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Towards therapeutic disease control in inflammatory bowel diseases

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Chapter six

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 $1-2 \times 10^6$ 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: MSC 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 (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 (UC). 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 multilineage 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} Furthermore in humans, transplantation of bone marrow (bm) derived MSCs has led to improvement of corticoid 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 Crohn's disease.¹⁵

In this clinical phase I study, we evaluated the safety and feasibility of intravenous infusion of autologous bmMSC in refractory CD patients. Additionally the functionality of the bmMSCs of the CD patients 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 Crohn's disease was assessed.

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 4 weeks) and

methotrexate, azathioprine, or 6-mercaptopurine (at least 12 weeks, with stable dosage regimen for at least 8 weeks). Infliximab was discontinued at least 8 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, 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 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 (MNC) were isolated by Ficoll density gradient (density 1.077 g/cm³) 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 cm². Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂ in 175 cm² 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 cm². 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 (LUMC Pharmacy, the Netherlands). Data on MSCs obtained from healthy donors matched for age and gender, was obtained from previous studies.^{10,16,17} 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 50-100cc 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 (Ansell, 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, Bac-

tec plus aerobic/F and Bactec plus anaerobic/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 5×10^4 cells/cm² 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 µM indomethacin, 5 µg/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 (St Louis, Missouri, USA)

MSC/peripheral blood mononuclear cell (PBMC) proliferation assay

Cultured MSCs from Crohn's disease patients were plated in flat bottom 96 well plates (Costar, Zwijndrecht, the Netherlands) and allowed to attach overnight. PBMCs were isolated from whole blood of CD patients before MSC infusion. PBMCs were stimulated anti-CD28/anti-CD3 coated Dynabeads (1 bead/5 cells, Invitrogen) per 1×10^6 cells and were seeded in IMDM with 5% human serum (Sanquin, Amsterdam, the Netherlands), 5% FBS and 100 IU/mL IL-2 (LUMC Pharmacy, Leiden, the Netherlands) per well. Proliferation was measured by ³H-thymidine incorporation. In order to test the effect of Crohn's disease medication on the immunosuppressive properties of MSCs, the proliferation assay was also performed in the presence of indicated medications: 10 µg/mL infliximab, 10 µg/mL adalimumab, 10 µM dexamethasone (Sigma-Aldrich), 1 µM azathioprine (Sigma-Aldrich), 4 µM 6-mercaptopurine (Sigma-Aldrich), and 100 µg/ml methotrexate (Pharmachemie BV Haarlem, the Netherlands) performed as described above.

Cell bead array cytometric assay

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

Study design

Patients received 2 doses of MSCs, 7 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 $1-2 \times 10^6$ 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

Table 1 Baseline characteristics of included patients.

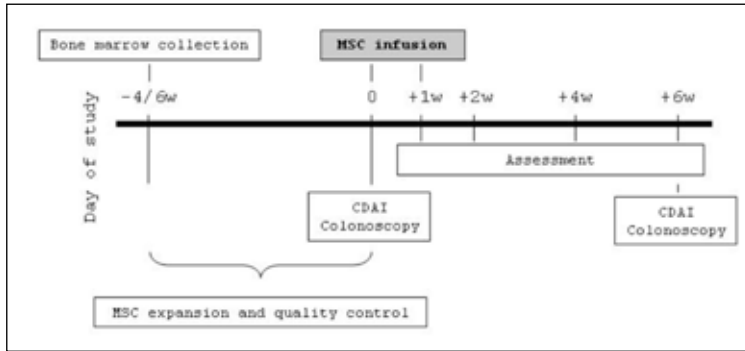
	Patient number									
	1	2	3	4	5	6	7	8	9	10
Age (y)	24	36	33	34	34	42	29	32	19	30
Sex	F	F	F	F	F	M	M	F	F	F
Disease duration (y)	10	5	15	3	11	4	7	12	3	10
CDAI at screening	442	346	283	316	337	237	316	280	255	277
Baseline CDAI	332	341	254	326	350	266	378	224	NA	304
Disease localization	ileo-colonic	colonic, ileum	colonic, ileum	ileum	ileum	colonic	colonic, ileum	colonic, ileum	no active disease	colonic
Perianal disease	y	n	y, perianal abscess (inactive)	n	y (inactive)	no	y (inactive)	no	no	y (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

<i>Prior medical therapies</i>	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, MTX, CZP, ADA	5-ASA, CS, AZA, IFX, MTX, CZP, ADA	5-ASA, CS, AZA, IFX, MTX, CZP, ADA	5-ASA, CS, ATB, AZA, IFX, MTX, CZP, ADA	5-ASA, CS, AZA, IFX, MTX, CZP, ADA, HSCT
	ileocolic resection	ileocolic resection	ileocolic resection	ileocolic resection	ileocolic resection	ileocolic resection	ileocolic resection	ileocolic resection	ileocolic resection	ileocolic resection	ileocolic resection
	no	no	no	no	no	no	no	no	no	no	no
	no	no	no	no	no	no	no	no	no	no	no
	no	no	no	no	no	no	no	no	no	no	no
	no	no	no	no	no	no	no	no	no	no	no
	no	no	no	no	no	no	no	no	no	no	no
	no	no	no	no	no	no	no	no	no	no	no
	no	no	no	no	no	no	no	no	no	no	no
	no	no	no	no	no	no	no	no	no	no	no
<i>Prior surgeries</i>	no	no	no	no	no	no	no	no	no	no	no

Abbreviations: F female, M male, n no, y yes, NA not applicable, ND not determined, 5-ASA mesalamine, CS corticosteroids, AZA azathioprine, 6-MP 6-mercaptopurine, ATB antibiotics, MTX methotrexate, IFX infliximab, CZP certolizumab pegol, ADA adalimumab, TAC tacrolimus, HSCT mobilisation phase of hematopoietic stem cell transplantation

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



Statistical analysis

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

Results

Patients

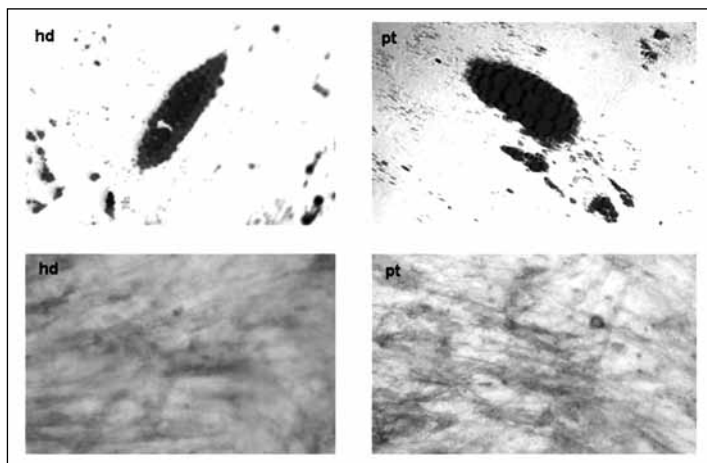
In total ten patients (eight females/two males, median age 32,5 and range 19-42 years) with moderate to severe Crohn's disease (median CDAI score at screening of 299,5 and range 255-442) were included in the study and underwent bone marrow aspiration under local anesthesia. Besides some local pain at 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 (supplementary Table 1).

One patient with a CDAI score of 255 at screening, showed no active disease on colonoscopy and was therefore excluded for further MSC administration (patient 9). The baseline median CDAI score of the 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 were ruled unlikely to be associated with MSC treatment and included AEs such as common cold and headache (Table 2).

Table 2 Adverse events in MSC treated patients (week 0-6).

	<i>n</i>	<i>Patient number</i>
<i>Likely related to MSC infusion</i>		
Allergic reaction	1	4
Typical taste and smell	9	all
Headache	3	1,4,7
<i>Unlikely related</i>		
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

Two serious adverse events (*) were reported due to worsening of disease requiring hospitalization

Figure 2 Mesenchymal stromal cells (MSCs) from Crohn's disease patients differentiate into mesenchymal lineages.

(A) Adipocyte differentiation was demonstrated in MSCs cultured from a healthy donor (hd) and from a patient with Crohn's disease (pt) after which cells were stained with Oil Red O to show lipid droplets in the cytoplasm of the cell.

(B) For osteoblast differentiation 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)

Table 3 Flow cytometric analysis of cultured bmMSCs from CD patients and healthy donors matched for gender and age (mean/median age CD patients 31,3/32,5 and hd 32,1/32,5).

<i>Patient number</i>		1	2	3	4	5	6	7	8	9	10	Average
HLA-DR		5,1	5,7	7,9	5,7	1,0	5,4	0,8	0,9	5,0	5,8	4,3
CD31		1,9	2,1	1,7	2,1	1,7	1,1	2,2	1,6	2,0	2,0	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		100,0	99,9	100,0	100,0	100,0	100,0	100,0	99,9	99,9	100,0	100,0
CD80		3,1	6,2	10,3	18,0	4,2	3,3	1,0	0,2	0,3	29,4	7,6
CD90		100,0	100,0	99,9	99,9	99,3	99,6	99,8	100,0	99,8	99,9	99,8
CD34		6,0	4,0	4,6	9,5	2,8	5,6	6,5	0,8	10,1	4,3	5,4
<i>Healthy donor number</i>												
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	94,0	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	100,0	100,0	99,9	99,9	100,0	100,0	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	99,0	99,8	100,0	99,9	99,7
CD34		4,2	1,7	2,9	4,0	7,1	19,2	6,6	16,5	5,1	4,0	7,1

bmMSC from refractory Crohn's disease patients are comparable to MSCs from healthy donors

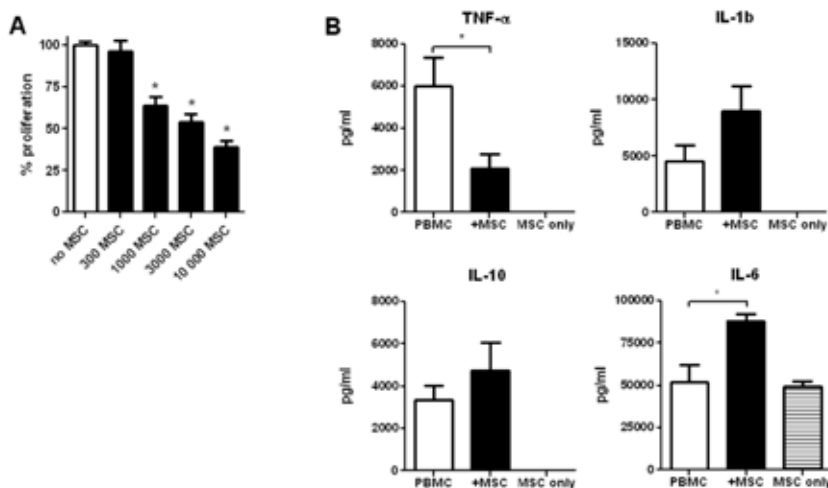
Approximately hundred milliliters of bone marrow was aspirated from each patient and bmMSCs were isolated and cultured. MSCs from Crohn's disease patients showed the typical spindle-shaped morphology and similar growth potential and yield compared to MSCs from healthy donors (Supplementary Figure 1). Depending on the number of cells needed and the yield of cells, MSCs were harvested in the first, second or third passage (Supplementary Table 1). All MSC cultures showed normal karyotyping. Immunophenotypical characterization was performed by FACS analysis, and showed similar phenotyping as described for healthy controls (Table 3).

Furthermore, CD MSCs were able to differentiate along the osteogenic and adipogenic lineages when cultured in appropriate culture medium (Figure 2).

bmMSCs from refractory Crohn's disease patients suppress immune responses *in vitro*

In the presence of autologous bmMSCs proliferation of PBMCs was reduced in a cell dose-dependent fashion (Figure 3A) 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 3B). MSC proliferation was not affected by co-medication (infliximab, adalimumab, dex-

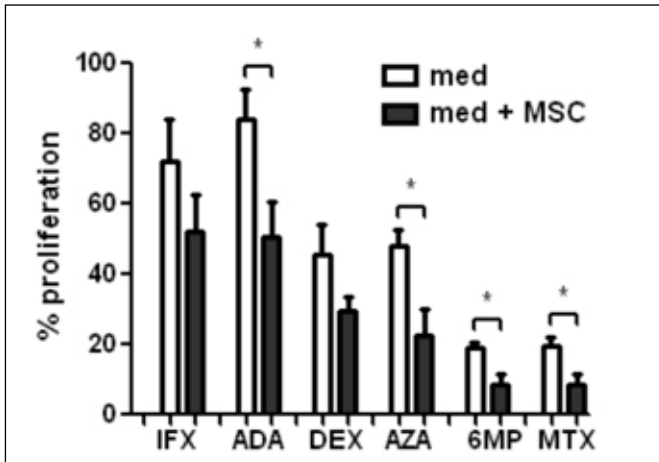
Figure 3 Mesenchymal stromal cells (MSCs) from Crohn's disease patients significantly inhibit the proliferation of peripheral blood mononuclear cells (PBMCs) and this inhibition is dose-dependent.



(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 ^3H -thymidine uptake in counts per 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. MSCs alone produce IL-6 (lower right). In the presence of MSCs, TNF- α production is lowered, whereas IL-1b and IL-10 production is increased. Bars represent the mean and SEM of data from 10 patients in triplo. * $p < 0.05$ for significant differences

amethasone, azathioprine, 6-mercaptopurine and methotrexate) *in vitro* (data not shown). However, MSCs do have an additive effect on inhibition of PBMC proliferation by various Crohn's disease medication *in vitro* (Figure 4).

Figure 4 Mesenchymal strom cells (MSCs) have an additive effect on the inhibition of PBMC proliferation by Crohn's disease medication.



In the presence of indicated Crohn's disease medication proliferation of 100 000 stimulated PBMCs added to 1000 MSC (black bars) compared to PBMC without MSCs (white bars) was measured. Proliferation measured by ^3H -thymidine uptake in counts per minute was expressed as a percentage relative to PBMC proliferation in control medium (set to 100%). Bars represent mean and SEM. * $p < 0.05$ for significant differences between test conditions based (two-sided t -test)

Clinical response

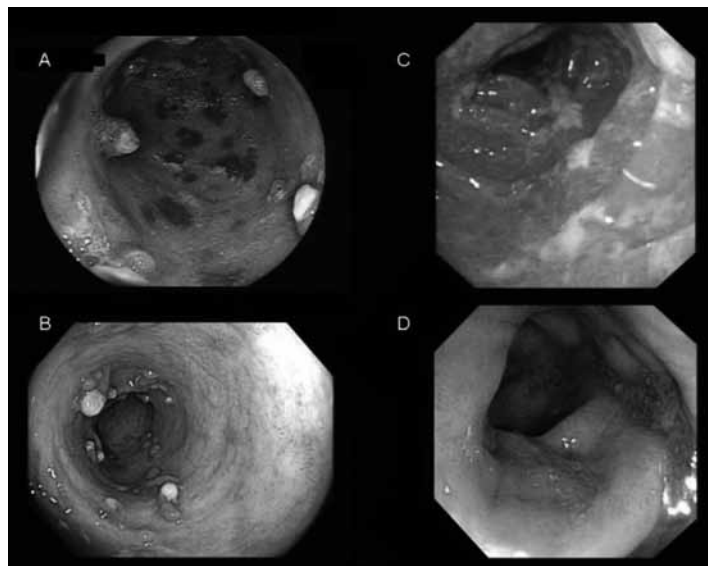
Clinical assessment was performed on all patients using CDAI scoring. Two patients were excluded before the primary endpoint was met. The first patient was a chronic severe steroid refractory patient on the waiting list for surgery. Although an initial drop of CDAI score was seen, this patient was excluded when presented with a CDAI >450 due to poor 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 5 patients, clinical response (defined as a drop in CDAI >70) was seen in 3 patients at week 6 (Table 4).

Table 4 Clinical scores of patients at week 0, 1, and 6.

		Patient number									
		1	2	3	4	5	6	7	8	10	
<i>CDAI (0-600)</i>											
wk 0*	First infusion	332	341								
wk 1	Second infusion			254	326	350	266	378	224	304	
wk 6	Primary endpoint	305	281	182	318	306	247	#452	167	ND	
		#473	185	179	267	314	160	ND	340	354	
<i>Surgical resection</i>											
	In week number	7	n	n	n	12	n	n	14	n	

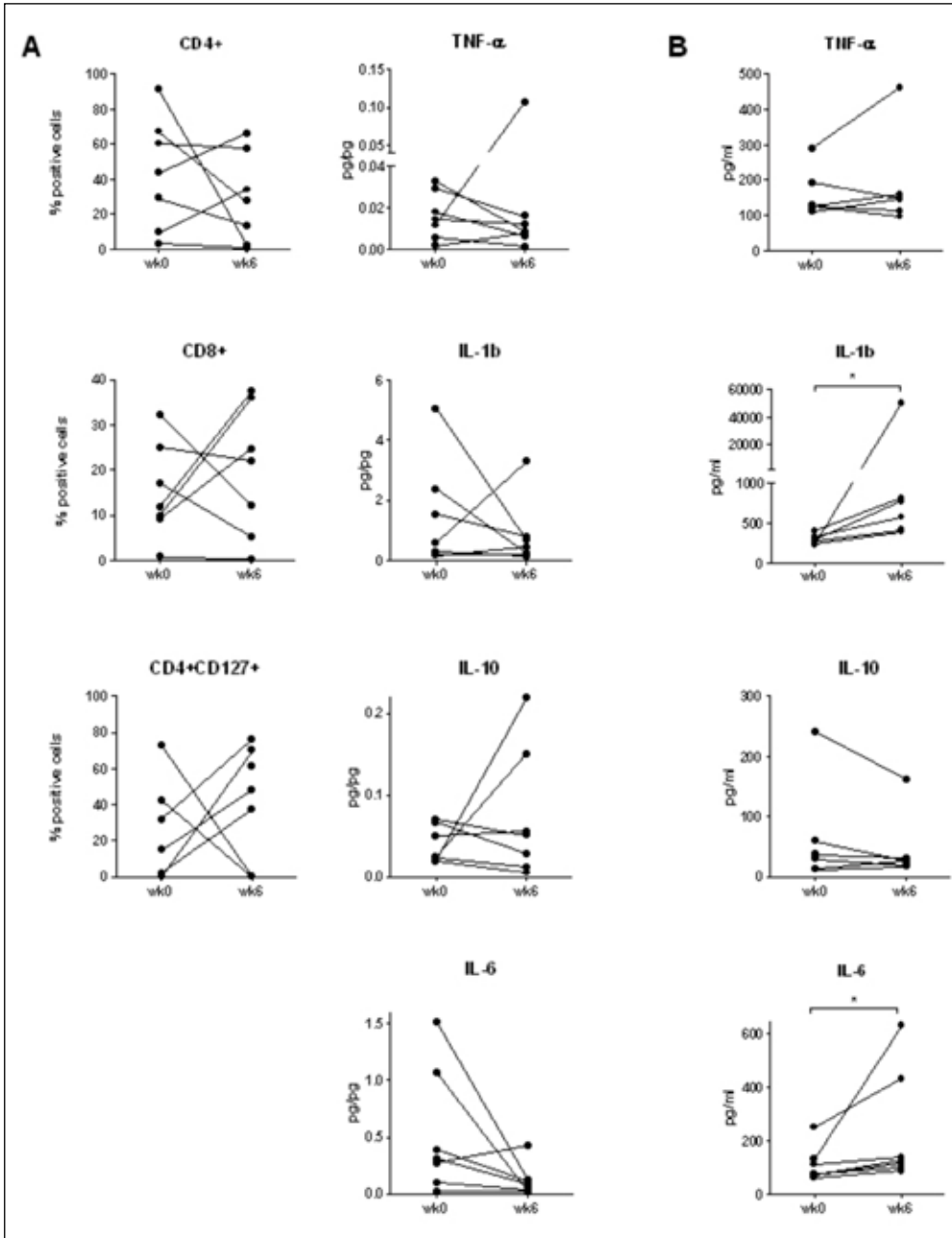
In the 6 month follow up period, three patients underwent surgery in indicated week after MSC infusion. *baseline, #withdrawn from study, ND not determined, n no

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 4). No significant differences in C-reactive protein (CRP) levels were seen.

Figure 5 Endoscopy 0 (upper panels) and at 6 weeks (lower panels) after mesenchymal stromal cell (MSC) treatment (two administrations of 2×10^6 autologous bmMSCs) shows clear mucosal healing.

Pictures A and B are from patient 2, pictures C and D from patient 3

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

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 (fig 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-1 β , 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 Crohn's disease patients is feasible and that MSCs cultured from Crohn's disease patients are similar to MSCs from healthy donors, in for example plastic adherence, spindle-shaped morphology, growth potential (Supplementary Figure 1), surface marker expression, lack of hematopoietic markers (Table 3) and differentiation capability (Figure 2). In addition, MSCs from refractory Crohn's disease patients are able to inhibit autologous PBMC proliferation and inhibit TNF- α production *in vitro* (Figure 3). 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 2).

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 4). 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 CD¹⁸ and plasmacytoid dendritic cells in dermal inflammation.¹⁹

The intravenous route of administration and target dose of $1-2 \times 10^6$ cells/kg bodyweight were based on experience with other protocols 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.*²⁰ 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 numbers²¹ and those from patients with multiple myeloma have been shown to be impaired and possibly contribute to the pathogenesis of the disease.²² In this study we demonstrate that MSCs obtained from refractory Crohn's disease patients show similar growth potential, yield and properties when studied *in vitro* in comparison to MSCs from healthy donors. Our data supports work published recently²³ and suggests that bmMSCs from patients with refractory Crohn's disease 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.

Another important issue is the effect of MSCs on concomitant medication.²⁴ To address this matter, we studied the effect of MSCs on various routinely used CD medications *in vitro*. We found that adding various CD medications to MSC cultures did not affect cell proliferation. Furthermore, adding MSCs to proliferating PBMCs in the presence of tested medications, gave a cumulative effect on the inhibition of PBMC proliferation (Figure 4), suggesting that MSCs can be used in addition to concomitant CD medication.

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^{25, 26} and promote metastasis.²⁷ Additionally, extensive *in vitro* expansion of cells may induce genetic instability. 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 karyotype the MSC product before clinical release to confirm normal karyotyping and did not observe any aberrancies.

In conclusion, our data suggests 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.

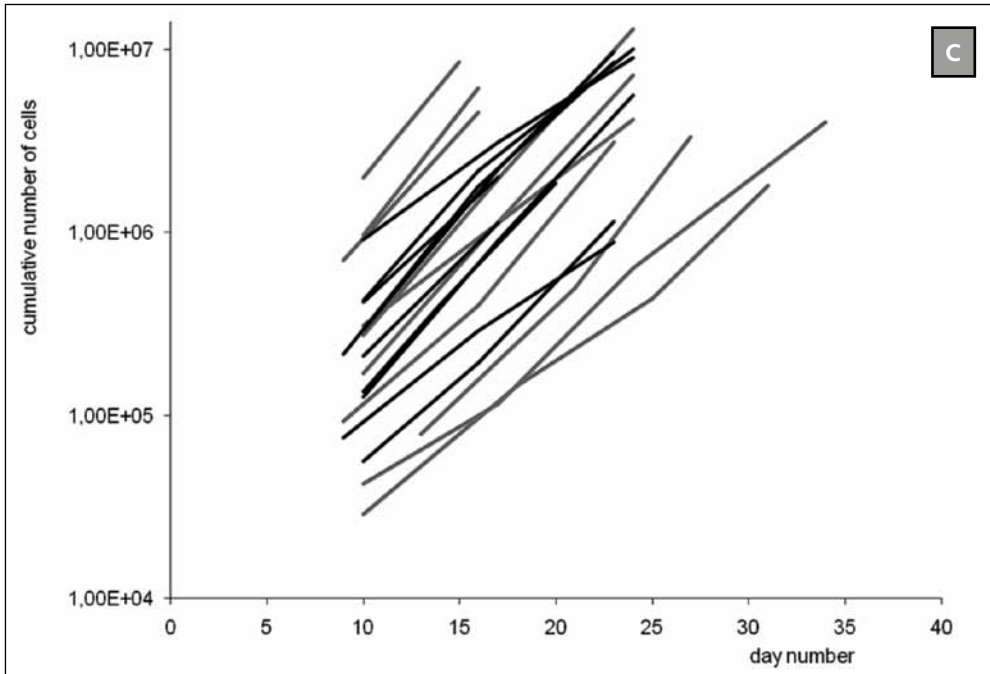
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Competing interests: None declared

Supplementary files

Supplementary figure 1.



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

Supplementary table 1.

Patient number	ml bone marrow collected	Days of culture	Passage number	Number of cells ($\times 10^6$)/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	2,0	300
4	114	16	1	2,0	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

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

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