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CHAPTER 3

Autologous bone marrow derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: results of a phase I study

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ABSTRACT

Background and aim

Mesenchymal stromal cells (MSCs) are pluripotent cells that have immunosuppressive effects both in vitro and in experimental colitis. Promising results of MSC therapy have been obtained in patients with severe graft versus host disease of the gut. Our objective was to determine the safety and feasibility of autologous bone marrow derived MSC therapy in patients with refractory Crohn's disease (CD).

Patients and intervention

Ten adult patients with refractory CD (8 females/2 males) underwent bone marrow aspiration under local anesthesia. Bone marrow MSCs were isolated and expanded ex vivo. MSCs were tested for phenotype and functionality in vitro. Nine patients received 2 doses of 1-2x10^6 cells/kg bodyweight, intravenously, 7 days apart. During follow up, possible side effects and changes in patients' Crohn's disease activity index (CDAI) scores were monitored. Colonoscopies were performed at week 0 and 6, and mucosal inflammation was assessed by using the Crohn's disease endoscopic index of severity (CDEIS).

Results

MSCs isolated from CD patients showed similar morphology, phenotype, and growth potential compared to MSCs from healthy donors. Importantly, immunomodulatory capacity was intact, as CD MSCs significantly reduced peripheral blood mononuclear cell proliferation in vitro. MSC infusion was without side effects, besides a mild allergic reaction probably due to the cryopreservant DMSO in one patient. Baseline median CDAI was 326 (range 224-378). Three patients showed clinical response (CDAI decrease ≥70 from baseline) 6 weeks post treatment, conversely three patients required surgery due to disease worsening.

Conclusions

Administration of autologous bone marrow derived MSCs appears safe and feasible in the treatment of refractory CD. No serious adverse events were detected during bone marrow harvesting and administration.

INTRODUCTION

Inflammatory bowel diseases (IBD) are chronic inflammatory disorders of the gastro-intestinal tract, including Crohn's disease (CD) and ulcerative colitis. Despite the improvements in IBD management with the introduction of anti-TNF compounds, remission often remains difficult to maintain. Many patients suffer from a poor quality of life due to disease relapse, repeated surgeries, extra intestinal manifestations and drug side effects. Therefore, novel therapeutic approaches need to be explored.

Mesenchymal stromal cells (MSCs) are nonhematopoietic stromal cells exhibiting multi-lineage differentiation capacity and the ability to mediate immunosuppressive and anti-inflammatory effects.[1-3] MSCs are easily isolated from various tissues[4-6], including the bone marrow, and are capable of ex vivo expansion. Moreover, MSCs can be cryopreserved without loss of phenotype or differentiation potential.[7] Systemic infusion of MSCs ameliorated the clinical and histopathologic severity of experimental colitis, abrogating body weight loss, diarrhea, and inflammation and increasing survival. [8, 9] Moreover, in humans, transplantation of bone marrow (bm) derived MSCs has led to improvement of corticosteroid refractory graft-versus-host disease (GvHD), including GvHD of the gut[10, 11] and MSCs obtained from adipose tissue induced healing in complex perianal fistulas in patients with CD.[12] Although the mechanisms underlying these effects are not fully elucidated, it has been shown that both cell-cell contact and the secretion of growth factors and cytokines are involved.[13, 14] The potential role of MSCs in the modulation of immune responses and tissue regeneration aroused interest to use MSCs as a novel cellular therapy to treat CD.[15]

In this clinical phase I study we evaluated the safety and feasibility of intravenous infusion of autologous bmMSCs in refractory CD patients. Additionally the functionality of these MSCs was studied focusing on culture potential, morphology, cell surface marker profiling, differentiation potential and immunosuppressive properties. Finally, the effect of bmMSCs on various drugs used to treat CD was assessed.

MATERIAL AND METHODS

Patient selection

On January 14th 2008, the Medical Ethical Committee of the Leiden University Medical Center (LUMC) and the Central Committee on Research involving Human Subject (CCMO, The Hague, the Netherlands) approved this phase I study on autologous bmMSCs in the treatment of refractory CD (registered in the Netherlands National Trial Register under study number NTR1360 *www.trialregister.nl*). All patients gave written informed consent. Criteria for patient inclusion were that patients were at least 18 years of age and had moderate to severe CD, as defined by a baseline Crohn's disease activity index (CDAI) score between 220 and 450. Furthermore, patients had to be refractory to the standard treatment options for CD. We defined refractory patients as patients that, at some time during the course of the disease, must have received steroids, immunosuppressive agents (for example, azathioprine, 6-mercaptopurine or methotrexate) or anti-TNF therapy which did not result in an adequate

response to treatment. The following medications were allowed: 5-aminosalicylates and corticosteroids (at a stable dosage regimen for at least four weeks) and methotrexate, azathioprine, or 6-mercaptopurine (at least twelve weeks, with stable dosage regimen for at least eight weeks). Infliximab was discontinued at least eight weeks prior to enrolment. All patients continued current treatment at the time of infusion. Before bone marrow harvest for MSC isolation and expansion, patients were thoroughly screened including medical history, physical examination, standard laboratory investigations and chest x-ray to rule out tuberculosis. Each patient was also screened for human immunodeficiency virus (HIV), syphilis, and hepatitis B and C virus. Patients were excluded if they had a history of lymphoproliferative disease or malignancy within the past five years, when they exhibited serious infections or when in need of immediate surgery. Colonoscopy was performed at baseline to confirm disease activity. Laboratory methods for clinical expansion of MSCs

MSC isolation and expansion

MSCs were expanded according to a common protocol devised by the European Group for Blood and Bone Marrow Transplantation (EBMT) developmental committee, as previously described.[16] Bone marrow was harvested by aspiration from the iliac crest from patients under local anesthesia in the outpatient clinic. Bone marrow mononuclear cells (MNC) 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 bovine serum (FBS, HyClone, Logan, UT) without any additional growth factors. MNCs 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. MSCs were passaged up to a maximum of three times. When sufficient MSCs were expanded, cells were harvested and cryopreserved in isotonic buffered salt solution supplemented with 10% dimethyl sulphoxide (DMSO, LUMC Pharmacy, the Netherlands). Data on MSCs obtained from healthy donors matched for age and gender were obtained from previous studies.[17-19] MSCs for these studies were sourced either from a family or non-related (third party) donor. Donors were informed about and consented to the possibility of the use of their MSCs for preclinical studies/analysis. All donors underwent routine donor control examination and screening tests, according to the standard procedures required for bone marrow donors. Following eligibility, donors donated 50-100ml of bone marrow under local anesthesia as described above.

Characterization of MSC products

Morphology was monitored twice a week throughout the culture period by light microscopy. *Cell viability* was determined at each passage and harvest procedure by trypan blue staining in a Bürker chamber. *Immunophenotyping* of cultured MSCs was performed using flow cytometry. The following markers were analyzed: HLA II (DR), CD73,

CD90, CD31, CD34, CD45, CD80 (Becton Dickinson, Franklin Lakes, NJ, USA), and CD105 (Ancell, Bayport, MN, USA). The samples were analyzed on a FACSCalibur™ using CellQuest Pro software (Becton Dickinson). *Absence of contamination by pathogens* was tested at culture initiation and harvest of the MSC product by aerobic and anaerobic cultures (Becton Dickinson, Bactec plus aerobe/F and Bactec plus anaerobe/F). *Genetic stability* of the expanded MSCs was tested by karyotype analysis using a standard G-banding procedure.

Clinical application of MSC products

Release criteria for clinical use of MSCs included product sterility, absence of visible cell clumps, spindle-shape morphology, expression of CD73, CD90, and CD105 surface molecules (>90%) and a normal karyotype in at least 20 observed metaphases.

Laboratory methods for supportive research

In vitro differentiation

MSCs were plated at $5x10^4$ cells/cm^2 in 24-well culture plates and kept in complete medium until 80-90% confluency was reached. For osteogenic differentiation cells were stimulated for 21 days 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. For adipogenic differentiation, cultures were stimulated for 21 days with complete medium supplemented with 0.5 mM 3-isobutyl-1-methylxanthine, 100 uM indomethacin, 5 ug/ml insulin and 10^{-6} M dexamethasone. Lipid droplets were revealed by staining with Oil Red O. Control MSCs were grown in non-conditioned medium. All chemicals were from Sigma-Aldrich.

MSC/peripheral blood mononuclear cell (PBMC) proliferation assay

Cultured MSCs from CD patients were plated in flat bottom 96 well plates (Costar) and allowed to attach overnight. PBMCs were isolated from whole blood of CD patients before MSC infusion. PBMCs were stimulated with anti-CD28/anti-CD3 coated Dynabeads (1 bead/5 cells, Invitrogen) per $1x10^6$ cells and were seeded in Iscove's Modified Dulbecco's Media (IMDM) with 5% human serum (Sanquin, the Netherlands), 5% FBS and 100 IU/mL IL-2 (LUMC Pharmacy, Leiden, the Netherlands) per well. Proliferation was measured by ^{3}H-thymidine incorporation.

Cell bead array cytometric assay.

Production of TNF-α, IL-1b, IL-10, and IL-6 in MSC/PBMC supernatants, colon biopsy homogenates and serum was determined using a cytometric bead array kit according to the manufacturer's instructions (BD Biosciences).

Study design

Patients received two doses of MSCs, seven days apart at week 0 and 1. Just before clinical application, cryopreserved cells were thawed and cells were infused intravenously at a target dose of $1\text{-}2x10^6$ cells/kg bodyweight. Patients were clinically assessed at weeks 0, 1, 2, 4, 6, and 14. At each visit, adverse events were ascertained, concomitant medications

were recorded and samples for clinical laboratory evaluations and the patients' CDAI score were obtained. Colonoscopies were performed at week 0 and 6, and mucosal inflammation was assessed using the Crohn's disease endoscopic index of severity (CDEIS). The study flow chart is depicted in figure 1.

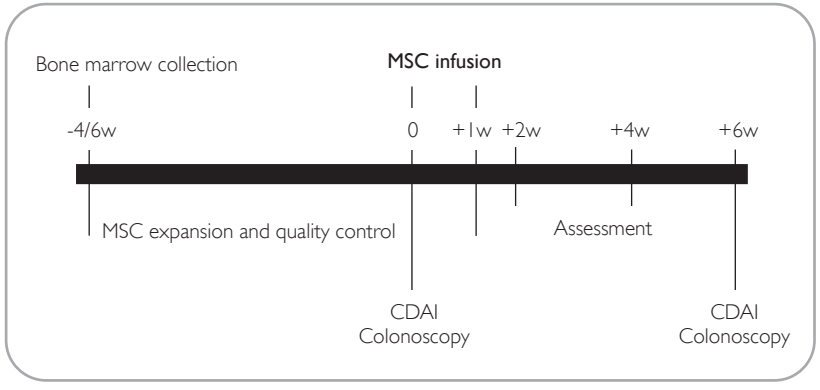

Figure 1. Study flow chart.

Statistical analysis

Data were analyzed using SPSS (version 16.0, SPSS Inc., Chicago, IL) or GraphPad (GraphPad software Inc., La Jolla, CA). Analyses included the Kruskall-Wallis test followed by Dunn's multiple comparisons, two-sided t-test and Wilcoxon signed-rank test for paired data. *P*-values <0.05 were considered significant.

RESULTS

Patients

In total ten patients (eight females/two males, median age 32.5 and range 19-42 years) with moderate to severe CD (median CDAI score at screening of 299.5 and range 255-442) were included in the study and underwent bone marrow aspiration under local anesthesia. Besides some local pain at the puncture site afterwards, bone marrow aspiration was well tolerated by all patients. Baseline characteristics are presented in table 1. The bone marrow aspiration procedure resulted in sufficient bone marrow to expand MSC up to the required therapeutic doses (Table 2). One patient (patient 9), with a CDAI score of 255 at screening, showed no active disease on colonoscopy and was therefore excluded for further MSC administration. The baseline median CDAI score of the remaining nine treated patients was 326 (range 224-378). During MSC infusion, patients were closely monitored. MSC infusion was successful and without relevant side effects. In one patient a transient mild allergic reaction occurred which was probably due to the cryopreservant DMSO. Moreover, all patients noticed the typical smell and taste due to the DMSO up to 48 hours after infusion. Other adverse events in the first 6 weeks of the protocol, such as common cold and headache (Table 3), were ruled unlikely to be associated with MSC treatment.

bmMSC from refractory CD patients are comparable to MSCs from healthy donors

Approximately hundred milliliters of bone marrow was aspirated from each patient and bmMSCs were isolated and cultured. MSCs from CD

Patient number	1	2	3	4	5	6	7	8	9	10
Age (y)	24	36	33	34	34	42	29	32	19	30
Sex	F	F	F	F	F	M	M	F	F	F
Disease duration (y)	10	5	15	3	11	4	7	12	3	10
CDAI at screening	442	346	283	316	337	237	316	280	255	277
Baseline CDAI	332	341	254	326	350	266	378	224	NA	304
Disease localization	ileocolonic	colonic, ileum ND	colonic, ileum ND	ileum	ileum	colonic	colonic, ileum ND	colonic, ileum ND	no active disease	colonic
Perianal disease	yes	no	yes, perianal abscess (inactive)	no	yes (inactive)	no	yes (inactive)	no	no	yes (inactive)
Extra-intestinal manifestations	arthralgias	arthralgias	no	arthralgias	no	no	cheilitis granulomatosa	no	no	no
Current medical therapies	CS 50mg, AZA	MTX, ADA	CS 10mg	CS 5mg	CS 40mg	MTX	CS 30mg, 6-MP	CS 5mg, MTX	NA	CS 5mg, MTX
Height (cm)	175	167	174	172	158	160	187.5	173	157	161
Weight (kg)	59.3	68.6	74	99.9	46	78	107.5	113.2	50.5	53.1
Current smoker / Smoking history	no / no	no / no	no / no	no / yes	no / no	no / yes	yes / yes	no / no	no / yes	no / yes
Prior medical therapies	CS, AZA, IFX, MTX, CZP, ADA	5-ASA, CS, AZA, IFX, MTX, ADA	5-ASA, CS, ATB, AZA, IFX, MTX, CZP, ADA	5-ASA, CS, 6MP, IFX, MTX, CZP, ADA	5-ASA, CS, AZA, IFX, MTX, CZP, ADA, TAC	CS, AZA, IFX, MTX, ADA	5-ASA, CS, AZA, IFX, CZP, ADA	5-ASA, CS, AZA, IFX, MTX, ADA	5-ASA, CS, ATB, AZA, IFX, MTX, CZP, ADA	5-ASA, CS, AZA, IFX, MTX, CZP, ADA, HSCT
Prior surgeries	ileocoecal resection	no	ileocoecal resection	ileocoecal resection	ileocoecal resection, colostoma on colon transversum	no	no	no	no	no

Table 1. Baseline characteristics of included patients. Abbreviations: F female, M male, NA not applicable, ND not determined, 5-ASA mesalamine, CS corticosteroids, AZA azathioprine, 6-MP 6-mercaptopurine, ATB antibiotics, MTX methotrexate, IFX infliximab, CZP certoluzimab pegol, ADA adalimumab, TAC tacrolimus, HSCT mobilisation phase of hematopoietic stem cell transplantation, y years.

Patient number	ml bone marrow collected	Days of culture	Passage number	Number of cells (x106)/kg/infusion	Total number of cells infused
1	120	15	1	1.9	220
2	97	24	2	1.9	260
3	106	24	2	2	300
4	114	16	1	2	400
5	106	24	2	1.6	150
6	111	31	3	0.9	146
7	96	23	2	1.1	240
8	109	34	3	1.5	346
9	100	16	1	NA	NA
10	102	27	2	2.1	220

Table 2. Overview of bone marrow collection, time needed for MSC culture, and final MSC product infused. NA not applicable.

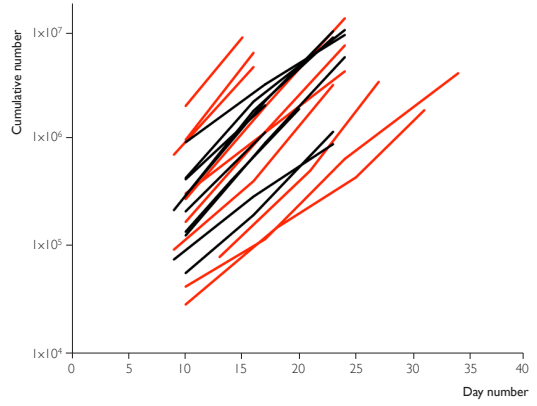

Figure 2. MSC expansion of CD patients (red) and healthy volunteers (black) expressed as theoretical cumulative cell number per ml harvested bone marrow.

Likely related to MSC infusion	n	Patient number
Allergic reaction	1	4
Typical taste and smell	9	all
Headache	3	1,4,7
Unlikely related		
Worsening CD*	2	1.7
Dizziness	1	1
Nausea	2	1.2
Vomiting	1	1
Bloating	1	3
Abdominal pain	3	3,5,8
Hemorrhoid	1	4
Fever	1	4
Lack of appetite	2	1.4
Fatigue	2	5.8
Diarrhea	1	8
Common cold	1	10
Otitis media acuta	1	2

Table 3. Adverse events in MSC treated patients (week 0-6). Two serious adverse events (*) were reported due to worsening of disease requiring hospitalization.

Patient number	1	2	3	4	5	6	7	8	9	10	Average
HLA-DR	5.1	5.7	7.9	5.7	1	5.4	0.8	0.9	5	5.8	4.3
CD31	1.9	2.1	1.7	2.1	1.7	1.1	2.2	1.6	2	2	1.8
CD73	99.9	99.7	99.9	99.6	99.3	99.7	99.9	99.2	90.4	98.1	98.6
CD45	1.3	1.6	1.9	2.1	3.6	1.3	0.9	1.2	1.4	1.3	1.7
CD105	100	99.9	100	100	100	100	100	99.9	99.9	100	100
CD80	3.1	6.2	10.3	18	4.2	3.3	1	0.2	0.3	29.4	7.6
CD90	100	100	99.9	99.9	99.3	99.6	99.8	100	99.8	99.9	99.8
CD34	6	4	4.6	9.5	2.8	5.6	6.5	0.8	10.1	4.3	5.4
Healthy donor number	1	2	3	4	5	6	7	8	9	10	Average
HLA-DR	10.5	3.7	0.6	4.2	12.8	10.1	1.4	9.1	5.3	3.1	6.1
CD31	2.6	1.7	2.5	1.7	2.8	2.9	1.7	3.1	0.4	1.8	2.1
CD73	99.4	97.4	99.7	99.2	99.7	96.7	94	96.4	99.4	99.4	98.1
CD45	2.6	0.7	1.4	1.9	1.2	0.6	1.8	1.2	0.6	1.9	1.4
CD105	99.9	99.9	99.8	100	100	99.9	99.9	100	100	99.9	99.9
CD80	1.5	6.6	3.6	6.2	2.2	19.6	19.8	2.2	2.3	3.8	6.8
CD90	99.9	98.9	99.9	99.8	99.9	99.9	99	99.8	100	99.9	99.7
CD34	4.2	1.7	2.9	4	7.1	19.2	6.6	16.5	5.1	4	7.1

Table 4. Flow cytometric analysis (%) of cultured bmMSCs from CD patients and healthy donors matched for gender and age (mean/median age patients 31.3/32.5 and healthy donors 32.1/32.5).

patients showed the typical spindle-shaped morphology and similar growth potential and yield compared to MSCs from healthy donors (Figure 2). Depending on the number of cells needed and the yield of cells, MSCs were harvested in the first, second or third passage (Table 2). All MSC cultures showed normal karyotyping. Immunophenotypical characterization was performed by FACS analysis and showed similar phenotyping as described for healthy controls (Table 4). Furthermore, CD MSCs were able to differentiate along the osteogenic and adipogenic lineages when cultured in appropriate culture medium (Figure 3).

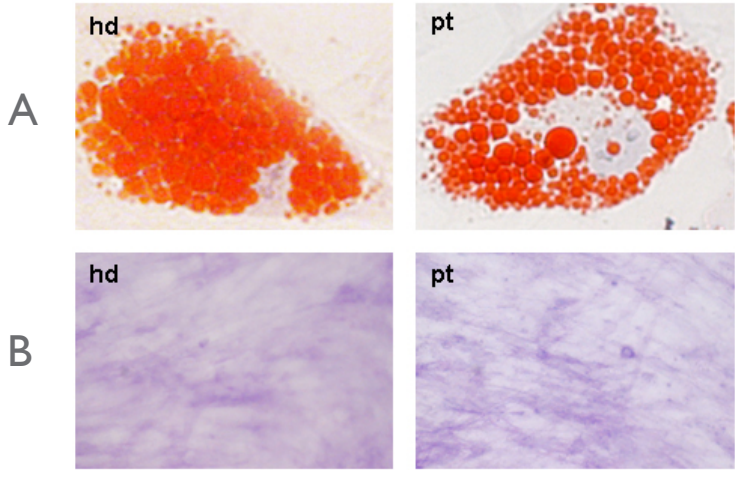

Figure 3. CD MSCs differentiate into mesenchymal lineages. (A) Adipocyte differentiation was demonstrated in MSCs cultured from a healthy donor (hd) and from a CD patient (pt) after which cells were stained with Oil Red O to show lipid droplets in the cytoplasm of the cell. (B) For osteoblast differentation MSCs were stained with Fast Blue to show alkaline phosphatase activity. Non-conditioned MSCs did not stain for Oil Red O. whereas Fast Blue gave slight background staining (not shown).

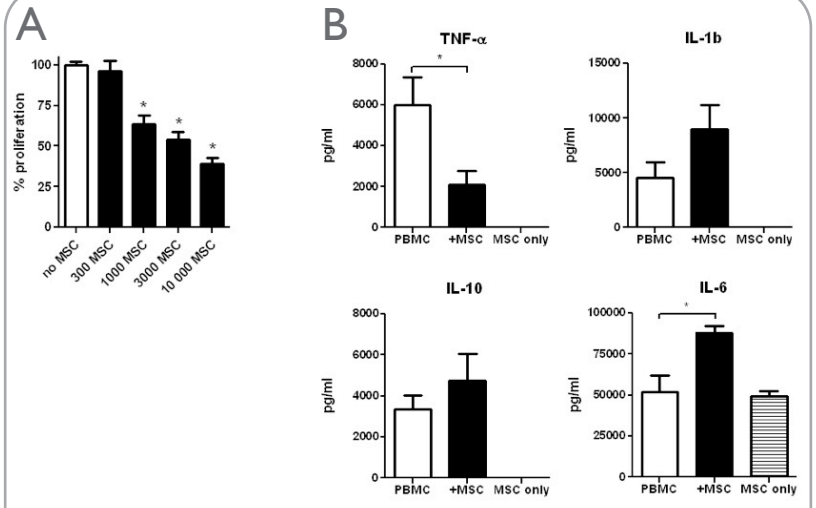

Figure 4. MSCs significantly inhibit the proliferation of PBMCs and this inhibition is dose-dependent. (A) 100 000 PBMCs cells were stimulated with anti-CD3/CD28 beads in the absence (white column) or presence (black columns) of indicated numbers of autologous MSCs. Proliferation measured by ^{3}H-thymidine uptake (counts/minute) was expressed as a percentage of PBMCs proliferation without MSCs for each individual patient. (B) Cytokine production in the supernatants of PBMC cultures and 10 000MSC/100 000 PBMC cocultures. Bars represent the mean and SEM of data from 10 patients in triplo. *P< 0.05 for significant differences

bmMSCs from CD patients suppress immune responses in vitro

In the presence of autologous bmMSCs proliferation of PBMCs was reduced in a cell dose-dependent fashion (Figure 4A) and a decreased TNF-α production was observed. An increase of IL-1b, IL-6 was seen, as well as an increase in the regulatory cytokine IL-10 (Figure 4B).

Clinical response

Clinical assessment was performed on all patients using CDAI scoring. Two patients were excluded before the primary endpoint was met. The first patient (patient 1) was a chronic severe steroid refractory patient on the waiting list for surgery. Although an initial drop of CDAI score was seen, patient was excluded when presented with a CDAI >450 due to poor

Patient number		1	2	3	4	5	6	7	8	10
wk 0*	First infusion	332	341	254	326	350	266	378	224	304
wk 1	Second infusion	305	281	182	318	306	247	#452	167	ND
wk 6	Primary endpoint	#473	185	179	267	314	160	ND	340	354
Surgical resection										
	In week number	7	NA	NA	NA	12	NA	NA	14	NA

Table 5. Clinical scores of patients at week 0, 1, and 6. In the 6 month follow up period, three patients underwent surgery in indicated week after MSC infusion. *baseline, #withdrawn from study. NA not applicable, ND not determined.

general condition and persistent rectal blood loss. Patient 7 was withdrawn from the study four days after the first MSC infusion because of continuing abdominal aches and bloody diarrhea. In this case infliximab treatment was resumed. CDAI scores improved in five patients, clinical response (defined as a drop in CDAI>70) was seen in three patients at week 6 (Table 5).

Remission (CDAI <150) was not achieved in any of the patients. Three patients had a reduction of 70 points in CDAI score, this decrease could in most cases be ascribed to solid stools and a decrease in soft stool frequency. In a period of 14 weeks, three patients required surgery due to disease worsening (Table 3). No significant differences in C-reactive protein (CRP) levels were seen.

Endoscopy

Endoscopic improvement, observed by a drop in CDEIS of 10.0 and 24.7 points, was seen in two patients with extensive CD localized in the colon (Figure 5). In the other five patients no significant endoscopic improvement was seen between baseline and six weeks post-infusion. At week 0 and 6, levels of CD4+, CD8+ and CD4+CD127+ populations were determined in biopsies of inflamed mucosa (Figure 6A, left panels). Lower CD4+ T-cells and higher CD4+CD127+ regulatory T-cells were observed at week 6 when compared to week 0. Cytokine levels of TNF-α, IL-1b, IL-10 and IL-6 were determined in mucosal biopsies and serum at week 0 and 6. In general, cytokine levels went down in the mucosa (Figure 6A right panels), whereas an increase of cytokine levels in the serum was seen (Figure 6B).

DISCUSSION

This phase I study shows that bone marrow harvesting and expansion of bmMSCs from refractory CD patients is feasible and that these MSCs are similar to MSCs from healthy donors in, for example, plastic adherence, spindle-shaped morphology, growth potential (Figure 2), surface marker

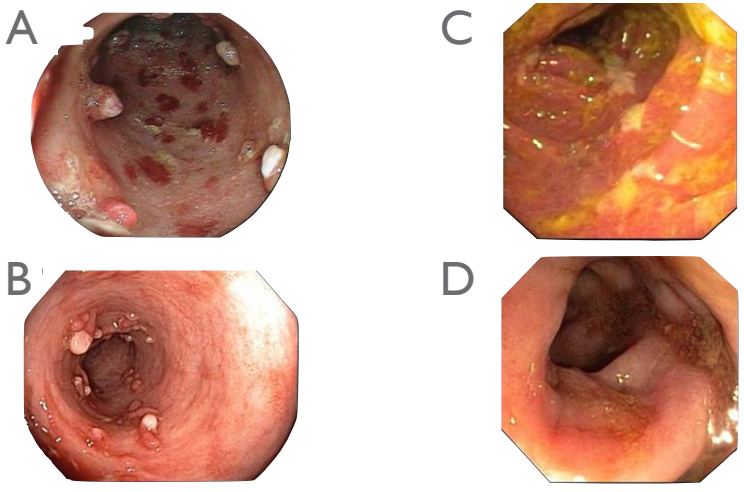

Figure 5. Endoscopy at week 0 (upper panels) and at 6 weeks (lower panels) after MSC treatment (two administrations of $2x10^6$ autologous bmMSCs) shows clear mucosal healing. Pictures A and B are from patient 2, pictures C and D from patient 3.

expression, lack of hematopoietic markers (Table 4), and differentiation capability (Figure 3). In addition, CD MSCs are able to inhibit autologous PBMC proliferation and inhibit TNF-α production in vitro (Figure 4). Furthermore, autologous bmMSC infusion appears to be safe as intravenous MSC infusions were clinically well tolerated. Reported adverse events directly related to MSC infusion were a mild and transient allergic reaction in one patient and the typical taste and smell of the cryopreservant DMSO noticed by all patients (Table 3).

Although the design of this study does not allow conclusions on efficacy, after two infusions with autologous bmMSCs, endoscopic improvement was seen in two patients (Figure 5), while three patients required surgery due to worsening of disease (Table 3). Patients included were chronic active patients refractory to all currently available medical therapeutic options. One could speculate that the immunomodulatory effect of MSCs might not be sufficient to induce clinical remission in this category of patients. Further (randomized) trials in also less refractory patients are therefore warranted

In order to study the biologic effects of systemic MSC infusion in refractory CD patients we analyzed CD4+CD127+ expression on T-cells obtained from colonic biopsies and determined cytokine production in both colon homogenates and serum. We observed a trend of lower CD4+ T-cells and higher CD4+CD127+ regulatory T-cells at week 6 when compared to week 0, although the number of patients in this study was not enough to reach statistical significance. In addition, cytokine levels went down in mucosal biopsies, indicating a decrease in intestinal inflammation (Figure 6A, right panels). The apparent reciprocal increase in serum cytokine levels (Figure 6B) may be the result of altered distribution of inflammatory cells. Due to the decreased local inflammation, leukocytes are no longer recruited to the intestine but remain in the circulation, thus increasing the systemic cytokine levels. Similar findings have been observed for regulatory T-cells in CD[20] and plasmacytoid dendritic cells in dermal inflammation.[21]

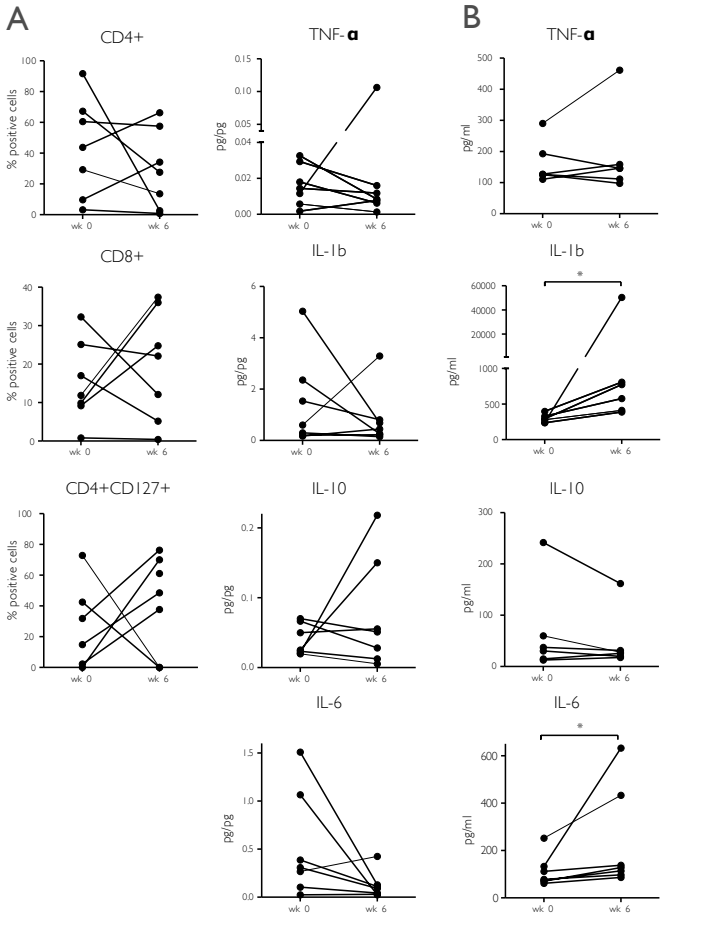

Figure 6 CD4+, CD8+, and CD4+CD127+ populations in biopsies of inflamed mucosa at week 0 and 6 (A, left panels). Cytokine levels of TNF-**α**, IL-1b, IL-10, and IL-6 were determined in mucosal biopsies (A, right panels) and serum (B) at week 0 and 6. Bars represent mean and SEM. *P< 0.05 for significant differences.

The intravenous route of administration and target dose of 1-2x10^6 cells/kg bodyweight were based on experience with protocols used in GvHD. Intravenous infusion of cells is an easy, minimal invasive and routinely performed procedure with proven safety so far. Although it has been suggested that MSCs home to sites of inflammation, it is unknown how many cells will eventually reach the intestine in CD patients. In a case report, Dinesen et al.[22] showed that MSCs administration via selective mesenteric artery cannulation was safe and feasible. This approach may possibly increase the number of cells reaching the affected organ.

In the case of autologous MSCs, an ongoing discussion is whether MSCs are affected by or may contribute to the underlying disease. For instance, MSCs from patients with systemic lupus erythematosus are difficult to expand in culture and yield low cell numbers[23] and those from patients with multiple myeloma have been shown to be impaired and possibly contribute to the pathogenesis of the disease.[24] In this study we demonstrate that MSCs obtained from refractory CD patients show similar growth potential, yield and properties when studied in vitro in comparison to MSCs from healthy donors. Our data support work published recently[25] and suggest that bmMSCs from refractory CD patients are not affected by the disease. Unfortunately, there is no golden standard test to assess the functionality of MSCs and it has not been demonstrated that in vitro effectiveness of MSCs can be translated to clinical effectiveness, making true extrapolation of this topic difficult.

A concern in cell based therapies with ex vivo expanded cells is the formation of tumors. Previous work indicated that in mice, MSCs stimulate

the growth of cancers[26, 27] and promote metastasis.[28] Additionally, extensive in vitro expansion of cells may induce genetic instability. However, two main works reporting transformation of human MSCs in culture were recently retracted as obtained data were based on tumor cell contaminated MSC cultures.[29] Although an increased risk on tumor formation has never been confirmed in humans, patients with a history of malignancy were excluded from this study. To minimize the risk of transformation of cells we have expanded MSCs in the absence of growth factors, plated MSCs in moderate cell concentrations and used only low passage numbers. Furthermore, we karyotyped the MSC product before clinical release to confirm normal karyotype and did not observe any aberrancies.

In conclusion, our data suggest that intravenous application of autologous bmMSCs is feasible and well tolerated. Furthermore, bmMSC administration may produce clinical benefits in severe refractory Crohn's disease. Therefore, further studies should be designed to examine MSCs as a potential treatment for Crohn's disease.

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ETHICAL APPROVAL

The study was approved by the Medical Ethical Committee of the LUMC and the Central Committee on Research involving Human Subject (CCMO, the Hague, the Netherlands). *www.trialregister.nl* identifier: NTR1360.

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