in the regenerative phase of colitis. A) Tissue ACE, B) ACE shedding, measured as the release of ACE protein into the incubation fluid C), and *Atgr1a* mRNA expression in the colon.

Supplement Figure 1 (S1). MSC characterization results. The MSCs used in the studies were characterized for (A) MSC phenotype, (B) ostgeogenic and adipogenic differentiation and (C) T-cell immunosuppression capacity *in vitro*. Figure A: filled histogram = specific labeling of the cells; empty histogram = negative control. Figure C: filled histogram = MSCs + T-cells; empty histogram = T-cells only (proliferation control).

Table 1. Macroscopic scores and colon lengths in the acute phase of the colitis. (To be placed after the paragraph "*Macroscopic signs of inflammation were not improved by MSCs in acute phase of DSS colitis*")

Group	Macroscopic Score	Colon length (cm)
Healthy control	0.0 ± 0.0	7.2 ± 0.3
3 % DSS + VE IV	2.8 ± 0.4	5.5 ± 0.2***
3 % DSS + MSC IV	3.3 ± 0.4*	5.3 ± 0.1***
3 % DSS + VE IP	3.9 ± 0.6**	4.9 ± 0.2***
3 % DSS + MSC IP	3.8 ± 0.7**	4.9 ± 0.2***

Macroscopic scores (scale 0-6) including stool consistency, edema and occult blood, and colon length of the mice after 7 days of DSS administration. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ compared with healthy controls. MSC = fresh mesenchymal stromal cells, VE = vehicle, IV = intravenously administered, IP = intraperitoneally administered.

Table 2. Macroscopic and histopathologic changes in the regenerative phase of colitis. (To be placed after the paragraph "*Severity of colitis not improved by MSCs in the regenerative phase of DSS colitis*")

Group	Macroscopic Score	Histopathology Score	Regeneration	Colon length (cm)	Colon/Animal weight (mg/g)
Healthy	0.0 ± 0.0	0.0 ± 0.0	-	8.1 ± 0.3	6.0 ± 0.6
DSS Control	2.1 ± 0.2**	5.2 ± 0.9**	2.0 ± 0.4	6.1 ± 0.3**	10.2 ± 1.3***
Fresh-MSC	2.0 ± 0.3**	5.2 ± 0.6**	3.3 ± 0.3	6.5 ± 0.3*	9.4 ± 0.6***
Cryo-MSC	1.8 ± 0.4**	5.8 ± 1.1**	2.4 ± 0.4	6.5 ± 0.5*	9.6 ± 1.6***

The macroscopic scores (stool consistency, edema and occult blood, scale 0-6), histopathology scores (inflammation and mucosal atrophy, scale 0-10), regeneration scores, colon length and colon weight normalized to bodyweight during the regenerative phase of colitis. Regeneration scores were similar between the groups. Colitis groups differed from healthy controls in all other parameters, but there were no statistically significant differences between the colitis groups. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared with healthy controls. DSS = dextran sodium sulfate, Fresh-MSC = fresh mesenchymal stromal cells, Cryo-MSC = cryopreserved MSCs.