Universiteit Leiden
The Netherlands

Stem cell therapy for inflammatory bowel disease

Duijvestein, M.

Citation

Duijvestein, M. (2012, February 9). *Stem cell therapy for inflammatory bowel disease*. Retrieved from https://hdl.handle.net/1887/18462

Version:	Corrected Publisher's Version
License:	Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from:	https://hdl.handle.net/1887/18462

Note: To cite this publication please use the final published version (if applicable).

Stem Cell Therapy for Inflammatory Bowel Disease

Marjolijn Duijvestein

The studies described in this thesis were performed at the Department of Gastroenterology and Hepatology of the Leiden University Medical Center, Leiden, the Netherlands.

Layout: Pepijn Duijvestein
Cover photograph: Marjolijn Duijvestein
Cover design: Nina Sickenga

ISBN/EAN: 978-90-818537-0-5
Copyright © 2012 M. Duijvestein, Amsterdam, the Netherlands. All rights reserved.
No part of this book may be reproduced or transmitted in any form or by any means, without prior written admission of the author.

Printed by: Haveka, Alblasserdam (www.haveka.nl/greening). This publication (cover and interior) is printed on recycled paper 'Revive®' with ink form renewable resources and alcohol free fountain solution. Recycled, EU Flower, FSC, ISCO 14001

Financial support for the publication of this thesis was kindly provided by
ABBOTT Immunology, Bayer HealthCare Pharmaceuticals, ChipSoft B.V., Dr. Falk Pharma Benelux B.V.
Dutch Digestive Foundation (Maag Lever Darm Stichting), EuroTec B.V., J.E. Jurriaanse Stichting, Ferring B.V.
Merck Sharp & Dohme B.V, Netherlands Society of Gastroenterology (NVGE), Olympus Nederland B.V.
Section Experimental Gastroenterology (SEG) of the Netherlands Society of Gastroenterology (NVGE), Tramedico B.V.

Stem Cell Therapy for Inflammatory Bowel Disease

Proefschrift

ter verkrijging van

de graad van Doctor aan de Universiteit Leiden,

op gezag van Rector Magnificus prof. mr. P. F. van der Heijden,

volgens besluit van het College voor Promoties

te verdedigen op donderdag 9 februari 2012

klokke 13.45 uur

door

Marjolijn Duijvestein

geboren te Tegucigalpa (Honduras)

in 1982

Promotiecommissie

Promotor:	prof. dr. D.W. Hommes
Co-promotores:	dr. H. Roelofs
	prof. dr. G.R. van den Brink (Academisch Medisch Centrum, Amsterdam)
Overige leden:	prof. dr. L. Egan (University of Galway, Galway, Ireland)
	prof. dr. W.E. Fibbe
	prof. dr. F. Koning
	prof. dr. C.L. Mummery
	prof. dr. A.J. Rabelink
	dr. P.C.F. Stokkers (Sint Lucas Andreas Ziekenhuis, Amsterdam)

TABLE OF CONTENTS

Chapter 1	Introduction *Journal of Crohn's and Colitis* 2008 Jun;2(2):99-106 and *Inflammatory Bowel Disease Monitor* 2010;11(2):57–64.	9
Chapter 2	Long-term follow-up of autologous hematopoietic stem cell transplantation for severe refractory Crohn`s disease *Journal of Crohn's and Colitis* 2011 Dec;5(6):543-9.	25
Chapter 3	Autologous bone marrow derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: results of a phase I study *Gut* 2010;59:1662-1669.	37
Chapter 4	Mesenchymal stromal cell function is not affected by drugs used in the treatment of inflammatory bowel disease *Cytotherapy* 2011 Oct;13(9):1066-73.	53
Chapter 5	Pretreatment with interferon-gamma enhances the therapeutic activity of mesenchymal stromal cells in animal models of colitis *Stem Cells* 2011 Oct;29(10):1549-58.	65
Chapter 6	Summary, discussion, and future perspectives	85
	Samenvatting, discussie en toekomstperspectieven	89
Chapter 7	List of publications	96
	Curriculum Vitae	99
	Acknowledgements	100
	Abbreviations	103

CHAPTER 1

Introduction

Adapted from *Journal of Crohn's and Colitis* 2008 Jun;2(2):99-106
and *Inflammatory Bowel Disease Monitor* 2010;11(2):57–64.

INFLAMMATORY BOWEL DISEASE

Inflammatory bowel disease (IBD) refers to chronic diseases that cause inflammation of the intestines. The most common inflammatory bowel diseases are ulcerative colitis (UC) and Crohn's disease (CD). These disorders have both distinct and overlapping pathologic and clinical characteristics. Ulcerative colitis is characterized by recurring episodes of inflammation limited to the mucosal layer of the colon. It almost invariably involves the rectum and may extend in a proximal and continuous fashion to involve other portions of the colon. Crohn's disease is characterized by chronic, relapsing transmural inflammation and ulceration, and may affect any part of the gastrointestinal tract from the oral cavity to the anus, but typically involves the ileum, colon or perianal region. Their pathogenesis remains incompletely understood.[1]

Epidemiology, clinical manifestation and diagnosis

Incidence rates range from 2.2 to 14.3 cases per 100 000 person-years for UC and 3.1 to 14.6 cases per 100 000 person-years for CD in North America.[2] In Europe, the overall incidence per 100 000 person-years is 5.6.[3] Prevalence rates range from 37 to 246 cases per 100 000 persons for UC and from 26 to 201 cases per 100 000 for CD. IBD can present at any age, although the peak incidence occurs between the ages of 15 and 30 years.[4] There is no gender specificity.

The major symptoms of IBD are abdominal pain, (bloody) diarrhea and generalized fatigue. Patients can also experience fever and weight loss. Frequent complications of CD are intestinal obstruction, fistula and abscess formation, and extra intestinal manifestations, such as cutaneous ulcerations, uveitis, and arthropathy. The diagnosis of IBD is established by the clinical features and can be confirmed by endoscopic, radiologic, and histopathologic examination.[5] Biopsy specimens from inflamed gut mucosa typically show inflammation, distorted crypt architecture, and crypt abscesses.

Treatment and prognosis

The choice of treatment of IBD depends on the location of the disease, its severity, and response to earlier therapy. Most clinicians initially treat patients with steroids, 5-aminosalicylic acid (5-ASA) agents, and antibiotics. Patients who are steroid dependent, and those with moderate to severe disease needing induction therapy with conventional corticosteroids, can be treated with azathioprine, mercaptopurine, and methotrexate. These so called immunomodulating drugs have been shown to be effective in inducing clinical remission, but their widespread use is limited by their toxicity. Infliximab and newer generation antibodies to tumor necrosis factor (TNF)-α (certulizomab, adalimumab) have been shown efficacious as well, but are not able to maintain remission in

most patients. Thereby, the treatment of IBD remains challenging for treating physicians.

In most patients with IBD, the course is chronic and intermittent. The disease responds less well to medical therapy with time and approximately 50-70% of the patients require surgical resection during the course of the disease. In the case of CD, surgery is accompanied by a high recurrence rate. On top of that, many patients have already had resections and are therefore at risk for developing short bowel syndrome. Consequently, there is an unmet need for more effective therapeutic strategies.

STEM CELL THERAPY

A classical definition of a stem cell is a cell that has the capacity for self-renewal and the ability to give rise to one or more types of differentiated progeny.[6, 7] Self-renewal is defined as the ability of a cell to proliferate while it maintains its proliferation and differentiation potential. Stem cells are known to exist in different tissues but their frequency, exact function and identity are generally not well understood. Both in animal models and in patients, it appears that bone marrow derived cells play a role in the healing process following intestinal injury[8] and that these cells may contribute to regeneration of various mucosal components.[9-11] The bone marrow contains at least two types of stem cells. One population consists of CD34 positive hematopoietic stem cells (HSC) committed to differentiate into all blood cell types, including the myeloid and lymphoid lineages. A second population of stem cells remains less well characterized. These non-hematopoietic stem cells are thought to support hematopoiesis and are variously known as mesenchymal stem cells, marrow stromal cells, and, more recently, mesenchymal stromal cells, all designated by the acronym MSC.[12]

Hematopoietic stem cell transplantation

Although more than fifty years ago hematopoietic stem cell transplantation (HSCT) was introduced as a treatment for injury, it is now principally used as treatment for hematologic and lymphoid cancers.[13] Evidence that HSCT is an effective treatment for autoimmune diseases comes from animal models[14, 15] and case reports from HSCT recipients with coexistent autoimmune diseases.[16] Ever since, autologous HSCT has been performed in more than 700 patients with autoimmune diseases,[17] the most frequent indications being systemic sclerosis, multiple sclerosis, rheumatoid arthritis and systemic lupus erythematosus.

HSCT includes conditioning (immune ablation with high dose chemotherapy, total body irradiation and/or anti-lymphocyte antibodies), in which the bone marrow cells of the host are completely eliminated, followed by the infusion of either autologous or allogeneic stem cells. The HSCs are either directly harvested from the marrow or mobilized from bone marrow or blood before being harvested by apheresis. High dose immune ablation is an intensive treatment with risks of severe complications which on rare occasions have been fatal. In autologous transplantation, the individual's own HSCs are harvested to be returned after conditioning. The graft is typically depleted of T-cells to avoid the

reinfusion of autoreactive T-cells. In allogeneic transplantation, the HSCs are harvested from a donor, usually a human leukocyte antigen (HLA) matched sibling. In addition to the complications associated with conditioning, allogeneic HSCT is associated with a much higher transplant-related morbidity, due to graft-versus-host disease (GvHD), aplastic anemia, and hematological malignancies, and also a higher mortality rate (15-25% vs. 3-5%[18]) due to a considerable risk on GvHD. In view of the risks related to allogeneic transplantation most patients treated for autoimmune disease have received autologous transplants.

Hematopoietic stem cell transplantation in IBD patients for other indications

The possibility that autologous HSCT could be an effective treatment for IBD was suggested by the improvement of the clinical course of disease in patients with CD that received autologous transplantation for other indications.[19-23] The first published abstract dates from 1993 and describes two year clinical remission in two patients with active IBD treated with autologous HSCT for breast cancer.[19] Long term clinical remission after autologous HSCT was reported in a patient with clinical disease control of CD up to seven years following transplantation for non-Hodgkin lymphoma.[21] Similar results were obtained in a 30-year-old patient with a ten year history of severe CD who developed Hodgkin's disease and remained in complete treatment-free remission of both diseases three years after autologous HSCT[24] and in an IBD patient who received HSCT for acute myeloid leukemia and had normal findings during ileo-colonoscopy at 1, 2, 3, and 5 years after transplantation.[23]

Similar to the first experience with autologous HSCT in IBD, the effect of allogeneic HSCT on IBD was initially described in patients treated for hematological malignancies. The first case report was published in 1998 and described a 35-year-old male free of symptoms and signs of CD eight years post allogeneic marrow transplantation for acute leukemia.[25] A second report in the same year described six patients that underwent allogeneic transplantation for leukemia between 1962 and 1982.[26] In this report, five out of six patients had active CD at time of transplantation. One patient died of septic complications 97 days after transplantation, the other five patients remained free of disease activity for more than one year post-transplantation. Only one out of these five patients relapsed during the follow up period of up to 15 years post-transplantation. Interestingly, the only patient that developed a mixed donor-host hematopoietic chimerism following allogeneic HSCT continued to have active CD. In a retrospective study by Ditschkowski et al., ten out of eleven patients remained free of symptoms following allogeneic HSCT for hematological malignancies with a median follow-up time of 34 months.[27] In another case report,[28] a 41-year-old man with CD underwent allogeneic HSCT for lymphoma. Following transplantation, his bowel symptoms ceased and he was able to stop all immunosuppressive drugs. Eighteen months after transplantation colonoscopy showed no evidence of CD activity. Remission of UC following allogeneic HSCT has also been described.[29, 30] Two patients, each with a long history of psoriasis and UC, received an allogeneic HSCT for leukemia and remained in full remission four and twelve years after transplantation.

The coincidental treatment of IBD with both autologous and allogeneic HSCT increased the interest in the possibility that stem cell transplantation could be of value in IBD treatment. Autologous HSCs are infused only to shorten the post-HSCT neutropenic interval, in contrast with allogeneic HSCT in which the recipient's immune and hematologic system is replaced with that of a healthy donor without the genetic predisposition to IBD. In this light it has been proposed that the risk of disease recurrence may be higher after autologous HSCT.[31]

Early studies on HSCT specifically for Crohn's disease

The first reports of autologous HSCT specifically given for the treatment of CD were published in 2003 and concerned five patients with severe disease activity refractory to conventional treatment and treatment with anti-TNFα antibodies, who received autologous HSCT.[32-34] No serious transplantation related complications were reported and all patients entered clinical remission. Some of the colonoscopies however, showed persistent mild inflammation up to one year post-transplantation. A larger phase I study on twelve patients with chronic active refractory CD also suggested that autologous HSCT can have a beneficial effect on CD activity. Besides fever, the autologous HSCT was well tolerated by the patients. Adverse effects included hematemesis from a Mallory-Weiss tear, a prolonged febrile course, clostridium difficile-induced diarrhea, and diarrhea after an upper respiratory tract infection. After fifteen months only one patient developed a recurrence of active CD. All others maintained in clinical and drug-free remission, but similar to the patients described in the reports above with persisting nonsymptomatic histologic and/or radiologic evidence of CD.[35] In **Chapter 2** of this thesis, the data on the clinical effect of HSCT in three patients with refractory CD can be found.

Potential risks of hematopoietic stem cell transplantation

HSCT may be an effective treatment for CD but is also associated with a high morbidity and mortality rate.[36] In 390 patients undergoing autologous HSCT for various autoimmune diseases, a mobilization associated mortality of 1.5% and an overall procedure related mortality of 9% were found.[37] Early toxicity is related to direct organ damage either from the agents used or due to infection and bleeding during the 10–12 days of bone marrow aplasia following the immunosuppressive conditioning period. Late toxicity relates to malignancy development due to the chemotherapy and/or radiation exposure. In addition, HSCT is associated with complications such as veno-occlusive disease of the liver and acute and chronic GvHD.[38] Although HSCT seems a reasonably successful treatment for CD it is clear that, given the considerable mortality rate of HSCT for autoimmune diseases, this treatment should only be considered in selected cases of CD. HSCT could be considered as a last resort in patients with debilitating disease refractory to all immunosuppressive drugs, including the different anti-TNFα compounds now available for treatment, and in patients in which surgery is not a treatment option.

MSC Transplantation

HSCT is thought to result in clinical remission in CD due to the combination of the immunosuppressive conditioning regimen and the replacement of the derailed lamina propria immune cells that maintain the disease. A novel emerging stem cell treatment may offer the benefit of immunosuppression without the need for conditioning chemotherapy, even when given as allogeneic transplant.

Mesenchymal stromal cells (MSC) reside in almost every type of connective tissue. Friedenstein and colleagues were the first to identify an adherent, fibroblast-like population of cells in the bone marrow.[39] Once isolated, these cells adhere to plastic, are capable of developing colony forming-units, and proliferate in vitro. In addition, MSCs are multipotent cells capable of differentiating into multiple lineages of the mesenchyme, including fat, bone, and cartilage tissue. MSCs consist of a heterogeneous population of cells and thus far no unique marker has been identified that allows reproducible isolation of precursors with predictable developmental potential. The isolation and characterization of these cells therefore still relies on their ability to adhere to plastic and their expansion potential. Isolated and expanded MSCs express CD73, CD90 and CD105 and are negative for hematopoietic stem cell markers (CD14, CD34, CD45), thereby distinguishing them from the hematopoietic stem cells.[40] Furthermore, MSCs do not express major histocompability complex (MHC) class II or co-stimulatory molecules and are poor antigen presenting cells that do not elicit a proliferative response in allogeneic lymphocytes, which suggests that MSCs are of low immunogenecity. Although MSCs are present in virtually all tissues, our current knowledge is based on MSCs isolated from accessible tissues (e.g. bone marrow, adipose tissue, and umbilical cord blood).

Immunomodulatory capacities of MSCs

The immunomodulatory functions of MSCs were examined in vitro by coculturing them with purified subpopulations of immune cells. It has been shown that MSCs suppress several functions of naïve and memory T-cells,[41-43] B-cells,[44] and natural killer cells[45, 46] as well as the differentiation, maturation, and function of dendritic cells (DCs).[47] Furthermore, expanded MSCs alter cytokine secretion profiles of DCs, naïve and effector T-cells, and natural killer cells to induce a more anti-inflammatory or tolerant phenotype.[48] Even though cell-cell contact plays a role in the interaction between MSCs and other immune cells, immunosuppressive mechanisms of MSCs are mainly mediated through soluble factors. MSCs constitutively produce transforming growth factor-β1 (TGF-β1), hepatocyte growth factor (HGF), interleukin (IL)-10, prostaglandin E_2 (PGE_2), soluble HLA-G5, and IL-6 (Figure 1). The latter inhibits monocyte differentiation towards DCs and MSC-DC interaction directs DC maturation towards an anti-inflammatory or regulatory phenotype, thereby decreasing their stimulation capability on T-cells.[47] Other factors, such as the enzyme indoleamine 2,3-dioxygenase (IDO), are released upon stimulation with inflammatory cytokines such as IFNγ and TNFα. IDO metabolizes tryptophan to kynurenin, which causes depletion of local tryptophan and accumulation of toxic breakdown products, thereby suppressing both CD4 and CD8

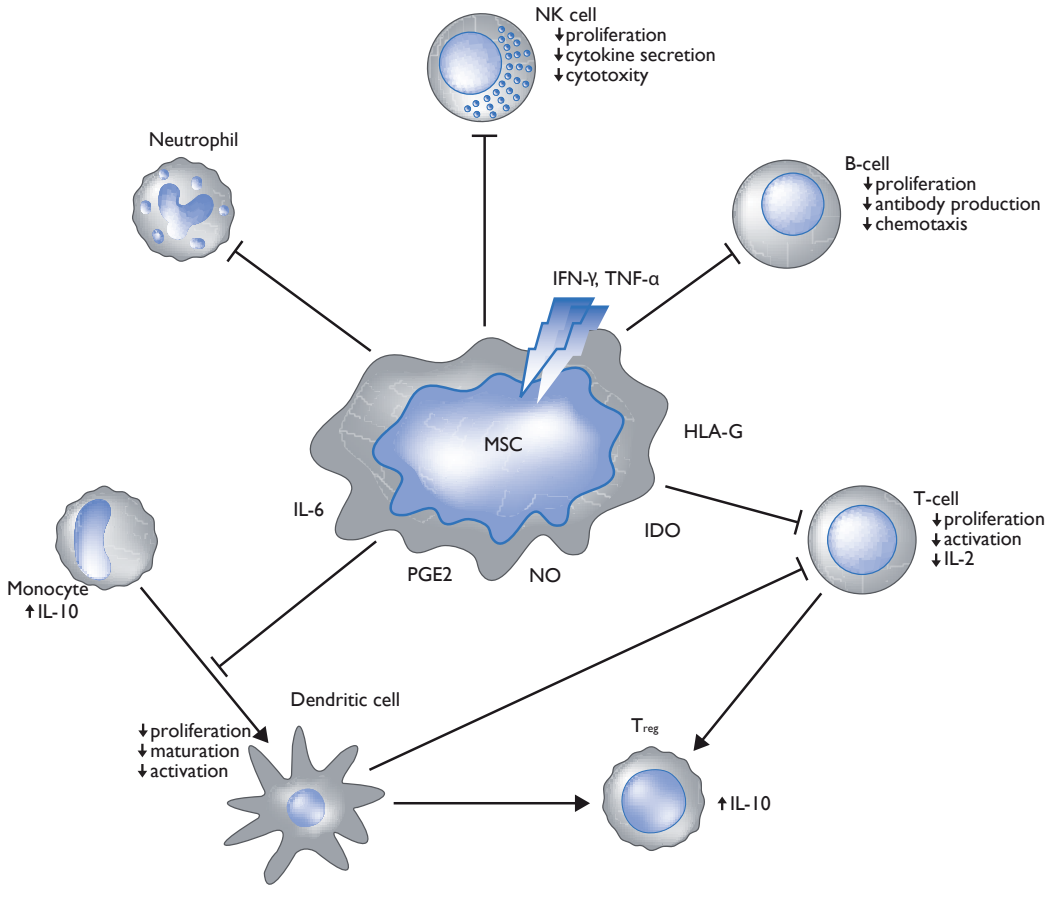


Figure 1. Schematic illustration of the immunomodulatory effects of MSCs on different cells of the immune system. After activation, MSCs secrete various factors, such as PGE2, IDO, and HLA-G. These factors regulate the function and proliferation of NK cells, B-cells, and T-cells. Constitutively secreted IL-6 plays a role in the inhibition of alloantigen-induced generation and maturation of DCs and neutrophil activation. DC: dendritic cell; HLA-G: human leukocyte antigen-G; IDO: indoleamine 2,3-dioxygenase; IL: interleukin; IFN-γ: interferon-γ; MSC: mesenchymal stromal cell; NO: nitric oxide; PGE2: prostaglandin E2; NK: natural killer; Treg: regulatory T cell; TNF-α: tumor necrosis factor-α.

T-lymphocytes proliferation with mitogens and specific antigens.[49, 50] Although the involvement of the enzyme IDO has been consistently reported, IDO alone is not responsible for inhibition of T-cell proliferation, as IDO inhibitors were not able to restore proliferation of peripheral blood mononuclear cells (PBMCs). Moreover, MSCs lacking both IFNγ receptor I and IDO still exert immunomodulatory activity.[51] In mice, MSCs express very little IDO and is the induction of inducible nitric oxidase synthase (iNOS) essential in T-cell proliferation inhibition.[52] Available data on the interaction between MSCs and B-cells is controversial. MSCs have been shown to inhibit as well as stimulate B-cell proliferation, depending on dose, source, and test system.[53] For example, MSCs decrease proliferation and immunoglobulin secretion of B-cells in a 1:1 ratio,[44] whereas in a lower concentration MSCs stimulates B-cell antibody secretion.[54]

Therapeutic applications of MSCs

Animal studies on models for tissue damage and autoimmune disease indicate that, similar to their immunosuppressive capacities in vitro, MSCs also display immunosuppressive capacities in vivo. For example, in a T-cell mediated experimental model of multiple sclerosis (MS) in mice, murine MSCs have been used to successfully treat experimental MS through the induction of peripheral T-cell tolerance.[55] Systemically infused MSCs first show a wide-ranging distribution followed by homing to injured tissues,[56] including the gut,[57] where they may participate in tissue repair. However, only a fraction of the systemically infused MSCs are traceable, and the fate of the remainder of the cells remains unknown. That intravenous infused MSCs entrapped as emboli are activated to secrete the anti-inflammatory protein TSG-6[58] and that MSC derived molecules have anti-inflammatory properties,[59] suggests that specific homing of MSCs to damaged tissues is not required for an effect.

In humans, the safety and feasibility of both local and systemic MSC administration has been studied in a variety of phase I and phase II trials. MSCs are low immunogenic and not restricted by MHC, therefore MSCs do not have to be human leukocyte antigen (HLA) matched to the recipient and can be infused without the need for conditioning chemotherapy, not even when given as allogeneic transplant. Both allogeneic and autologous MSCs are therefore currently under investigation. A great advantage of the allogeneic MSCs is their immediate availability. Furthermore, the age and fitness of the donor is controlled. MSC number and functionality decreases with age[60, 61] and an ongoing discussion is whether MSCs might contribute to or if the MSCs are affected by the underlying disease. For instance, MSCs from patients with systemic lupus erythematosus yield low cell numbers and are difficult to grow in culture.[62] Moreover, MSCs from patients with multiple myeloma are functionally defective and possibly contribute to the pathogenesis of the disease.[63,64] Rationale for autologous application comes from data that MSCs may under certain conditions also be subject to immune rejection. In a nonmyeloablated host, allogeneic MSCs are able to mount a T-cell memory response and consequently are eliminated.[65] Comparable loss of immune privilege has been reported by others.[66]

The initial clinical trials were in patients with osteogenesis imperfecta[67], followed by trials in which the immunosuppressive effects of the MSCs were used either to reduce the incidence of GvHD after allogeneic HSCT[68] or as treatment of active disease, including GvHD of the gut.[69] Based on their ability to moderate T-cell response, MSCs are currently under evaluation in a range of (autoimmune) diseases (Table 1).

Safety issues and concerns

In the limited number of patients treated with MSCs in the last decade, few adverse events have been attributed to MSC administration. Although acute toxicity appears low, little is known about long-term unwanted side effects. Also, as mentioned before, safety concerns remain concerning immunogenecity and the dysfunction of MSCs due to the underlying disease. Moreover, potential hazards include the possibility of malignant transformation, ectopic tissue formation and the possible xenogenic transmission of disease and antibody formation when fetal bovine serum (FBS) is added to the culture medium. In fact, reports showed that extensively in vitro expanded stem cells may be prone to malignant transformation.[70] It has been demonstrated by some groups that MSCs stimulate the growth of cancers and promote metastasis in rodents,[71-73] although an increased risk of tumor formation has never been confirmed in humans.[74] Moreover, two main works reporting transformation of human MSCs in culture were recently retracted as obtained data were based on tumor cell contaminated MSC cultures.[75] Although reassuring, safety issues remain important and it is therefore essential to carefully characterize MSCs passaged in vitro to maximize safety for the recipient. Furthermore, patients should be thoroughly screened before MSC administration as the cells might enhance the growth of unknown cancer.

MSCs for IBD

Data from experimental colitis models

MSCs have been studied in both dextran sulfate sodium (DSS) and trinitrobenzene sulfuric acid (TNBS) colitis, in mice as well as in rats

Autoimmune diseases
- Diabetes mellitus, type 1 and 2
- Systemic sclerosis (SSc)
- Systemic lupus erythematosus (SLE)
- Primary Sjögren's syndrome (pSS)

Orthopedics
- Fractures
- Arthrosis and arthritis
- Chondral and meniscal lesions
- Articular cartilage defects

Neurology
- Amyotrophic lateral sclerosis (ALS)
- Ischemic cerebral stroke
- Multiple sclerosis (MS)
- Parkinson's disease

Cardiology
- Myocardial ischemia
- Dilated cardiomyopathy
- Chronic ischemic left ventricular dysfunction secondary to myocardial infarction

Table 1. Overview of indications for MSC therapy. Registered trials were found using the International Clinical Trials Registry Platform (ICTRP), available on www.who.int/ictrp.

(Table 2). Different sources of MSCs have been used; i.e. bone marrow (bmMSCs), adipose tissue (atMSCs) and, though not commonly used, gingiva (gMSCs). Interestingly, MSCs obtained from both syngeneic and allogeneic sources have been applied and also human MSCs were studied in wild type mice (xenogenic). Systemic route of administration was either via the tail vein (rat) or intraperitoneally (i.p.) in mice. The latter prederred in mice as MSCs, due to their large size, frequently entrap in the pulmonary circulation which can cause acute death due to asphyxia.[76] Local administration was also studied by injecting the MSCs into the colonic submucosa.[77]

The first two articles published in 2008 showed beneficial effects of the bmMSC in both DSS[78] and TNBS[77] colitis in rats. These data were further supported by two articles from the same group on atMSCs. In their first article, the authors showed that systemic infusion of MSCs obtained from adipose tissue ameliorated the clinical and histopathologic severity of TNBS colitis, abrogating body weight loss, diarrhea and inflammation, and increasing survival.[79] A second paper supported these data by showing that systemic infusion of atMSCs protects against experimental DSS colitis and sepsis. The therapeutic effect was associated with down-regulation of the Th1-driven inflammatory responses.[80] Zhang et al. nicely demonstrated that also MSCs from human gingiva, a tissue source easily accessible from the oral cavity, have similar immunomodulatory and anti-inflammatory properties as bmMSCs. In addition, they showed that a comparable therapeutic effect was mediated in the acute model of DSS colitis. This effect was achieved by the suppression of inflammatory infiltrates and inflammatory cytokines/mediators, by the increased infiltration of regulatory T-cells, and by the expression of anti-inflammatory cytokine IL-10 at the colonic sites.[81]

Recent data suggest that resting MSCs do not have significant immunomodulatory activity, but that the immunosuppressive function of MSCs has to be elicited by interferon-gamma (IFN-γ).[49, 50] In **Chapter 5**,

Hematology
- Myelodysplastic syndromes
- Graft-versus-host disease (GvHD)

Dermatology
- Epidermolysis bullosa
- Burn injury
- Diabetic foot

Nephrology
- Diabetic chronic kidney disease
- Lupus nephritis
- Chronic allograft nephropathy
- Allograft rejection after renal transplantation

Other
- Osteogenesis imperfecta
- Liver cirrhosis
- Periodontitis
- Inflammatory bowel disease (IBD)

Ref.	Species	Colitis model	Donor source	Tissue source	Cell number	Route	Timing	Sarifice	Outcome of MSC treatment
81	C57BL/6	7 days 3% DSS	human	bm and g	2×10^6	i.p.	one day after DSS initiation	day 10	Amelioration of colitis. Suppression of inflammatory infiltrates and inflammatory cytokines/mediators. Increased infiltration of regulatory T cells and the expression of anti-inflammatory cytokine IL-10 at the colonic sites.
80	C57Bl/6	acute colitis: 7 days 5% DSS	human, allogeneic and syngeneic	at	10^5–5×10^6	i.p.	day 2	day 5-14	Amelioration of the clinical and histopathological severity of colitis. Less weight loss, diarrhoea and inflammation, and increase in survival. Downregulation of the Th1-driven inflammatory responses.
	C57Bl/6	chronic colitis: two cycles of 7 days with 3% DSS, followed by a 10-day period without DSS supplementation		at	1×10^6	i.p.	day 7 each cycle	day 10 or 26	
91	male Lewis rats	DSS colitis with bone marrow hypoplasia: busulphan i.p. day 0, 1% DSS day 5 - 10	SDTG (CAG-EGFP) rat	bm	2×10^4/g	tail vein	day 7	day 10	Less severe colitis due to restoration of epithelial barrier integrity, no changes in cytokine expression
78	male Lewis rats	7 days 4% DSS	syngeneic	bm	5×10^6	tail vein	day 0, 2, and 4	day 7	Reduction in bloody stools, weight loss, colon shortening, and microscopic injuries. Decrease in mRNA expression of TNF-alpha, IL-1beta, and COX-2 in the rectum of MSC treated rats. Suppression of VEGF, HGF, and b-FGF. Green-fluorescent-labeled MSCs in lamina propria of inflamed regions.

Ref.	Species	Colitis model	Donor source	Tissue source	Cell number	Route	Timing	Sarifice	Outcome of MSC treatment
79	BALB/c	3 or 5 mg TNBS in 50%EtOH intrarectally	human, allogeneic and syngeneic	at	10^5–10^6 cells	i.p.	12 hours after TNBS installation	day 3 and 10	Protection against colitis, reduction in histopathologic signs and infiltration of macrophages, lymphocytes, and neutrophils. Reduced levels of inflammatory cytokines (TNF-α, IFN-γ, IL-6, IL-1β, and IL-12) and chemokines. Abrogation of established colitis. Increased levels of the anti-inflammatory/regulatory cytokine IL-10. Reduction of disease recurrence.
	BALB/c	4 or 5 mg TNBS in 50%EtOH intrarectally	human	at	1×10^6	i.p.	2 consecutive days starting day 6	day 14	
	BALB/c	1.5 mg TNBS day 0 and 9	human	at	1×10^6	i.p.	12 hours after the first infusion of TNBS	day 14	
77	male Sprague-Dawley rats	0.15 M TNBS in 35% EtOH	syngeneic	bm	1×10^7	injected into the colonic submucosa	immediately after the TNBS-induced colon injury	day 6	The engrafted MSCs survived and accelerated healing of TNBS-induced colitis. After the implantation, the MSCs became potential sources of VEGF and TGF-β1, angiogenic and immunomodulating factors, in colon tissues.

Table 2. Published studies with mesenchymal stromal cells in experimental models of colitis. A literature search in Pubmed was performed using the following keywords, alone or in combination: 'mesenchymal stem cell', 'mesenchymal stromal cell', 'colitis', 'crohn's disease' and 'inflammatory bowel disease'.

the effects of IFN-γ prestimulation of MSCs (IMSCs) was assessed in vitro and in animal models of colitis, demonstrating an enhanced therapeutic activity of MSCs after IFN-γ exposure.

Clinical trials in patients with Crohn's disease

Currently, multiple trials on MSCs for the treatment of CD are registered in the public registries for clinical trials (Table 3). The indication is either active luminal disease, for which MSCs are injected intravenously, or fistulizing disease, for which MSCs are injected locally in fistula tracts. Cells are isolated from bone marrow or from adipose tissue and from either the patient itself (autologous) or from a healthy donor (allogeneic). At least three companies are currently investigating the application of MSCs in Crohn's disease, i.e. Anterogen (Korea), Cellerix (Spain) and Osiris (USA).

Completed trials

Safety of local application of atMSCs in the treatment of fistulizing CD was demonstrated in a phase I clinical trial in which in total nine fistulas in four patients were inoculated with autologous atMSCs. Although the results are preliminary and follow-up is short, they are interesting as after 8 weeks 75 percent of these fistulas were considered healed and no adverse effects were observed in any of these patients.[82] This phase I study was followed by a multicenter, randomized, controlled trial sponsored by Cellerix to evaluate the efficacy and safety of atMSCs. Forty-nine patients with complex perianal fistula from cryptoglandular disease (n=35) or CD (n=14) were included. Patients received fibrin glue or 20 million cells plus fibrin glue intralesionally. Fistula healing was evaluated at 8 weeks. If not healed, a second dose of fibrin glue or 40 million cells plus fibrin glue was administered, with healing evaluated 8 weeks later. Healing was defined as absence of drainage and complete reepithelization of the external openings. The proportion of patients whose fistulas were healed was significantly higher with atMSCs than with fibrin glue alone in the CD as well as the non CD patients.[83] Osiris Therapeutics uses Prochymal™, MSCs obtained from the bone marrow of healthy adult volunteer donors. Although a significant decrease of the Crohn's disease Activity Index (CDAI) score was observed in the phase I trial, [84] the company recently terminated a phase III trial because of a high placebo response rate. Unfortunately, the results have not yet been published in peer reviewed journals. In **Chapter 3** the feasibility and safety of the intravenous application of autologous MSCs obtained from the bone marrow (bmMSC) of CD patients, was assessed in a phase I trial.[85]

Ongoing studies

The Royal Perth Hospital (Australia) has just launched a multicenter phase II trial in 20 patients to evaluate the safety and efficacy of weekly intravenous infusion for 4 weeks with allogeneic bmMSCs. The University Hospital La Paz in Madrid is performing a phase I/IIa trial on allogeneic atMSCs in the local treatment of recto-vaginal fistula in CD. The Leiden University Medical Center (LUMC) is currently investigating the safety and preliminary efficacy of allogeneic bmMSCs in the induction of response for active fistulizing CD in a dose escalation study.

Main trial ID	Title	Indication	Cell type and source	Dose	Delivery route	Time of delivery	Primary endpoint	Phase and design	Patient number	Site/company
Recruiting										
NCT01011244	A Phase II Clinical Study of ADIPOPLUS (Autologous Cultured Adipose-derived Stem Cell) for the Treatment of Crohn's Fistula to Evaluate Safety and Efficacy	Fistula	autologous/at		in fistula site		1) Efficacy: more than half closure of fistula (week 8) 2)Safety: Clinically measured abnormality of laboratory tests and adverse events	Phase II, uncontrolled		Anterogen Co., Ltd.
NCT01090817	A Multicentre Australian Phase 2 Study to Evaluate Safety and Efficacy of Mesenchymal Stromal Cells for Treating Biologic Refractory Crohn's Disease	Luminal	allogeneic/bm	$2x10^6$/kg recipient weight	intravenous	weekly for 4 weeks	Clinical response to MSC: Reduction of Crohn's disease Activity score by 100 points or more at six weeks post start of therapy	Phase II, non-randomized, historical controls	20	Royal Perth Hospital
NCT00999115	Clinical Trial in Phase I-IIa to Study the Feasibility and Security of the Allogenic Use of Adipose-derived Stem Cells for the Local Treatment of Recto-vaginal Fistula in Crohn´s Disease	Recto-vaginal fistula	allogeneic/at	$20x10^6$ cells	intralesional injection	at baseline with a possible second administration of $40x10^6$	Percentage of subjects in whom the external openings of the treated rectovaginal fistula have closed (12 weeks)	Phase I-IIa	10	Biomedica del Hospital Universitario la Paz
NCT01020825	A Prospective Study for the Assessment of the Long-term Safety and Efficacy of Cx401 in Patients Taking Part in the FATT-1 Trial	Perianal fistula	autologous/at	20 and $40x10^6$	intralesional injection		Cumulative incidence of adverse events (clinical or laboratory) attributed to the study therapy in the preceding FATT-1 randomized trial (CX401 or fibrin glue) (6 months)	Prospective, observational	150	Cellerix
NCT01144962	Dose-escalating Therapeutic Study of Allogeneic Bone Marrow Derived Mesenchymal Stem Cells for the Treatment of Fistulas in Patients With Refractory Perianal Crohn's Disease	Perianal fistula	allogeneic/bm	10, 30, $90x10^6$ or placebo	intralesional injection	baseline	1) The number of adverse and serious adverse events and 2) a reduction in the number of draining fistulas (12 weeks)	Phase I-II, dose escalation	21	Leiden University Medical Center
Active, not recruiting										
NCT00482092	A Phase III, Multicenter, Placebo-Controlled, Randomized, Double-Blind Study to Evaluate the Safety and Efficacy of PROCHYMAL[tm] (ex Vivo Cultured Adult Human Mesenchymal Stem Cells) Intravenous Infusion for the Induction of Remission in Subjects Experiencing Treatment-Refractory Moderate-to-Severe Crohn's Disease	Luminal	allogeneic/bm	600 or 1200 $x10^6$ or placebo	intravenous	over 4 infusions in 2 weeks	Disease remission (CDAI at or below 150) (28 days)	Phase III, multicenter, placebo-controlled, randomized, double-blind	270	Osiris Therapeutics
Completed										
NCT00543374	A Phase III, Multicenter, Placebo-controlled, Randomized, Double-blind Durability and Retreatment Study to Evaluate the Safety and Efficacy of PROCHYMAL™ (ex Vivo Cultured Adult Human Mesenchymal Stem Cells) Intravenous Infusion for the Maintenance and Re-induction of Clinical Benefit and Remission in Subjects Experiencing Treatment-refractory Moderate-to-severe Crohn's Disease	Luminal	allogeneic/bm	NA	NA	NA	1) Duration of clinical benefit 2) Re-induction of clinical benefit (6 months)	Observational, double blind, randomized, placebo-controlled		Osiris Therapeutics
NTR1360	Bone Marrow Derived Mesenchymal Stem Cells for the Treatment of Refractory Crohn's Diseas	Luminal	autologous/bm	$2x10^6$/kg body weight	intravenous	2 infusions, one week apart	1) Safety: rate of (serious) adverse events in the study population 2) Feasibility: determination of the number of expanded MSCs in relation to the amount of bone marrow collected, number of passages required and time to reach study target doses (week 6)	Phase I, open label	10	Leiden University Medical Center
NCT00294112	A Phase II, Open-Label, Randomized Study to Evaluate the Safety and Efficacy of PROCHYMAL™ IBD (ex Vivo Cultured Adult Human Mesenchymal Stem Cells) Intravenous Infusion for the Treatment of Subjects Experiencing Moderate-to-Severe Crohn's Disease That is Refractory to Steroids and Immune Suppressants	Luminal	allogeneic/bm	2 or $8x10^6$/kg body weight	intravenous	2 infusions, one week apart	Crohn's disease activity index (28 days)	Phase I, open label, randomized	10	Osiris Therapeutics
NCT00992485	A Phase I Dose Escalation Clinical Study of ADIPOPLUS (Autologous Cultured Adipose-derived Stem Cell) for the Treatment of Crohn's Fistula to Evaluate Safety and Efficacy	Fistula	autologous/at	Escalating doses	in fistula site		1) Efficacy: closure of fistula (week 8) 2) Safety: Clinically measured abnormality of laboratory tests and adverse events	Phase I, dose escalation	9	Anterogen Co., Ltd.
NCT01144962	Dose-escalating Therapeutic Study of Allogeneic Bone Marrow Derived Mesenchymal Stem Cells for the Treatment of Fistulas in Patients With Refractory Perianal Crohn's Disease	Perianal fistula	allogeneic/bm	10, 30, $90x10^6$ or placebo	intralesional injection	baseline	1) The number of adverse and serious adverse events and 2) a reduction in the number of draining fistulas (12 weeks)	Phase I-II, dose escalation	21	Leiden University Medical Center

Table 3. Registered clinical trials on mesenchymal stromal cells in Crohn's disease. Both recruiting and completed trials are listed (sources: clinicaltrials.gov and trialregister.nl).

Safety issues and concerns

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[62] and those from patients with multiple myeloma have been shown to be impaired and possibly contribute to the pathogenesis of the disease.[86] **Chapter 3** therefore evaluates MSCs obtained from refractory CD patients, focusing on growth potential, yield, and functional properties.

Previous studies showed that immunosuppressive drugs can be harmful to hematopoietic stem cells or endothelial progenitor cell proliferation, thereby affecting their functional capacities.[87-90] We hypothesized that likewise, immunosuppressive agents might have an effect on MSC function and could, therefore, change the outcome of MSC therapy and affect safety. **Chapter 4** investigates the interaction between MSCs and immunosuppressive drugs frequently used in the treatment of IBD.

REFERENCES

1. Sandler RS. Epidemiology of Inflammatory Bowel Disease. In: Targan SR and Shanahan F, eds. Inflammatory Bowel Disease; from bench to bedside. 1994 ed. Baltimore: Wiliams & Wilkins, 2007:5-30.
2. Loftus EV, Jr., Schoenfeld P, Sandborn WJ. The epidemiology and natural history of Crohn's disease in population-based patient cohorts from North America: a systematic review. Aliment Pharmacol Ther 2002;16:51-60.
3. Shivananda S, Lennard-Jones J, Logan R, Fear N, Price A, Carpenter L, van Blankenstein M. Incidence of inflammatory bowel disease across Europe: is there a difference between north and south? Results of the European Collaborative Study on Inflammatory Bowel Disease (EC-IBD). Gut 1996;39:690-697.
4. Abdullah BA, Gupta SK, Croffie JM, Pfefferkorn MD, Molleston JP, Corkins MR, Fitzgerald JF. The role of esophagogastroduodenoscopy in the initial evaluation of childhood inflammatory bowel disease: a 7-year study. J Pediatr Gastroenterol Nutr 2002;35:636-640.
5. Glickman RM. Inflammatory Bowel Disease: Ulcerative Colitis and Crohn's Disease. In: Fauci AS, Braynwald E, Isselbacher KJ, Wilson JD, Martin JB, Kasper DL, Hauser SL, and Longo DL, eds. Harrison's Principles of Internal Medicine. 14th edition ed. McGraw-Hill, 2007:1633-1646.
6. Morrison SJ, Shah NM, Anderson DJ. Regulatory mechanisms in stem cell biology. Cell 1997;88:287-298.
7. Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 1997;276:71-74.
8. Okamoto R, Yajima T, Yamazaki M, Kanai T, Mukai M, Okamoto S, Ikeda Y, Hibi T, Inazawa J, Watanabe M. Damaged epithelia regenerated by bone marrow-derived cells in the human gastrointestinal tract. Nat Med 2002;8:1011-1017.
9. Brittan M, Hunt T, Jeffery R, Poulsom R, Forbes SJ, Hodivala-Dilke K, Goldman J, Alison MR, Wright NA. Bone marrow derivation of pericryptal myofibroblasts in the mouse and human small intestine and colon. Gut 2002;50:752-757.
10. Peppelenbosch MP, van Deventer SJ. T-cell apoptosis and inflammatory bowel disease. Gut 2004;53:1556-1558.
11. Komori M, Tsuji S, Tsujii M, Murata H, Iijima H, Yasumaru M, Nishida T, Irie T, Kawano S, Hori M. Involvement of bone marrow-derived cells in healing of experimental colitis in rats. Wound Repair Regen 2005;13:109-118.
12. Horwitz EM, Le Blanc K, Dominici M, Mueller I, Slaper-Cortenbach I, Marini FC, Deans RJ, Krause DS, Keating A. Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. Cytotherapy 2005;7:393-395.
13. Copelan EA. Hematopoietic stem-cell transplantation. N Engl J Med 2006;354:1813-1826.
14. Ikehara S. Treatment of autoimmune diseases by hematopoietic stem cell transplantation. Exp Hematol 2001;29:661-669.
15. Van Bekkum DW. Stem cell transplantation in experimental models of autoimmune disease. J Clin Immunol 2000;20:10-16.
16. Marmont AM. Stem cell transplantation for autoimmune disorders. Coincidental autoimmune disease in patients transplanted for conventional indications. Best Pract Res Clin Haematol 2004;17:223-232.
17. Van Laar JM, Tyndall A. Adult stem cells in the treatment of autoimmune diseases. Rheumatology (Oxford) 2006;45:1187-1193.
18. Tyndall A, Gratwohl A. Blood and marrow stem cell transplants in autoimmune disease. A consensus report written on behalf of the European League Against Rheumatism (EULAR) and the European Group for Blood and Marrow Transplantation (EBMT). Br J Rheumatol 1997;36:390-392.
19. Castro J, Bentch H.L., Smith L, Kalter S, Bachier C, Meneghatti C, Moore A, Oliversen S, LeMaistre CF. Prolonged clinical remission in patients with inflammatory bowel disease (IBD) after high dose chemotherapy (HDC) and autologous blood stem cell transplantation. ASH Annual Meeting Abstracts 1996;88:133a.
20. Hawkey CJ, Snowden JA, Lobo A, Beglinger C, Tyndall A. Stem cell transplantation for inflammatory bowel disease: practical and ethical issues. Gut 2000;46:869-872.
21. Kashyap A, Forman SJ. Autologous bone marrow transplantation for non-Hodgkin's lymphoma resulting in long-term remission of coincidental Crohn's disease. Br J Haematol 1998;103:651-652.
22. Musso M, Porretto F, Crescimanno A, Bondi F, Polizzi V, Scalone R. Crohn's disease complicated by relapsed extranodal Hodgkin's lymphoma: prolonged complete remission after unmanipulated PBPC autotransplant. Bone Marrow Transplant 2000;26:921-923.
23. Soderholm JD, Malm C, Juliusson G, Sjodahl R. Long-term endoscopic remission of crohn disease after autologous stem cell transplantation for acute myeloid leukaemia. Scand J Gastroenterol 2002;37:613-616.
24. Musso M, Porretto F, Crescimanno A, Bondi F, Polizzi V, Scalone R. Crohn's disease complicated by relapsed extranodal Hodgkin's lymphoma: prolonged complete remission after unmanipulated PBPC autotransplant. Bone Marrow Transplant 2000;26:921-923.
25. Talbot DC, Montes A, Teh WL, Nandi A, Powles RL. Remission of Crohn's disease following allogeneic bone marrow transplant for acute leukaemia. Hosp Med 1998;59:580-581.
26. Lopez-Cubero SO, Sullivan KM, McDonald GB. Course of Crohn's disease after allogeneic marrow transplantation. Gastroenterology 1998;114:433-440.
27. Ditschkowski M, Einsele H, Schwerdtfeger R, Bunjes D, Trenschel R, Beelen DW, Elmaagacli AH. Improvement of inflammatory bowel disease after allogeneic stem-cell transplantation. Transplantation 2003;75:1745-1747.
28. Hawkey CJ. Stem cell transplantation for Crohn's disease. Best Pract Res Clin Haematol 2004;17:317-325.

29. Lin Yin JA, Jowitt SN. Resolution of immune-mediated diseases following allogeneic bone marrow transplantation for leukaemia. Bone Marrow Transplant 1992;9:31-33.
30. Lin Yin JA. Transplantation for new indications: autoimmune diseases. In: Atkinson K, ed. Clinical Bone Marrow Transplantation. Cambridge: Cambridge University Press, 1994:699-703.
31. Hinterberger W, Hinterberger-Fischer M, Marmont A. Clinically demonstrable anti-autoimmunity mediated by allogeneic immune cells favorably affects outcome after stem cell transplantation in human autoimmune diseases. Bone Marrow Transplant 2002;30:753-759.
32. Burt RK, Traynor A, Oyama Y, Craig R. High-dose immune suppression and autologous hematopoietic stem cell transplantation in refractory Crohn disease. Blood 2003;101:2064-2066.
33. Craig RM, Traynor A, Oyama Y, Burt RK. Hematopoietic stem cell transplantation for severe Crohn's disease. Bone Marrow Transplant 2003;32 Suppl 1:S57-S59.
34. Kreisel W, Potthoff K, Bertz H, Schmitt-Graeff A, Ruf G, Rasenack J, Finke J. Complete remission of Crohn's disease after high-dose cyclophosphamide and autologous stem cell transplantation. Bone Marrow Transplant 2003;32:337-340.
35. Oyama Y, Craig RM, Traynor AE, Quigley K, Statkute L, Halverson A, Brush M, Verda L, Kowalska B, Krosnjar N, Kletzel M, Whitington PF, Burt RK. Autologous hematopoietic stem cell transplantation in patients with refractory Crohn's disease. Gastroenterology 2005;128:552-563.
36. Armitage JO. Bone marrow transplantation. N Engl J Med 1994;330:827-838.
37. Tyndall A, Passweg J, Gratwohl A. Haemopoietic stem cell transplantation in the treatment of severe autoimmune diseases 2000. Ann Rheum Dis 2001;60:702-707.
38. Tabbara IA, Zimmerman K, Morgan C, Nahleh Z. Allogeneic hematopoietic stem cell transplantation: complications and results. Arch Intern Med 2002;162:1558-1566.
39. Friedenstein AJ, Deriglasova UF, Kulagina NN, Panasuk AF, Rudakowa SF, Luria EA, Ruadkow IA. Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. Exp Hematol 1974;2:83-92.
40. Dominici M, Le BK, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;8:315-317.
41. Bartholomew A, Sturgeon C, Siatskas M, Ferrer K, McIntosh K, Patil S, Hardy W, Devine S, Ucker D, Deans R, Moseley A, Hoffman R. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. Exp Hematol 2002;30:42-48.
42. Glennie S, Soeiro I, Dyson PJ, Lam EW, Dazzi F. Bone marrow mesenchymal stem cells induce division arrest anergy of activated T-cells. Blood 2005;105:2821-2827.
43. Krampera M, Glennie S, Dyson J, Scott D, Laylor R, Simpson E, Dazzi F. Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T-cells to their cognate peptide. Blood 2003;101:3722-3729.
44. Corcione A, Benvenuto F, Ferretti E, Giunti D, Cappiello V, Cazzanti F, Risso M, Gualandi F, Mancardi GL, Pistoia V, Uccelli A. Human mesenchymal stem cells modulate B-cell functions. Blood 2006;107:367-372.
45. Sotiropoulou PA, Perez SA, Gritzapis AD, Baxevanis CN, Papamichail M. Interactions between human mesenchymal stem cells and natural killer cells. Stem Cells 2006;24:74-85.
46. Spaggiari GM, Capobianco A, Becchetti S, Mingari MC, Moretta L. Mesenchymal stem cell-natural killer cell interactions: evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation. Blood 2006;107:1484-1490.
47. Nauta AJ, Kruisselbrink AB, Lurvink E, Willemze R, Fibbe WE. Mesenchymal stem cells inhibit generation and function of both CD34+-derived and monocyte-derived dendritic cells. J Immunol 2006;177:2080-2087.
48. Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 2005;105:1815-1822.
49. Ren G, Zhang L, Zhao X, Xu G, Zhang Y, Roberts AI, Zhao RC, Shi Y. Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. Cell Stem Cell 2008;2:141-150.
50. Krampera M, Cosmi L, Angeli R, Pasini A, Liotta F, Andreini A, Santarlasci V, Mazzinghi B, Pizzolo G, Vinante F, Romagnani P, Maggi E, Romagnani S, Annunziato F. Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells. Stem Cells 2006;24:386-398.
51. Gieseke F, Schutt B, Viebahn S, Koscielniak E, Friedrich W, Handgretinger R, Muller I. Human multipotent mesenchymal stromal cells inhibit proliferation of PBMCs independently of IFNgammaR1 signaling and IDO expression. Blood 2007;110:2197-2200.
52. Ren G, Zhang L, Zhao X, Xu G, Zhang Y, Roberts AI, Zhao RC, Shi Y. Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. Cell Stem Cell 2008;2:141-150.
53. Le BK, Ringden O. Immunomodulation by mesenchymal stem cells and clinical experience. J Intern Med 2007;262:509-525.
54. Rasmusson I, Le BK, Sundberg B, Ringden O. Mesenchymal stem cells stimulate antibody secretion in human B cells. Scand J Immunol 2007;65:336-343.
55. Zappia E, Casazza S, Pedemonte E, Benvenuto F, Bonanni I, Gerdoni E, Giunti D, Ceravolo A, Cazzanti F, Frassoni F, Mancardi G, Uccelli A. Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy. Blood 2005;106:1755-1761.
56. Wu GD, Nolta JA, Jin YS, Barr ML, Yu H, Starnes VA, Cramer DV. Migration of mesenchymal stem cells to heart allografts during chronic rejection. Transplantation 2003;75:679-685.
57. Chapel A, Bertho JM, Bensidhoum M, Fouillard L, Young RG, Frick J, Demarquay C, Cuvelier F, Mathieu E, Trompier F, Dudoignon N, Germain C, Mazurier C, Aigueperse J, Borneman J, Gorin NC, Gourmelon P, Thierry D. Mesenchymal stem cells home to injured tissues when co-infused with hematopoietic cells to treat a radiation-induced multi-organ failure syndrome. J Gene Med 2003;5:1028-1038.
58. Lee RH. Intravenous hMSCs improve myocardial infarction in mice because cells embolized in lung are activated to secrete the anti-inflammatory protein TSG-6. Cell Stem Cell. 2009 Jul 2;5(1):54-63.
59. Parekkadan B, Upadhyay R, Dunham J, Iwamoto Y, Mizoguchi E, Mizoguchi A, Weissleder R, Yarmush ML. Bone Marrow Stromal Cell Transplants Prevent Experimental Enterocolitis and Require Host CD11b(+) Splenocytes. Gastroenterology 2010.
60. Stolzing A, Jones E, McGonagle D, Scutt A. Age-related changes in human bone marrow-derived mesenchymal stem cells: consequences for cell therapies. Mech Ageing Dev 2008;129:163-173.
61. Caplan AI. Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. J Cell Physiol 2007;213:341-347.
62. Sun LY, Zhang HY, Feng XB, Hou YY, Lu LW, Fan LM. Abnormality of bone marrow-derived mesenchymal stem cells in patients with systemic lupus erythematosus. Lupus 2007;16:121-128.
63. Corre J, Mahtouk K, Attal M, Gadelorge M, Huynh A, Fleury-Cappellesso S, Danho C, Laharrague P, Klein B, Reme T, Bourin P. Bone marrow mesenchymal stem cells are abnormal in multiple myeloma. Leukemia 2007;21:1079-1088.
64. Arnulf B, Lecourt S, Soulier J, Ternaux B, Lacassagne MN, Crinquette A, Dessoly J, Sciaini AK, Benbunan M, Chomienne C, Fermand JP, Marolleau JP, Larghero J. Phenotypic and functional characterization of bone marrow mesenchymal stem cells derived from patients with multiple myeloma. Leukemia 2007;21:158-163.
65. Nauta AJ, Westerhuis G, Kruisselbrink AB, Lurvink EG, Willemze R, Fibbe WE. Donor-derived mesenchymal stem cells are immunogenic in an allogeneic host and stimulate donor graft rejection in a nonmyeloablative setting. Blood 2006;108:2114-2120.
66. Eliopoulos N, Stagg J, Lejeune L, Pommey S, Galipeau J. Allogeneic marrow stromal cells are immune rejected by MHC class I- and class II-mismatched recipient mice. Blood 2005;106:4057-4065.
67. Horwitz EM, Prockop DJ, Fitzpatrick LA, Koo WW, Gordon PL, Neel M, Sussman M, Orchard P, Marx JC, Pyeritz RE, Brenner MK. Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. Nat Med 1999;5:309-313.

68. Lazarus HM, Koc ON, Devine SM, Curtin P, Maziarz RT, Holland HK, Shpall EJ, McCarthy P, Atkinson K, Cooper BW, Gerson SL, Laughlin MJ, Loberiza FR, Jr., Moseley AB, Bacigalupo A. Cotransplantation of HLA-identical sibling culture-expanded mesenchymal stem cells and hematopoietic stem cells in hematologic malignancy patients. Biol Blood Marrow Transplant 2005;11:389-398.

69. Le Blanc K., Rasmusson I, Sundberg B, Gotherstrom C, Hassan M, Uzunel M, Ringden O. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. Lancet 2004;363:1439-1441.

70. Rubio D, Garcia-Castro J, Martin MC, de la FR, Cigudosa JC, Lloyd AC, Bernad A. Spontaneous human adult stem cell transformation. Cancer Res 2005;65:3035-3039.

71. Tolar J, Nauta AJ, Osborn MJ, Panoskaltsis MA, McElmurry RT, Bell S, Xia L, Zhou N, Riddle M, Schroeder TM, Westendorf JJ, McIvor RS, Hogendoorn PC, Szuhai K, Oseth L, Hirsch B, Yant SR, Kay MA, Peister A, Prockop DJ, Fibbe WE, Blazar BR. Sarcoma derived from cultured mesenchymal stem cells. Stem Cells 2007;25:371-379.

72. Djouad F, Plence P, Bony C, Tropel P, Apparailly F, Sany J, Noel D, Jorgensen C. Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. Blood 2003;102:3837-3844.

73. Karnoub AE, Dash AB, Vo AP, Sullivan A, Brooks MW, Bell GW, Richardson AL, Polyak K, Tubo R, Weinberg RA. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. Nature 2007;449:557-563.

74. Centeno CJ, Schultz JR, Cheever M, Robinson B, Freeman M, Marasco W. Safety and complications reporting on the re-implantation of culture-expanded mesenchymal stem cells using autologous platelet lysate technique. Curr Stem Cell Res Ther 2010;5:81-93.

75. Vogel G. Cell biology. To scientists' dismay, mixed-up cell lines strike again. Science 2010;329:1004.

76. Schrepfer S, Deuse T, Reichenspurner H, Fischbein MP, Robbins RC, Pelletier MP. Stem cell transplantation: the lung barrier. Transplant Proc 2007;39:573-576.

77. Hayashi Y, Tsuji S, Tsujii M, Nishida T, Ishii S, Iijima H, Nakamura T, Eguchi H, Miyoshi E, Hayashi N, Kawano S. Topical implantation of mesenchymal stem cells has beneficial effects on healing of experimental colitis in rats. J Pharmacol Exp Ther 2008;326:523-531.

78. Tanaka F, Tominaga K, Ochi M, Tanigawa T, Watanabe T, Fujiwara Y, Ohta K, Oshitani N, Higuchi K, Arakawa T. Exogenous administration of mesenchymal stem cells ameliorates dextran sulfate sodium-induced colitis via anti-inflammatory action in damaged tissue in rats. Life Sci 2008;83:771-779.

79. Gonzalez MA, Gonzalez-Rey E, Rico L, Buscher D, Delgado M. Adipose-derived mesenchymal stem cells alleviate experimental colitis by inhibiting inflammatory and autoimmune responses. Gastroenterology 2009;136:978-989.

80. Gonzalez-Rey E, Anderson P, Gonzalez MA, Rico L, Buscher D, Delgado M. Human adult stem cells derived from adipose tissue protect against experimental colitis and sepsis. Gut 2009;58:929-939.

81. Zhang Q, Shi S, Liu Y, Uyanne J, Shi Y, Shi S, Le AD. Mesenchymal stem cells derived from human gingiva are capable of immunomodulatory functions and ameliorate inflammation-related tissue destruction in experimental colitis. J Immunol 2009;183:7787-7798.

82. Garcia-Olmo D, Garcia-Arranz M, Herreros D, Pascual I, Peiro C, Rodriguez-Montes JA. A phase I clinical trial of the treatment of Crohn's fistula by adipose mesenchymal stem cell transplantation. Dis Colon Rectum 2005;48:1416-1423.

83. Garcia-Olmo D, Herreros D, Pascual I, Pascual JA, Del-Valle E, Zorrilla J, De-La-Quintana P, Garcia-Arranz M, Pascual M. Expanded adipose-derived stem cells for the treatment of complex perianal fistula: a phase II clinical trial. Dis Colon Rectum 2009;52:79-86.

84. Newman RE, Yoo D, LeRoux MA, nilkovitch-Miagkova A. Treatment of inflammatory diseases with mesenchymal stem cells. Inflamm Allergy Drug Targets 2009;8:110-123.

85. Duijvestein M, Vos AC, Roelofs H, Wildenberg ME, Wendrich BB, Verspaget HW, Kooy-Winkelaar EM, Koning F, Zwaginga JJ, Fidder HH, Verhaar AP, Fibbe WE, van den Brink GR, Hommes DW. Autologous bone marrow-derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: results of a phase I study. Gut 2010;59:1662-1669.

86. Arnulf B, Lecourt S, Soulier J, Ternaux B, Lacassagne MN, Crinquette A, Dessoly J, Sciaini AK, Benbunan M, Chomienne C, Fermand JP, Marolleau JP, Larghero J. Phenotypic and functional characterization of bone marrow mesenchymal stem cells derived from patients with multiple myeloma. Leukemia 2007;21:158-163.

87. Buhler L, Kurilla-Mahon B, Chang Q, Abraham S, Alwayn IP, Appel JZ, III, Newman D, Awwad M, White-Scharf ME, Sackstein R, Sachs DH, Cooper DK, Down JD. Cryopreservation and mycophenolate therapy are detrimental to hematopoietic progenitor cells. Transplantation 2002;74:1159-1166.

88. Chen TG, Chen JZ, Wang XX. Effects of rapamycin on number activity and eNOS of endothelial progenitor cells from peripheral blood. Cell Prolif 2006;39:117-125.

89. Guo J, Zeng Y, Liang Y, Wang L, Su H, Wu W. Cyclosporine affects the proliferation and differentiation of neural stem cells in culture. Neuroreport 2007;18:863-868.

90. Miriuka SG, Rao V, Peterson M, Tumiati L, Delgado DH, Mohan R, Ramzy D, Stewart D, Ross HJ, Waddell TK. mTOR inhibition induces endothelial progenitor cell death. Am J Transplant 2006;6:2069-2079.

91. Yabana T, Arimura Y, Tanaka H, Goto A, Hosokawa M, Nagaishi K, Yamashita K, Yamamoto H, Adachi Y, Sasaki Y, Isobe M, Fujimiya M, Imai K, Shinomura Y. Enhancing epithelial engraftment of rat mesenchymal stem cells restores epithelial barrier integrity. J Pathol. 2009 Jul;218(3):350-9.

CHAPTER 2

Long-term follow-up of autologous hematopoietic stem cell transplantation for severe refractory Crohn`s disease

Journal of Crohn's and Colitis 2011 Dec;5(6):543-9.

Daniel W. Hommes,[1] Marjolijn Duijvestein,[1] Zuzana Zelinkova,[2]
Pieter C.F. Stokkers,[3] Maartje Holsbergen-De Ley,[1] Jaap Stoker,[4]
Carlijn Voermans,[5] Marinus H.J. van Oers,[6] Marie José Kersten[6]

[1]Department of Gastroenterology and Hepatology, Leiden University Medical Center, Leiden, the Netherlands
[2]Department of Gastroenterology and Hepatology, Erasmus Medical Center, Rotterdam, the Netherlands
[3]Department of Gastroenterology and Hepatology, Academic Medical Center, Amsterdam, the Netherlands
[4]Department of Radiology, Academic Medical Center, Amsterdam, the Netherlands,
[5]Department of Experimental Immunohematology, Sanquin Research, Amsterdam and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands
[6]Department of Hematology, Academic Medical Center, Amsterdam, the Netherlands.

ABSTRACT

Although new therapeutic strategies have been developed to control Crohn`s disease, medical treatment for refractory cases is not able to prevent extensive and/or repeated surgery. Recently, several cases have been reported of successful remission induction in Crohn's disease (CD) patients by means of hematopoietic stem cell transplantation (HSCT). Here we report our long-term (4 to 6 years) outcome in three patients.

Patients

Three patients (two male, one female) with active severe CD were planned to undergo autologous HSCT. All patients were intolerant or refractory to conventional therapies, including anti-TNFα antibodies. Patients either refused surgery or surgery was considered not to be a feasible alternative due to the extensive disease involvement of the small intestine.

Methods

Peripheral blood stem cells were mobilized using a single infusion of cyclophosphamide 4g/m^2, followed on day 4 by subcutaneous injections with granulocyte colony-stimulating factor (G-CSF) 5μg/kg twice daily until leukapheresis. CD34+ cells were isolated after leukapheresis by magnetic cell sorting. In two of the patients a second round of stem cell mobilization using G-CSF only was required either because of low yield or because of insufficient recovery after CD34 selection. Prior to transplantation, immune ablation was achieved using cyclophosphamide 50 mg/kg/day (4 days), antithymocyte globulin 30 mg/kg/day (3 days) and prednisolone 500 mg (3 days). Endoscopy, barium small bowel enteroclysis and MRI enterography were performed.

Results

All three patients successfully completed stem cell mobilization and two of them subsequently underwent conditioning and autologous HSCT with CD34+ cell selection. Treatment was well tolerated, with acceptable toxicity. Now, 5 and 6 years post-transplantation, these patients are in remission under treatment. The third patient went into remission after mobilization and therefore she decided not to undergo conditioning and HSCT transplantation. After a successful pregnancy she relapsed two years later. Since then, she suffers from refractory CD for which we are now reconsidering conditioning and transplantation.

Conclusion

Autologous HSCT appears to be safe and can be an alternative strategy for CD patients with severe and therapy resistant disease.

INTRODUCTION

Crohn's disease is an inflammatory disorder of potentially any part of the gastrointestinal tract, leading to various intestinal but also extra-intestinal symptoms. It is thought that dysregulation of the normally controlled immune response to commensal bacteria in genetically susceptible patients drives the disease.[1] Although medical therapy of inflammatory bowel disease (IBD) has improved due to an extensive repertoire of immunosuppressive drugs and the introduction of anti-tumor necrosis factor-α (TNFα) compounds, the clinical course of the disease cannot be adequately controlled in a substantial group of patients. These patients may not respond or may become refractory to their medication, or they may develop treatment limiting toxicities.[2] The course of the disease is chronic and intermittent and the disease responds less well to medical therapy over time. Half of the patients require surgical resection during the course of the disease.[3] Unfortunately, surgery is accompanied by a high recurrence rate and many patients are at risk to develop short bowel syndrome. Therefore, there is an unmet need for more effective therapeutic strategies.

Evidence that hematopoietic stem cell transplantation (HSCT) would be an effective treatment for autoimmune diseases firstly came from several animal models[4, 5] and from incidental case reports on HSCT recipients with coexistent autoimmune disease.[6] Already more than 15 years ago a case report on the positive effect of HSCT on active IBD was published, followed by many others showing improvement of the clinical course of disease after HSCT indicated for other (malignant) diseases.[7] In the last couple of years, several cases have been reported of successful remission induction of patients with (therapy refractory) CD by means of an autologous HSCT. The most extensive experience on autologous HSCT applied specifically for CD has been reported by Oyama and colleagues.[8, 9] All twelve treated patients went into remission with a Crohn's disease activity index (CDAI) score of <150. There was no inpatient mortality and neutropenic fever was the most important complication. Recently published long-term follow-up data showed a clinical relapse-free survival of 63% at 3 years and 36% at 5 years. The percentage of patients in remission (CDAI < 150), steroid free, or medication free at any post transplant evaluation interval over 5 years post transplant was respectively 70%, 80% and 60%.[8]

Supported by these reports we have mobilized stem cells in three patients with severe refractory CD, for whom surgery was not a feasible option due to extensive disease activity, and performed autologous HSCT in two of these patients. Here, we report on the follow-up period of four to six years.

MATERIAL AND METHODS

Patient selection

Three patients with severe refractory CD were selected for an autologous HSCT procedure. The considerations for selection included longstanding refractory disease and exhaustion of the full range of therapeutic strategies such as anti-TNFα antibodies. In addition, surgery was unattractive because of unwillingness of the patients and the likelihood of either

developing short bowel syndrome (patient 1) or a definite ileostomy (patients 2 and 3). All cases were reviewed prior to selection by an independent gastroenterologist. The protocol was approved by the Medical Ethics Committee of the Academic Medical Center (AMC), Amsterdam, the Netherlands. All patients gave written informed consent.

HSCT protocol

Mobilization: Prior to cyclophosphamide treatment, semen was cryopreserved of the male patients. The female patient was offered treatment with the luteinizing hormone-releasing hormone analague gosereline, in an attempt to prevent chemotherapy-induced gonadal toxicity and premature ovarian failure.[10] Peripheral blood stem cells were mobilized using a regimen consisting of a single intravenous dose of cyclophosphamide 4g/m^2 followed on day 4 by granulocyte colony-stimulating factor (G-CSF) twice daily, 5μg/kg/day. Hyperhydration and mesna prophylaxis were given in order to prevent hemorrhagic cystitis. Partial bowel decontamination was obtained using oral ciprofloxacin and fluconazol. In addition, all patients received antibiotic prophylaxis consisting of clarithromycin. Peripheral blood stem cells (target >8 x10^6 CD34+ cells/kg body weight) were collected by large volume leukapheresis using a COBE Spectra leukapheresis machine (COBE BCT, Lakewood CO) as soon as the white blood cell count was >2x10^9/l and CD34+ cells were detectable in the peripheral blood. CD34+ cells were isolated within 48 hours after leukapheresis by magnetic cell sorting (CliniMACS CD34 selection procedure, Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The CD34+ cells were cryopreserved in dimethyl sulfoxide (DMSO) and stored in the vapor phase of liquid nitrogen until transplantation. *Conditioning:* Prior to transplantation, immune ablation was achieved using an immunosuppressive conditioning regimen consisting of cyclophosphamide 50 mg/kg/day intravenously and equine antithymocyte globulin (Lymphoglobulin, hATG, Genzyme Corporation, Boston, MA, USA) at a dosage of 30 mg/kg/day (on days -4, -3, -2). Premedication with methylprednisolone at a dose of 500 mg/day was administered intravenously for three consecutive days from day 4 and prednisolone was subsequently tapered over a period of 14 days. Hematopoietic stem cells were thawed and infused intravenously on day 0. *Transplantation:* Patients were hospitalized and nursed in clean rooms. Following transplantation, partial bowel decontamination was obtained using oral ciprofloxacin and fluconazol, and patients received antibiotic prophylaxis consisting of metronidazol and intravenous penicillin starting on day 5 until recovery of neutropenia. Supportive care were given when indicated. The hemoglobin level was maintained above 9.7 g/dl and platelet counts above 10 x10^9/l. All blood products, excluding the peripheral blood progenitor cells, were irradiated prior to infusion.

Evaluation of clinical response

Clinical assessment during HSCT and follow-up included medical history, physical examination and standard biochemical tests (including CRP). Endoscopy and both barium small bowel enteroclysis and MRI enterography were performed at baseline and after 3 and 6 months where indicated.

RESULTS

HSCT was offered to 3 patients (2 male, 1 female) with a median age of 28 (range 27-51). Patients were resistant or intolerant to conventional medication, including anti-TNFα therapy. All drugs, including steroids, were tapered after the mobilization procedure. Patient disease characteristics are described in table 1.

Safety and efficacy of stem cell mobilization

In general, the stem cell mobilization procedure with cyclophosphamide and G-CSF was safe. Besides minor expected side-effects such as nausea which were successfully managed, there were two major complications: in patient 1 a transient deterioration of kidney function was observed due to decreased oral intake because of nausea, which responded rapidly to intravenous fluids. Patient 2 had an episode of culture-negative neutropenic fever, which was empirically treated with broad-spectrum antibiotics. The blood cultures remained negative and the fever resolved promptly.

In patient 1, a sufficient number of stem cells was harvested (28.9×10^6 CD34+ cells/kg) in only one leukapheresis procedure. Despite a rather low recovery after CD34+ selection of 27%, a large enough amount of stem cells for reinfusion (7.87×10^6/kg) with a purity of 97% was obtained. In patient 2 and 3 however, a second stem cell mobilization procedure was required. In patient 2 the initial yield was sufficient (10.6×10^6 CD34+ cells/kg), but due to low recovery the yield after CD34 selection was borderline (2.5×10^6 CD34+ cells/kg). Therefore, a second mobilization procedure using only G-CSF 5 μg/kg twice daily for 5 days was performed two months later. The second mobilization yielded a total 2.1×10^6 CD34+ cells/kg, with 50% recovery after CD34 selection and a purity of 94%. In patient 3 the first attempt at stem cell mobilization with cyclophosphamide priming failed with a total yield after two leukapheresis procedures of 1.85×10^6 CD34+ cells/kg. Because of the low yield, CD34 selection was not performed. A second attempt at stem cell mobilization was done three months later using only G-CSF 5 μg/kg twice daily for 5 days. This resulted after two days in a yield of 5.84×10^6 CD34+ cells/kg, with a recovery of 53% and a purity of 89.7%. A third leukapheresis yielded 1.7×10^6 CD34+ cells/kg with a recovery of 66.5% and a purity of 79.7% after CD34 selection.

During the stem cell mobilization phase patient 1 required one transfusion of red blood cells (RBC) (3 units), patient 2 required two transfusions (in total 7 units of RBC) and patient 3 received one platelet transfusion (pooled platelets from five donors).

Safety of hematopoietic stem cell pretreatment and transfusion

During HSCT conditioning, patient 1 developed an allergic reaction (fever and hypotension) to antithymocyte globulin (ATG), that resolved with fluid challenge. Patient 2 had a quick and transient deterioration of kidney function due to hypotension after the first dose of ATG. In patient 1 and 2 respectively, a total of 5.9×10^6 and 3.5×10^6 CD34+ cells/kg were reinfused. The posttransplantation course was uneventful with an episode of rotavirus-associated diarrhea in both patients, clostridium difficile-

Patient	1	2	3
Basal demographic and clinical characteristics			
Sex	male	male	female
Year of birth	1953	1978	1978
Diagnosis	2003	2004	1999
Disease extension	stomach, duodenum, jejunum, ileum	colon including rectum	colon including rectum
Montreal			
Disease pattern	stricturing and penetrating	penetrating	penetrating
Extraintestinal disease manifestations	no	stomatitis	artralgia
Risk factors	none	previous smoker	previous smoker
Previous treatments			
5-asa	yes	yes	yes, intolerant
Corticosteroids	yes	yes	yes, intolerant
Antibiotics	yes	yes	yes
Azathioprine	yes	yes	yes, leucopenia
Infliximab	yes	yes	yes
Methotrexate	yes	yes	yes
visilizumab	no	yes	yes
Adalimumab	no	no	no
Surgery	no	no	no
HSCT			
Age at HSCT	51	27	28
Year of HSCT	2004	2005	2006
Crohn's disease medication			
At time of mobilization	prednisone 15 mg	prednisone 30 mg	none
At time of transplantation	prednisone 60 mg	prednisone 40 mg	not applicable

Table 1. Basal demographic, medical history. and clinical characteristics at time of HSCT.

associated diarrhea and an episode of fever caused by a central venous catheter-associated coagulase-negative staphylococci (CNS) bacteremia in patient 2. Recovery of neutrophils to >0.5x10^9/l was reached at day 12 and 14 in patient 1 and 2, and recovery of platelets >50x10^9/l was seen at day 14 and 9 respectively. Patient 1 required transfusion with six units of RBC and 3 platelet transfusions; patient 2 required six units of RBC and 1 platelet pool. Both patients discontinued their immunosuppressive therapies post HSCT and regained normal appetite and oral intake.

Clinical evaluation of HSCT treatment and follow-up

At a median follow-up of 62 (range 58-75) months all patients are alive. The two patients (Patient 1 and 2) who underwent the complete HSCT protocol are in clinical remission under immunosuppressive therapy. The patient (patient 3) who underwent stem cell mobilization only, remained in clinical remission and off of all immunosuppressive drugs for more than two years. A detailed description of the follow-up on these patients is described below:

Patient 1:

Patient 1 is a 51-year-old male with diagnosed CD of the stomach and almost the entire small bowel dependent on parenteral nutrition. This patient was offered autologous HSCT because he was unresponsive to immunosuppressive drugs (steroids in combination with azathioprine/ methotrexate) and biologic therapy (infliximab). Surgery would have put him at great risk of developing a short bowel syndrome as the whole small intestine was affected by the disease. After mobilization, an initial clinical response was observed. However, the patient experienced a clinical and endoscopic relapse within ten weeks. After immune ablation a total of 5.9x10^6/kg CD34+ cells/kg was reinfused and the patient could be discharged on day 15 after HSCT. In week 8 following transplantation, the patient was clinically in a partial remission, gaining weight (4 kg) without need for supplementary parenteral nutrition.

Follow-up: At 8 weeks clear improvement of the inflammatory lesions was observed endoscopically (Figure 1A). Also, barium enteroclysis (Figure 1B) and MRI enterography (Figure 1C) showed regression of small bowel stenosis. A sharp drop in CRP levels was seen after the mobilization phase, which further decreased during follow-up (Figure 1D). Two months after transplant the patient unexpectedly developed leukopenia (1.6x10^9/l), which resolved spontaneously within 2 months. Six months post-transplant the patient developed abdominal pain and gastric ulcers were detected during gastroscopy. Biopsies did not reveal Helicobacter pylori, cytomegalovirus, Epstein–Barr virus or other infectious agents and the ulcers had vanished two months later. MRI performed at 10 weeks showed no improvement compared to the baseline MRI, which showed bowel wall thickening, stenosis and inflammation of the horizontal part of the duodenum and jejunum. At the MRI performed at 6 months persistence of stenotic segments of the duodenum was seen. However, inflammatory activity evidently decreased and the stenosis in the jejunum disappeared (Figure 1D). As expected, residual strictures in the duodenum persisted which were dilated three (1x), twelve (3x), and 36 (1x) months after transplantation.

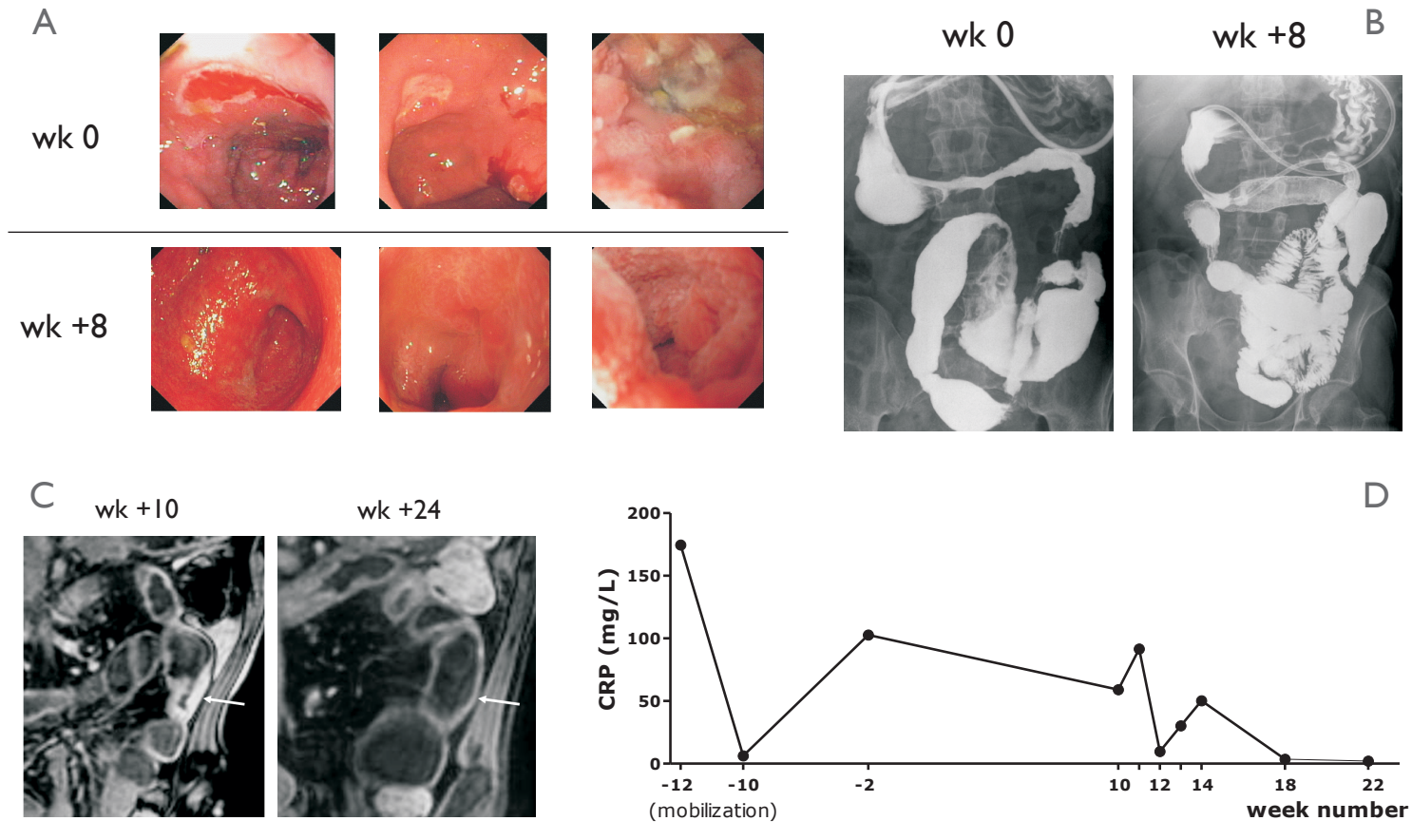


Figure 1. A Endoscopic changes after HSCT of patient 1. Duodenal ulcerations at the week 0 endoscopy (upper panels) and healing of duodenal mucosa at week 8 after HSCT (lower panels). B Barium small bowel enteroclysis of patient 1 at baseline and 8 weeks after HSCT shows regression of small bowel stenosis. C MRI enterography at week 10 and week 24 post HSCT. Coronal fat saturated T1-weighted sequence after intravenous contrast administration shows a reduction in the extension of a stenosis in the jenunum (arrow). D CRP levels during the mobilization phase and indicated number of weeks post HSCT.

Twelve months post-transplantation azathioprine was started (150 mg/day) and switched to certoluzimab two years post-transplantation because of leukopenia and thrombocytopenia. Repeat gastroscopy with enteral balloon dilatation of his proximal strictures was last performed in 2006. In 2007, he was admitted for unexplained ascites. Extensive work-up excluded common causes such as primary sclerosing cholangitis, portal hypertension, and hepatitis. The ascites resolved spontaneously without continued use of diuretics.

At present, the patient is doing relatively well. His weight has improved over the years and he now only sporadically vomits following meals. During his six years of follow up he did not have a luminal relapse of his CD and certolizumab treatment will be continued.

Patient 2

The next patient is a 27-year-old male patient with a short but complicated history of refractory CD of the colon unresponsive to immunosuppresive drugs (azathioprine and methotrexate), failing on infliximab and experimental anti-CD3 (visilizumab) therapy. In July of 2005 he underwent stem cell mobilization followed by HSCT in October of the same year. After HSCT, the stool frequency dropped from bloody and watery >10/day to semisolid without blood 6-7 times daily before hospital discharge. Dramatic improvement of the colonic mucosa was observed during colonoscopy at 10 weeks. The ulcerated areas had regained epithelium and no active inflammation was seen.

Follow-up: Six months after HSCT, 6-MP (mercaptopurine 100 mg) was started combined with infliximab. After having experienced no clinical complaints and having a high quality of life for five years, he relapsed with a mildly active distal colitis in the beginning of 2010. He was switched from infliximab to adalimumab, continuing 6-MP, and three months following his change of therapy he was again in complete clinical remission, with normalized CRP levels.

Patient 3

The third case is a 28-year-old female patient with a similar history. She has a completely refractory (included failure of anti-TNFα therapy) CD of her colon complicated by arthritis and perianal abscesses, needing multiple hospital admissions yearly. Patient refused colectomy and was therefore planned to undergo HSCT. In this patient, complete clinical remission was achieved after the stem cell mobilization procedure. For this reason the stem cell transplantation itself was deferred.

Follow-up: The patient remained in complete clinical and endoscopic remission without any medication for two years and had an uncomplicated pregnancy 1.5 years after mobilization. In 2008, she relapsed with a severe active Crohn's colitis for which she was treated with infliximab and methotrexate. After three infusions she experienced an allergic response to infliximab and, despite proper anti-allergic medication, a loss of response. Transplantation of the mobilized and stored CD34+ cells was proposed to the patient, which she declined because of cyclophosphamide associated toxicity. Consequently, she was included in a phase I trial on the safety and feasibility of autologous mesenchymal stromal cell infusion in refractory CD, for which conditioning is not needed (see Chapter 3 of this thesis).[11] No response was achieved and currently she experiences

persistent but tolerable activity with weekly adalimumab treatment in combination with steroids. As no medical therapeutic options are left we are now reconsidering conditioning and transplantation.

DISCUSSION

Our first experience of autologous HSCT for CD, employing similar protocols for mobilization, conditioning and supportive care as used in other published articles on HSCT for CD[12] is encouraging, as our patients have, since the onset of their disease, never been in remission for as long as after HSCT. The effect of HSCT was only partially successful in resolving Crohn's lesions at endoscopy. There was no transplant-related mortality and adverse effects were manageable. In one patient, an unexplained prolonged leukopenia following transplantation was observed which occurred two months after initial neutrophil recovery and which was not associated with infectious complications.

We did observe some difficulties with stem cell mobilization and CD34+ selection. In two of the patients, the initial procedure yielded an insufficient amount of CD34+ cells either before or after selection. This may in part have been due to the prior use of methotrexate in both patients, combined with thalidomide in one of the patients, even though this medication was stopped two months before the cyclophosphamide mobilization treatment. A subsequent second attempt at stem cell mobilization using G-CSF only was successful, although two leukapheresis procedures were required for both patients. The recovery of CD34+ cells after selection using the CliniMACS selection procedure was rather low (median 48.5%, range 23-66.5%). Whether this is related to the underlying disease is unknown, but similar low recovery rates were not reported in the series published by Oyama et al.[9]

Recently, safety and efficacy of autologous HSCT with unselected peripheral blood stem cells was demonstrated in four previously refractory CD patients.[13] In our study, peripheral blood cells were used after CD34+ cell selection in order to deplete activated T-cells to reduce the likelihood of relapse related to the reinfusion of autoreactive T-cells.[14] The last case is a good example of the role of the immunosuppressive effects of the stem cell mobilization regime which includes a high dose of cyclophosphamide. In 390 patients undergoing autologous HSCT for various autoimmune diseases a mobilization associated mortality of 1.5% and an overall procedure related mortality of 9% were found.[15] To evaluate the efficacy of HSC mobilization followed by high dose immune ablation and autologous stem cell transplantation versus HSC mobilization only, a multicenter, prospective, randomized phase III study has been initiated by the European Crohn's and Colitis Organisation (ECCO) in collaboration with the European Group for Blood and Marrow Transplantation (EBMT).

Not only autologous but also allogeneic HSCT has been shown to maintain remission in CD patients.[16] Theoretically, due to the additional graft versus autoimmunity effect in allogeneic HSCT this approach could give a longer lasting effect than autologous HSCT in the treatment of CD, even after withdrawal of prophylactic immunosuppression.[17] Allogeneic HSCT is, however, associated with graft-versus-host disease and has a considerably higher morbidity and mortality than autologous HSCT.[18]

Therefore, allogeneic HSCT at this point does not seem to be a viable option for a nonmalignant disease with very low mortality rates.

In conclusion, autologous HSCT is a relatively well tolerated and safe procedure. Given the considerable mortality rate of HSCT for autoimmune diseases, this treatment should only be considered in highly selected patients with severe and therapy resistant disease, or for patients for whom surgery is not a treatment option. Larger clinical trials are and should be conducted as more information is warranted.

ACKNOWLEDGMENTS

The authors thank the staff of the Laboratory for Stem Cell Transplantation at Sanquin Research (Amsterdam, the Netherlands) for their assistance.

REFERENCES

1. Cho JH. The genetics and immunopathogenesis of inflammatory bowel disease. *Nat Rev Immunol* 2008;8:458-466.
2. D'Haens G, Baert F, van AG, Caenepeel P, Vergauwe P, Tuynman H, De VM, van DS, Stitt L, Donner A, Vermeire S, Van de Mierop FJ, Coche JC, van der WJ, Ochsenkuhn T, van Bodegraven AA, Van Hootegem PP, Lambrecht GL, Mana F, Rutgeerts P, Feagan BG, Hommes D. Early combined immunosuppression or conventional management in patients with newly diagnosed Crohn's disease: an open randomised trial. *Lancet* 2008;371:660-667.
3. Peyrin-Biroulet L, Loftus EV, Jr., Colombel JF, Sandborn WJ. The natural history of adult Crohn's disease in population-based cohorts. *Am J Gastroenterol* 2010;105:289-297.
4. Ikehara S. Treatment of autoimmune diseases by hematopoietic stem cell transplantation. *Exp Hematol* 2001;29:661-669.
5. Van Bekkum DW. Stem cell transplantation in experimental models of autoimmune disease. *J Clin Immunol* 2000;20:10-16.
6. Marmont AM. Stem cell transplantation for autoimmune disorders. Coincidental autoimmune disease in patients transplanted for conventional indications. *Best Pract Res Clin Haematol* 2004;17:223-232.
7. Duijvestein M, van den Brink GR, Hommes DW. Stem cells as potential novel therapeutic strategy for inflammatory bowel disease. *J Crohns Colitis* 2080;2:99-106.
8. Burt RK, Craig RM, Milanetti F, Quigley K, Gozdziak P, Bucha J, Testori A, Halverson A, Verda L, de Villiers WJ, Jovanovic B, Oyama Y. Autologous nonmyeloablative hematopoietic stem cell transplantation in patients with severe anti-TNF refractory Crohn's disease: long-term follow-up. *Blood* 2010.
9. Oyama Y, Craig RM, Traynor AE, Quigley K, Statkute L, Halverson A, Brush M, Verda L, Kowalska B, Krosnjar N, Kletzel M, Whitington PF, Burt RK. Autologous hematopoietic stem cell transplantation in patients with refractory Crohn's disease. *Gastroenterology* 2005;128:552-563.
10. Badawy A, Elnashar A, El-Ashry M, Shahat M. Gonadotropin-releasing hormone agonists for prevention of chemotherapy-induced ovarian damage: prospective randomized study. *Fertil Steril* 2009;91:694-697.
11. Duijvestein M, Vos AC, Roelofs H, Wildenberg ME, Wendrich BB, Verspaget HW, Kooy-Winkelaar EM, Koning F, Zwaginga JJ, Fidder HH, Verhaar AP, Fibbe WE, van den Brink GR, Hommes DW. Autologous bone marrow-derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: results of a phase I study. *Gut* 2010;59:1662-1669.
12. Oyama Y, Craig RM, Traynor AE, Quigley K, Statkute L, Halverson A, Brush M, Verda L, Kowalska B, Krosnjar N, Kletzel M, Whitington PF, Burt RK. Autologous hematopoietic stem cell transplantation in patients with refractory Crohn's disease. *Gastroenterology* 2005;128:552-563.
13. Cassinotti A, Annaloro C, Ardizzone S, Onida F, Della VA, Clerici M, Usardi P, Greco S, Maconi G, Porro GB, Deliliers GL. Autologous haematopoietic stem cell transplantation without CD34+ cell selection in refractory Crohn's disease. *Gut* 2008;57:211-217.
14. Scime R, Cavallaro AM, Tringali S, Santoro A, Rizzo A, Montalbano L, Casa A, Cottone M. Complete clinical remission after high-dose immune suppression and autologous hematopoietic stem cell transplantation in severe Crohn's disease refractory to immunosuppressive and immunomodulator therapy. *Inflamm Bowel Dis* 2004;10:892-894.
15. Tyndall A, Passweg J, Gratwohl A. Haemopoietic stem cell transplantation in the treatment of severe autoimmune diseases 2000. *Ann Rheum Dis* 2001;60:702-707.
16. Lopez-Cubero SO, Sullivan KM, McDonald GB. Course of Crohn's disease after allogeneic marrow transplantation. *Gastroenterology* 1998;114:433-440.
17. Hinterberger W, Hinterberger-Fischer M, Marmont A. Clinically demonstrable anti-autoimmunity mediated by allogeneic immune cells favorably affects outcome after stem cell transplantation in human autoimmune diseases. *Bone Marrow Transplant* 2002;30:753-759.
18. Tabbara IA, Zimmerman K, Morgan C, Nahleh Z. Allogeneic hematopoietic stem cell transplantation: complications and results. *Arch Intern Med* 2002;162:1558-1566.

CHAPTER 3

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

Gut 2010;59:1662-1669.

Marjolijn Duijvestein,[1] Anne Christine W. Vos,[1] Helene Roelofs,[2]
Manon E. Wildenberg,[1] Barbara B. Wendrich,[1] Henricus W. Verspaget,[1]
Engelina M.C. Kooy-Winkelaar,[2] Frits Koning,[2] Jaap Jan Zwaginga,[2]
Herma H. Fidder,[1] Auke P. Verhaar,[1] Willem E. Fibbe,[2]
Gijs R. van den Brink,[1] Daniel W. Hommes[1]

[1]Department of Gastroenterology and Hepatology, Leiden University Medical Center, Leiden, the Netherlands
[2]Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, the Netherlands

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 $1\text{-}2 \times 10^6$ 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

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 5×10^4 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 1×10^6 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 $1\text{-}2\times10^6$ 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.

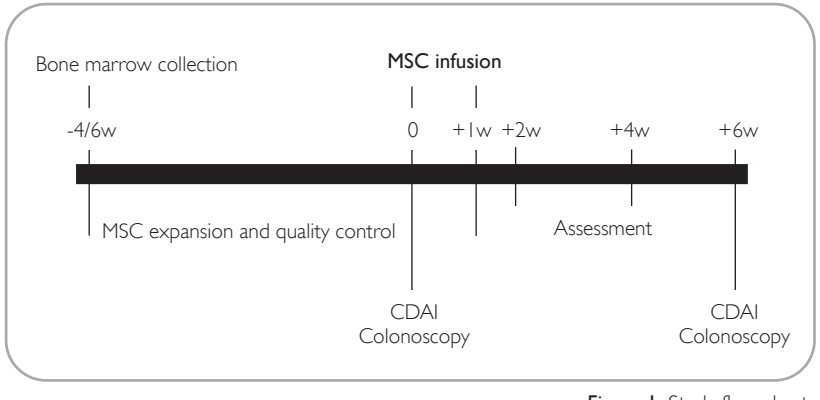


Figure 1. Study flow chart.

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.

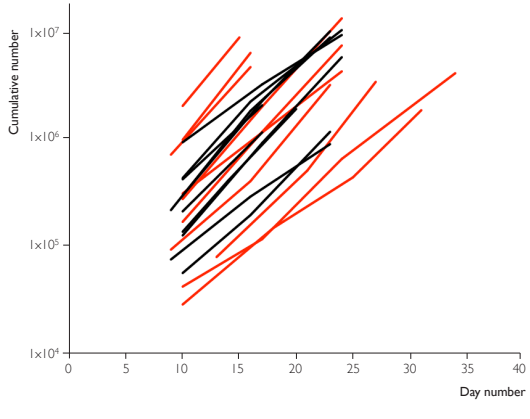


Figure 2. MSC expansion of CD patients (red) and healthy volunteers (black) expressed as theoretical cumulative cell number per ml harvested bone marrow.

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).

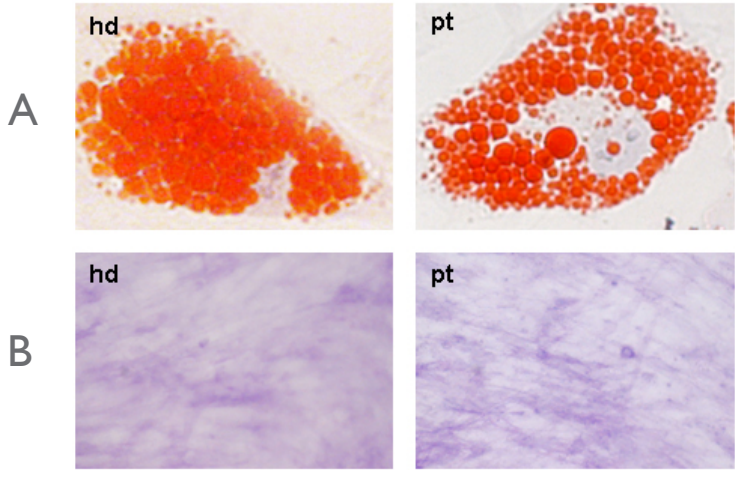


Figure 3. CD MSCs differentiate into mesenchymal lineages. (A) Adipocyte differentiation was demonstrated in MSCs cultured from a healthy donor (hd) and from a CD patient (pt) after which cells were stained with Oil Red O to show lipid droplets in the cytoplasm of the cell. (B) For osteoblast differentation 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).

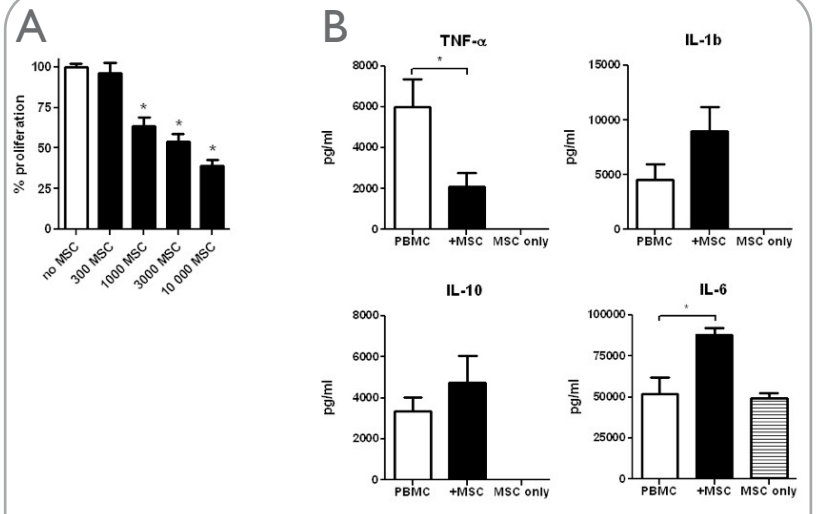


Figure 4. MSCs significantly inhibit the proliferation of PBMCs and this inhibition is dose-dependent. (A) 100 000 PBMCs cells were stimulated with anti-CD3/CD28 beads in the absence (white column) or presence (black columns) of indicated numbers of autologous MSCs. Proliferation measured by ^{3}H-thymidine 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

3

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

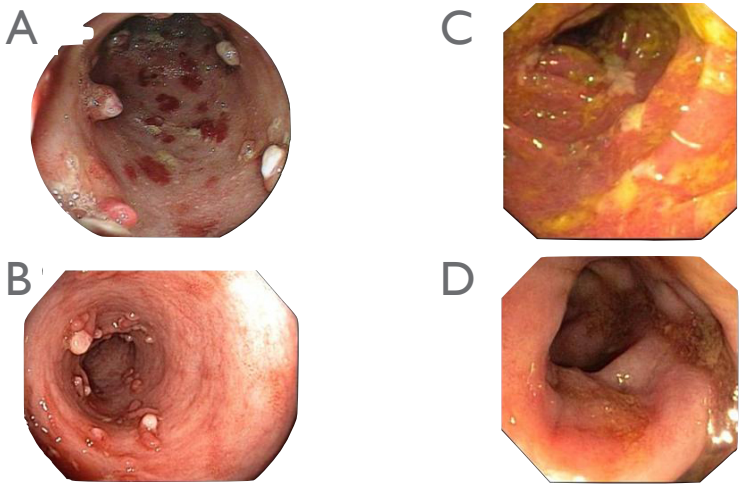


Figure 5. Endoscopy at week 0 (upper panels) and at 6 weeks (lower panels) after MSC treatment (two administrations of $2x10^6$ autologous bmMSCs) shows clear mucosal healing. Pictures A and B are from patient 2, pictures C and D from patient 3.

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]

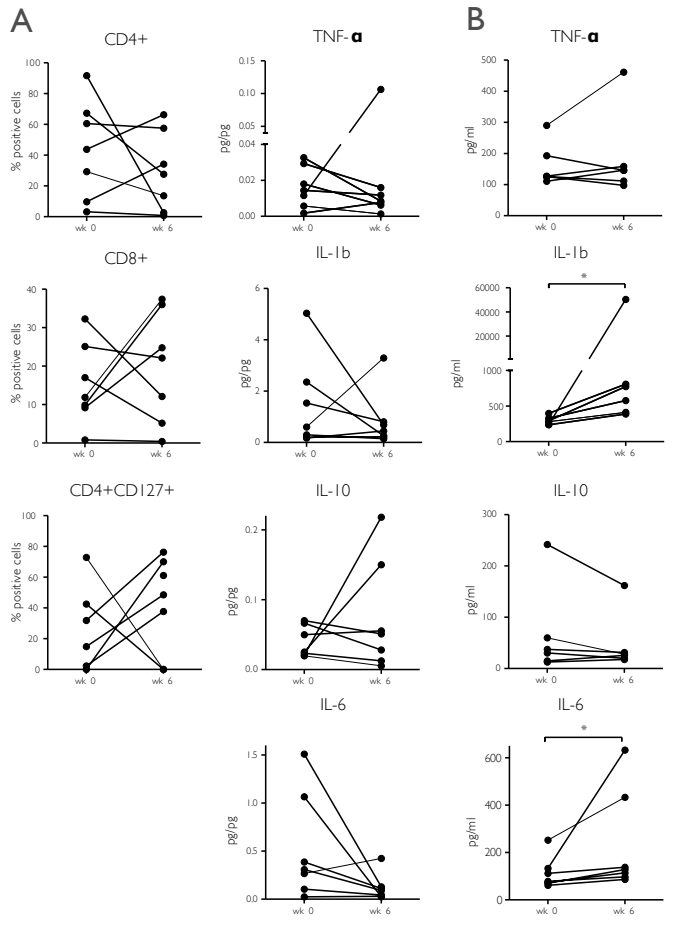


Figure 6 CD4+, CD8+, and CD4+CD127+ populations in biopsies of inflamed mucosa at week 0 and 6 (A, left panels). Cytokine levels of TNF-α, IL-1b, IL-10, and IL-6 were determined in mucosal biopsies (A, right panels) and serum (B) at week 0 and 6. Bars represent mean and SEM. *$P < 0.05$ for significant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

ACKNOWLEDGMENTS

We would like to thank Akin Inderson and Stefanie Kraus for performing bone marrow punctions. Marthe Verwey and Maartje Holsbergen-De Ley for collecting patient data and the technicians of the stem cell laboratory of the LUMC for MSC expansion. This work was supported by grants from the Dutch Digestive Diseases Foundation (MLDS, W07-17). Digest Science Grant, and a grant from ZonMW (TAS).

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.

REFERENCES

1. Aggarwal S, Pittenger MF, Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 2005;105:1815-1822.
2. Di Nicola M, Carlo-Stella C, Magni M, Milanesi M, Longoni PD, Matteucci P, Grisanti S, Gianni AM. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood 2002;99:3838-3843.
3. Krampera M, Glennie S, Dyson J, Scott D, Laylor R, Simpson E, Dazzi F. Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. Blood 2003;101:3722-3729.
4. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 2002;13:4279-4295.
5. Tondreau T, Meuleman N, Delforge A, Dejeneffe M, Leroy R, Massy M, Mortier C, Bron D, Lagneaux L. Mesenchymal stem cells derived from CD133-positive cells in mobilized peripheral blood and cord blood: proliferation, Oct4 expression, and plasticity. Stem Cells 2005;23:1105-1112.
6. Erices A, Conget P, Minguell JJ, Mesenchymal progenitor cells in human umbilical cord blood. Br J Haematol 2000;109:235-242.
7. Bruder SP, Jaiswal N, Haynesworth SE. Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. J Cell Biochem 1997;64:278-294.
8. Gonzalez-Rey E, Anderson P, Gonzalez MA, Rico L, Buscher D, Delgado M. Human adult stem cells derived from adipose tissue protect against experimental colitis and sepsis. Gut 2009;58:929-939.
9. Gonzalez MA, Gonzalez-Rey E, Rico L, Buscher D, Delgado M. Adipose-derived mesenchymal stem cells alleviate experimental colitis by inhibiting inflammatory and autoimmune responses. Gastroenterology 2009;136:978-989.
10. Le Blanc K, Frassoni F, Ball L, Locatelli F, Roelofs H, Lewis I, Lanino E, Sundberg B, Bernardo ME, Remberger M, Dini G, Egeler RM, Bacigalupo A, Fibbe W, Ringden O. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. Lancet 2008;371:1579-1586.
11. Le Blanc K, Rasmusson I, Sundberg B, Gotherstrom C, Hassan M, Uzunel M, Ringden O. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. Lancet 2004;363:1439-1441.
12. Garcia-Olmo D, Garcia-Arranz M, Herreros D. Expanded adipose-derived stem cells for the treatment of complex perianal fistula including Crohn's disease. Expert Opin Biol Ther 2008;8:1417-1423.
13. Ren G, Zhang L, Zhao X, Xu G, Zhang Y, Roberts AI, Zhao RC, Shi Y. Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. Cell Stem Cell 2008;2:141-150.
14. Parekkadan B, van Pol D, Suganuma K, Carter EA, Berthiaume F, Tilles AW, Yarmush ML. Mesenchymal stem cell-derived molecules reverse fulminant hepatic failure. PLoS One 2007;2:e941.
15. Duijvestein M, van den Brink GR, Hommes DW. Stem cells as potential novel therapeutic strategy for inflammatory bowel disease. J Crohns Colitis 2080;2:99-106.
16. Le Blanc K, Frassoni F, Ball L, Locatelli F, Roelofs H, Lewis I, Lanino E, Sundberg B, Bernardo ME, Remberger M, Dini G, Egeler RM, Bacigalupo A, Fibbe W, Ringden O. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. Lancet 2008;371:1579-1586.

17. Bernardo ME, Ball LM, Cometa AM, Roelofs H, Zecca M, Avanzini MA, Bertaina A, Vinti L, Lankester A, Maccario R, Ringden O, Le Blanc K, Egeler RM, Fibbe WE, Locatelli F. Co-infusion of ex vivo-expanded, parental MSCs prevents life-threatening acute GVHD, but does not reduce the risk of graft failure in pediatric patients undergoing allogeneic umbilical cord blood transplantation. Bone Marrow Transplant 2010.
18. Le Blanc K, Frassoni F, Ball L, Locatelli F, Roelofs H, Lewis I, Lanino E, Sundberg B, Bernardo ME, Remberger M, Dini G, Egeler RM, Bacigalupo A, Fibbe W, Ringden O. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. Lancet 2008;371:1579-1586.
19. Ball LM, Bernardo ME, Roelofs H, Lankester A, Cometa A, Egeler RM, Locatelli F, Fibbe WE. Co-transplantation of ex vivo expanded mesenchymal stem cells accelerates lymphocyte recovery and may reduce the risk of graft failure in haploidentical hematopoietic stem cell transplantation. Blood 2007.
20. Li Z, Arijs I, D'Haens G, Vermeire S, Noman M, Bullens D, Coorevits L, Sagaert X, Schuit F, Rutgeerts P, Ceuppens JL, Van Assche G. Reciprocal changes of Foxp3 expression in blood and intestinal mucosa in IBD patients responding to infliximab. Inflamm Bowel Dis 2010.
21. Nestle FO, Conrad C, Tun-Kyi A, Homey B, Gombert M, Boyman O, Burg G, Liu YJ, Gilliet M. Plasmacytoid predendritic cells initiate psoriasis through interferon-alpha production. J Exp Med 2005;202:135-143.
22. Dinesen L, Wang A, Vianello F, Tait P, Leen E, Davis J, Dazzi F, Ghosh S. Mesenchymal stem cells administered via novel selective mesenteric artery cannulation for the treatment of severe refractory Crohn's disease. J Crohns Colitis 2010;3:Pages S51-S52.
23. Sun LY, Zhang HY, Feng XB, Hou YY, Lu LW, Fan LM. Abnormality of bone marrow-derived mesenchymal stem cells in patients with systemic lupus erythematosus. Lupus 2007;16:121-128.
24. Arnulf B, Lecourt S, Soulier J, Ternaux B, Lacassagne MN, Crinquette A, Dessoly J, Sciaini AK, Benbunan M, Chomienne C, Fermand JP, Marolleau JP, Larghero J. Phenotypic and functional characterization of bone marrow mesenchymal stem cells derived from patients with multiple myeloma. Leukemia 2007;21:158-163.
25. Bocelli-Tyndall C, Bracci L, Spagnoli G, Braccini A, Bouchenaki M, Ceredig R, Pistoia V, Martin I, Tyndall A. Bone marrow mesenchymal stromal cells (BM-MSCs) from healthy donors and auto-immune disease patients reduce the proliferation of autologous- and allogeneic-stimulated lymphocytes in vitro. Rheumatology (Oxford) 2007;46:403-408.
26. Studeny M, Marini FC, Champlin RE, Zompetta C, Fidler IJ, Andreeff M. Bone marrow-derived mesenchymal stem cells as vehicles for interferon-beta delivery into tumors. Cancer Res 2002;62:3603-3608.
27. Djouad F, Plence P, Bony C, Tropel P, Apparailly F, Sany J, Noel D, Jorgensen C. Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. Blood 2003;102:3837-3844.
28. Karnoub AE, Dash AB, Vo AP, Sullivan A, Brooks MW, Bell GW, Richardson AL, Polyak K, Tubo R, Weinberg RA. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. Nature 2007;449:557-563.
29. Vogel G. Cell biology. To scientists' dismay, mixed-up cell lines strike again. Science 2010;329:1004.

CHAPTER 4

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

Cytotherapy 2011 Oct;13(9):1066-73.

Marjolijn Duijvestein,[1] Ilse Molendijk,[1] Helene Roelofs,[2]
Anne Christine W. Vos,[1] Auke P. Verhaar,[1] Marlies E.J. Reinders,[3]
Willem E. Fibbe,[2] Hein W. Verspaget,[1] Gijs R. van den Brink,[1,4]
Manon E. Wildenberg,[1] Daniel W. Hommes [1]

[1]Department of Gastroenterology and Hepatology, Leiden University Medical Center, Leiden, the Netherlands
[2]Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, the Netherlands
[3]Department of Nephrology, Leiden University Medical Center, Leiden, the Netherlands,
[4]current address: Tytgat Institute for Liver and Intestinal Research and Department of Gastroenterology and Hepatology, Academic Medical Center, Amsterdam, the Netherlands

ABSTRACT

Background and aim

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

Methods

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

Results

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

Conclusions

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

INTRODUCTION

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

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

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

MATERIALS AND METHODS

MSC isolation and expansion

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

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

IBD drugs

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

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

Flow cytometric analysis

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

Cell viability by MTT assay

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

In vitro differentiation

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

MSC/peripheral blood mononuclear cell (PBMC) proliferation assay

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

Statistical Analysis

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

Ethical Considerations

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

RESULTS

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

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

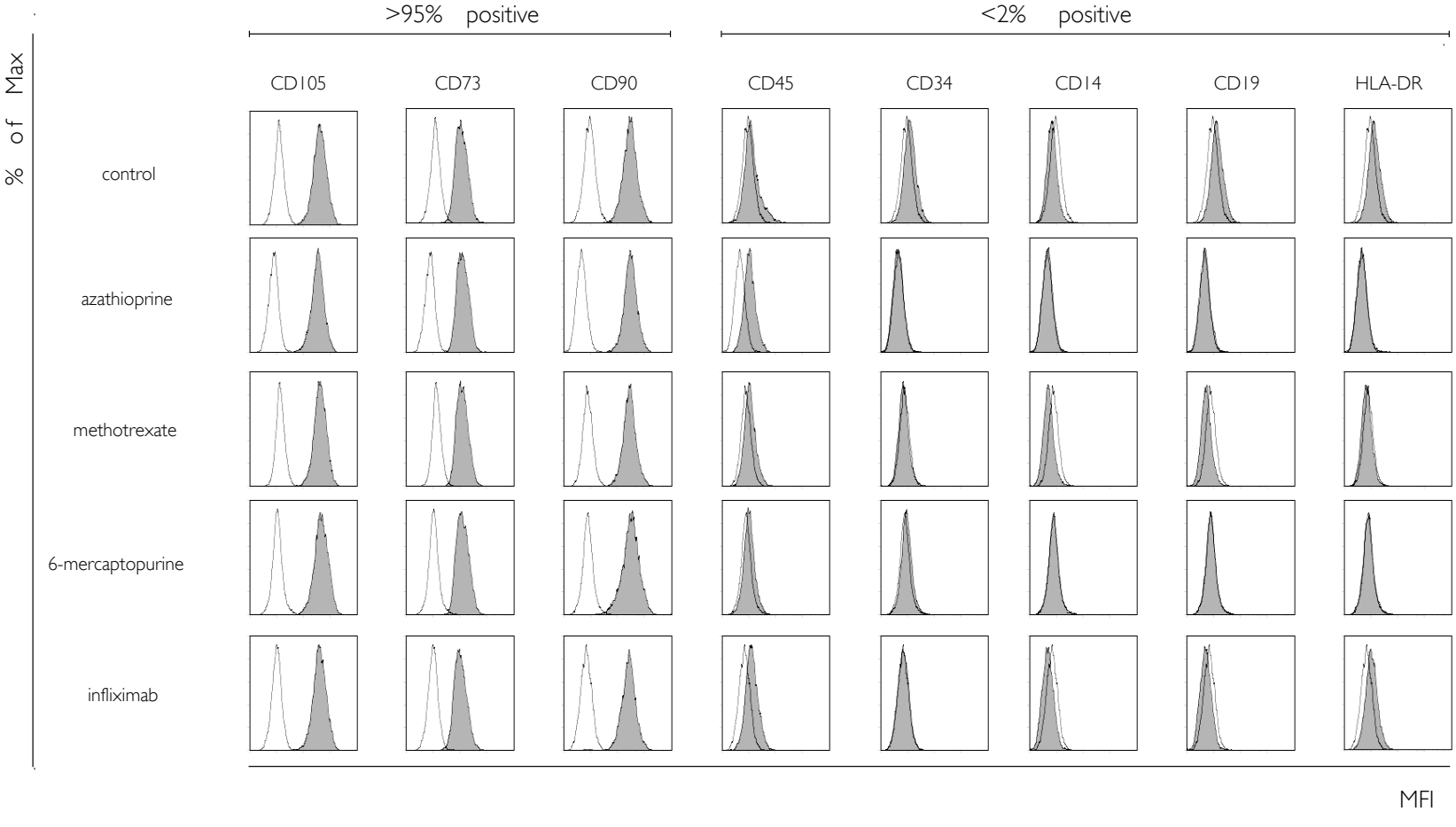


Figure 1. Effect of IBD drugs on MSC phenotype. Surface markers on control (upper panels) and IBD drug-treated MSCs (lower panels) were analyzed by flow cytometry. Markers are shown (solid histogram) compared to isotype control (line).

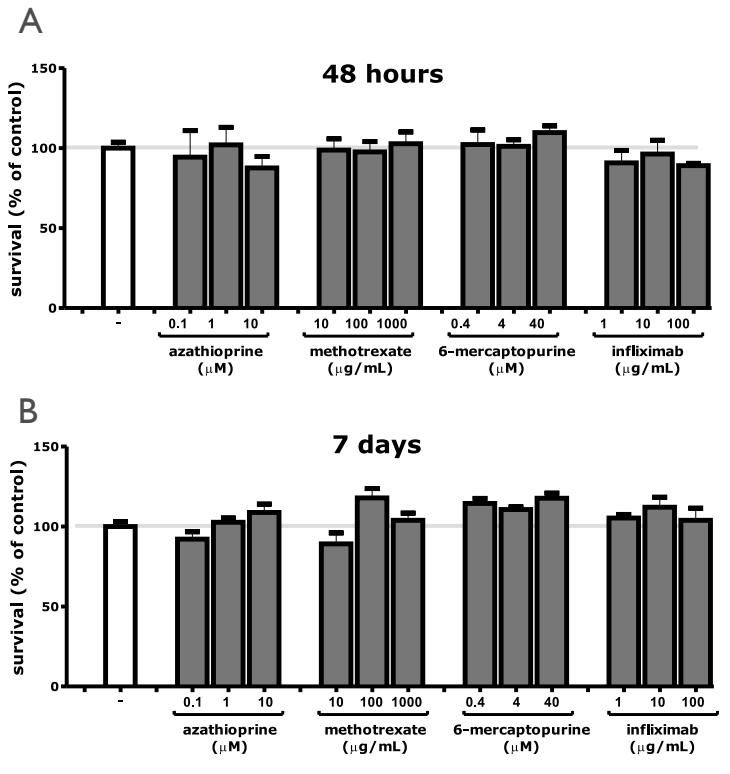


Figure 2. Cell viability of MSCs exposed to IBD drugs for 48 hours (A) and 7 days (B) as measured by MTT assay and expressed as a percentage of untreated control.

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

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

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

IBD drugs combined with MSCs retain immunosuppressive properties on activated PBMCs

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

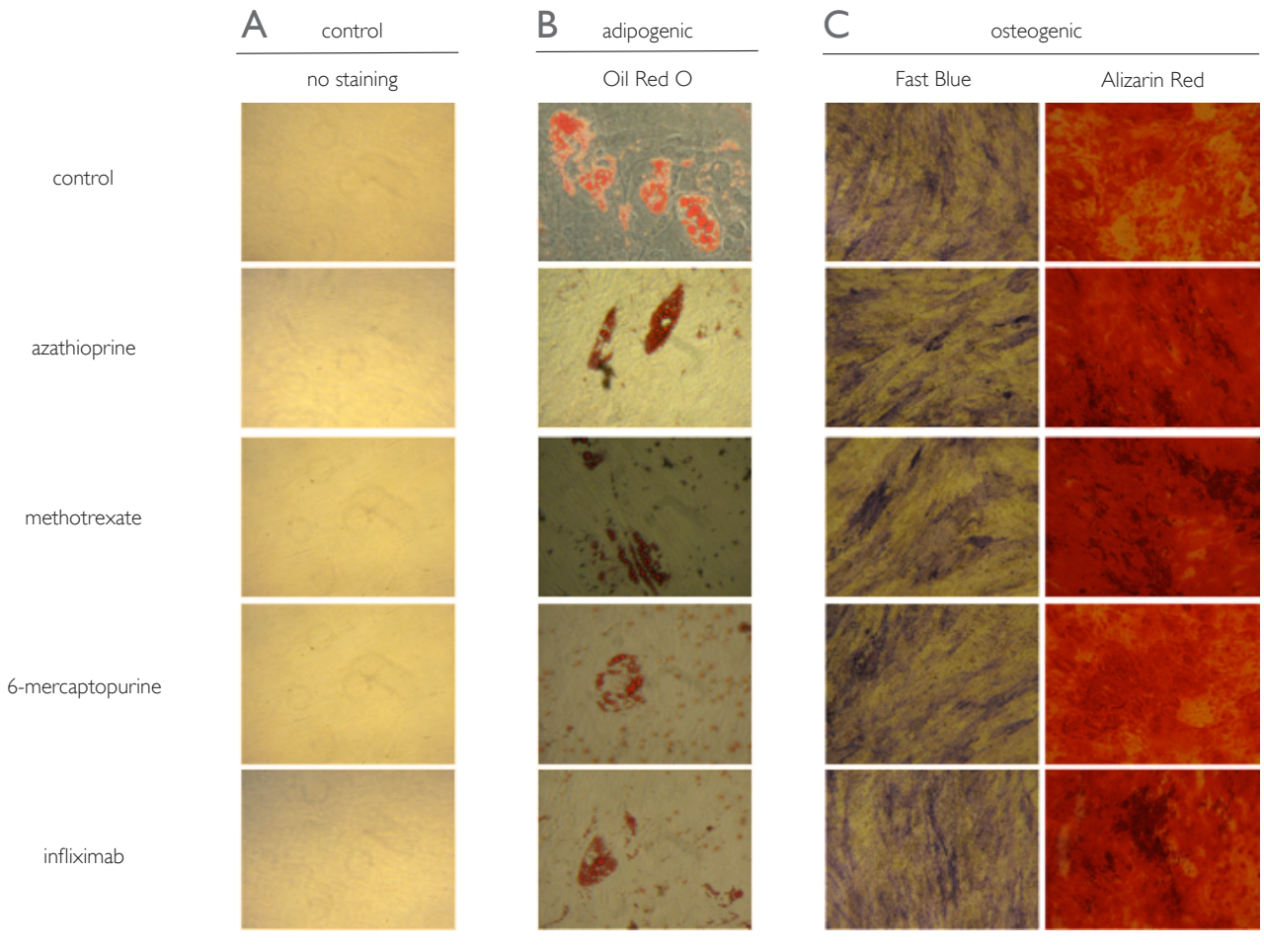


Figure 3. Differentiation capacity of MSCs into adipocytes and osteoblasts cultured in the presence of IBD drugs. A Control culture-expanded MSC morphology in the presence of IBD drug (no staining). B Lipid droplets in the cell cytoplasm of adipocytes were stained with Oil Red O. Magnifications 32x.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

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

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

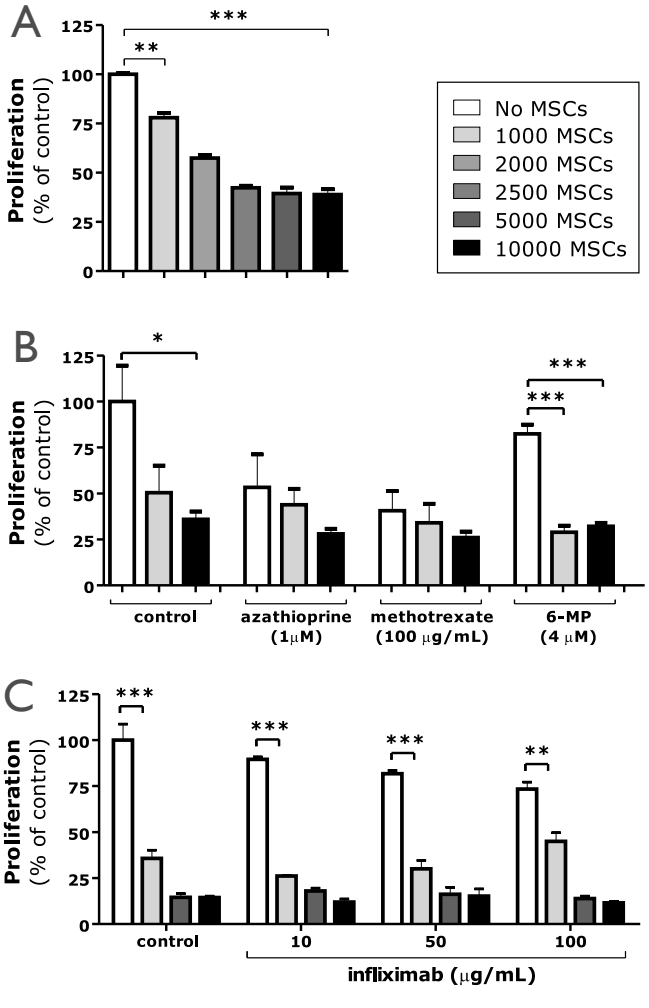


Figure 4. MSC and IBD drug interactions on PBMC proliferation. A MSCs inhibit the proliferation of activated PBMCs in a dose-dependent fashion. PBMCs (100 000 cells) were stimulated with αCD3/αCD28 beads and cocultured with different numbers of MSCs. B Effect of IBD drugs on the proliferation of PBMCs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

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

DISCUSSION

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

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

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

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

ACKNOWLEDGEMENTS

The authors would like to thank the technicians of the stem cell laboratory of the LUMC for MSC expansion.

FINANCIAL SUPPORT

This study was funded in part by the ZonMW Translational Adult Stem Cell (TAS) program and in part by the Digest Science Foundation.

REFERENCES

1. Duijvestein M, Vos AC, Roelofs H, Wildenberg ME, Wendrich BB, Verspaget HW, Kooy-Winkelaar EM, Koning F, Zwaginga JJ, Fidder HH, Verhaar AP, Fibbe WE, van den Brink GR, Hommes DW. Autologous bone marrow-derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: results of a phase I study. Gut 2010;59:1662-1669.
2. Dominici M, Le BK, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;8:315-317.
3. Garcia-Olmo D, Garcia-Arranz M, Herreros D, Pascual I, Peiro C, Rodriguez-Montes JA. A phase I clinical trial of the treatment of Crohn's fistula by adipose mesenchymal stem cell transplantation. Dis Colon Rectum 2005;48:1416-1423.
4. Garcia-Olmo D, Herreros D, Pascual I, Pascual JA, Del-Valle E, Zorrilla J, De-La-Quintana P, Garcia-Arranz M, Pascual M. Expanded adipose-derived stem cells for the treatment of complex perianal fistula: a phase II clinical trial. Dis Colon Rectum 2009;52:79-86.
5. Garcia-Olmo D, Herreros D, Pascual M, Pascual I, De-La-Quintana P, Trebol J, Garcia-Arranz M. Treatment of enterocutaneous fistula in Crohn's Disease with adipose-derived stem cells: a comparison of protocols with and without cell expansion. Int J Colorectal Dis 2009;24:27-30.
6. Chen TG, Chen JZ, Wang XX. Effects of rapamycin on number activity and eNOS of endothelial progenitor cells from peripheral blood. Cell Prolif 2006;39:117-125.
7. Miriuka SG, Rao V, Peterson M, Tumiati L, Delgado DH, Mohan R, Ramzy D, Stewart D, Ross HJ, Waddell TK. mTOR inhibition induces endothelial progenitor cell death. Am J Transplant 2006;6:2069-2079.
8. Guo J, Zeng Y, Liang Y, Wang L, Su H, Wu W. Cyclosporine affects the proliferation and differentiation of neural stem cells in culture. Neuroreport 2007;18:863-868.
9. Buhler L, Kurilla-Mahon B, Chang Q, Abraham S, Alwayn IP, Appel JZ, III, Newman D, Awwad M, White-Scharf ME, Sackstein R, Sachs DH, Cooper DK, Down JD. Cryopreservation and mycophenolate therapy are detrimental to hematopoietic progenitor cells. Transplantation 2002;74:1159-1166.
10. Le Blanc K, Frassoni F, Ball L, Locatelli F, Roelofs H, Lewis I, Lanino E, Sundberg B, Bernardo ME, Remberger M, Dini G, Egeler RM, Bacigalupo A, Fibbe W, Ringden O. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. Lancet 2008;371:1579-1586.
11. Ben-Horin S, Goldstein I, Fudim E, Picard O, Yerushalmi Z, Barshack I, Bank I, Goldschmid Y, Meir SB, Mayer L, Chowers Y. Early preservation of effector functions followed by eventual T-cell memory depletion: a model for the delayed onset of the effect of thiopurines. Gut 2009;58:396-403.
12. Tiede I, Fritz G, Strand S, Poppe D, Dvorsky R, Strand D, Lehr HA, Wirtz S, Becker C, Atreya R, Mudter J, Hildner K, Bartsch B, Holtmann M, Blumberg R, Walczak H, Iven H, Galle PR, Ahmadian MR, Neurath MF. CD28-dependent Rac1 activation is the molecular target of azathioprine in primary human CD4+ T lymphocytes. J Clin Invest 2003;111:1133-1145.
13. Kurnik D, Loebstein R, Fishbein E, Almog S, Halkin H, Bar-Meir S, Chowers Y. Bioavailability of oral vs. subcutaneous low-dose methotrexate in patients with Crohn's disease. Aliment Pharmacol Ther 2003;18:57-63.
14. Moshkowitz M, Oren R, Tishler M, Konikoff FM, Graff E, Brill S, Yaron M, Gilat T. The absorption of low-dose methotrexate in patients with inflammatory bowel disease. Aliment Pharmacol Ther 1997;11:569-573.
15. Bergan S, Rugstad HE, Bentdal O, Endresen L, Stokke O. Kinetics of mercaptopurine and thioguanine nucleotides in renal transplant recipients during azathioprine treatment. Ther Drug Monit 1994;16:13-20.
16. Rutgeerts P, D'Haens G, Targan S, Vasiliauskas E, Hanauer SB, Present DH, Mayer L, Van Hogezand RA, Braakman T, DeWoody KL, Schaible TF, Van Deventer SJ. Efficacy and safety of retreatment with anti-tumor necrosis factor antibody (infliximab) to maintain remission in Crohn's disease. Gastroenterology 1999;117:761-769.
17. Ball LM, Bernardo ME, Roelofs H, Lankester A, Cometa A, Egeler RM, Locatelli F, Fibbe WE. Co-transplantation of ex vivo expanded mesenchymal stem cells accelerates lymphocyte recovery and may reduce the risk of graft failure in haploidentical hematopoietic stem cell transplantation. Blood 2007.
18. Bernardo ME, Ball LM, Cometa AM, Roelofs H, Zecca M, Avanzini MA, Bertaina A, Vinti L, Lankester A, Maccario R, Ringden O, Le Blanc K, Egeler RM, Fibbe WE, Locatelli F. Co-infusion of ex vivo-expanded, parental MSCs prevents life-threatening acute GVHD, but does not reduce the risk of graft failure in pediatric patients undergoing allogeneic umbilical cord blood transplantation. Bone Marrow Transplant. 2011 Feb;46(2):200-7.
19. Chan JL, Tang KC, Patel AP, Bonilla LM, Pierobon N, Ponzio NM, Rameshwar P. Antigen-presenting property of mesenchymal stem cells occurs during a narrow window at low levels of interferon-gamma. Blood 2006;107:4817-4824.
20. Bartholomew A, Sturgeon C, Siatskas M, Ferrer K, McIntosh K, Patil S, Hardy W, Devine S, Ucker D, Deans R, Moseley A, Hoffman R. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. Exp Hematol 2002;30:42-48.
21. Bocelli-Tyndall C, Bracci L, Spagnoli G, Braccini A, Bouchenaki M, Ceredig R, Pistoia V, Martin I, Tyndall A. Bone marrow mesenchymal stromal cells (BM-MSCs) from healthy donors and auto-immune disease patients reduce the proliferation of autologous- and allogeneic-stimulated lymphocytes in vitro. Rheumatology (Oxford) 2007;46:403-408.
22. Krampera M, Glennie S, Dyson J, Scott D, Laylor R, Simpson E, Dazzi F. Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. Blood 2003;101:3722-3729.
23. Wright DC, Deol HS, Tuch BE. A comparison of the sensitivity of pig and human peripheral blood mononuclear cells to the antiproliferative effects of traditional and newer immunosuppressive agents. Transpl Immunol 1999;7:141-147.
24. Hornung N, Stengaard-Pedersen K, Ehrnrooth E, Ellingsen T, Poulsen JH. The effects of low-dose methotrexate on thymidylate synthetase activity in human peripheral blood mononuclear cells. Clin Exp Rheumatol 2000;18:691-698.
25. Krampera M, Cosmi L, Angeli R, Pasini A, Liotta F, Andreini A, Santarlasci V, Mazzinghi B, Pizzolo G, Vinante F, Romagnani P, Maggi E, Romagnani S, Annunziato F. Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells. Stem Cells 2006;24:386-398.
26. Ren G, Zhang L, Zhao X, Xu G, Zhang Y, Roberts AI, Zhao RC, Shi Y. Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. Cell Stem Cell 2008;2:141-150.
27. Nesbitt A, Fossati G, Bergin M, Stephens P, Stephens S, Foulkes R, Brown D, Robinson M, Bourne T. Mechanism of action of certolizumab pegol (CDP870): in vitro comparison with other anti-tumor necrosis factor alpha agents. Inflamm Bowel Dis 2007;13:1323-1332.
28. Gonzalez-Rey E, Anderson P, Gonzalez MA, Rico L, Buscher D, Delgado M. Human adult stem cells derived from adipose tissue protect against experimental colitis and sepsis. Gut 2009;58:929-939.
29. Gonzalez MA, Gonzalez-Rey E, Rico L, Buscher D, Delgado M. Adipose-derived mesenchymal stem cells alleviate experimental colitis by inhibiting inflammatory and autoimmune responses. Gastroenterology 2009;136:978-989.

30. Zhang Q, Shi S, Liu Y, Uyanne J, Shi Y, Shi S, Le AD. Mesenchymal stem cells derived from human gingiva are capable of immunomodulatory functions and ameliorate inflammation-related tissue destruction in experimental colitis. J Immunol 2009;183:7787-7798.
31. Le Blanc K, Rasmusson I, Gotherstrom C, Seidel C, Sundberg B, Sundin M, Rosendahl K, Tammik C, Ringden O. Mesenchymal stem cells inhibit the expression of CD25 (interleukin-2 receptor) and CD38 on phytohaemagglutinin-activated lymphocytes. Scand J Immunol 2004;60:307-315.
32. Maccario R, Moretta A, Cometa A, Montagna D, Comoli P, Locatelli F, Podesta M, Frassoni F. Human mesenchymal stem cells and cyclosporin a exert a synergistic suppressive effect on in vitro activation of alloantigen-specific cytotoxic lymphocytes. Biol Blood Marrow Transplant 2005;11:1031-1032.
33. Ringden O, Le Blanc K. Mesenchymal stem cells combined with cyclosporine inhibits cytotoxic T cells. Biol Blood Marrow Transplant 2006;12:693-694.
34. Hoogduijn MJ, Crop MJ, Korevaar SS, Peeters AM, Eijken M, Maat LP, Balk AH, Weimar W, Baan CC. Susceptibility of human mesenchymal stem cells to tacrolimus, mycophenolic acid, and rapamycin. Transplantation 2008;86:1283-1291.
35. Travis SP, Stange EF, Lemann M, Oresland T, Bemelman WA, Chowers Y, Colombel JF, D'Haens G, Ghosh S, Marteau P, Kruis W, Mortensen NJ, Penninckx F, Gassull M. European evidence-based Consensus on the management of ulcerative colitis: Current management. J Crohns Colitis 2008;2:24-62.
36. Dignass A, Van AG, Lindsay JO, Lemann M, Soderholm J, Colombel JF, Danese S, D'Hoore A, Gassull M, Gomollon F, Hommes DW, Michetti P, O'Morain C, Oresland T, Windsor A, Stange EF, Travis SP. The second European evidence-based Consensus on the diagnosis and management of Crohn's disease: Current management. J Crohns Colitis 2010;4:28-62.

CHAPTER 5

Pretreatment with interferon-gamma enhances the therapeutic activity of mesenchymal stromal cells in animal models of colitis

Stem Cells 2011 Oct;29(10):1549-58.

Marjolijn Duijvestein,[1] Manon E. Wildenberg,[1,7] Mick M. Welling,[2]
Simone Hennink,[1] Ilse Molendijk,[1] Vanessa van Zuylen,[3] Tjalling Bosse,[4]
Anne Christine W. Vos,[1] Eveline S.M. de Jonge-Muller,[1] Helene Roelofs,[3]
Louise van der Weerd,[2,5] Hein W. Verspaget,[1] Willem E. Fibbe,[3]
Anje A. te Velde,[6] Gijs R. van den Brink,[1,7] Daniel W. Hommes[1]

[1]Department of Gastroenterology and Hepatology, Leiden University Medical Center, Leiden, the Netherlands
[2]Department of Radiology, Division of Nuclear Medicine, Leiden University Medical Center, Leiden, the Netherlands
[3] Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, the Netherlands
[4]Department of Pathology, Leiden University Medical Center, Leiden, the Netherlands
[5]Department of Anatomy & Embryology, Leiden University Medical Center, Leiden, the Netherlands
[6]Center for Experimental and Molecular Medicine, Academic Medical Center, Amsterdam, the Netherlands
[7]Tytgat Institute for Liver and Intestinal Research, Academic Medical Center, Amsterdam, the Netherlands

ABSTRACT

Mesenchymal stromal cells (MSCs) are currently under investigation for the treatment of inflammatory disorders, including Crohn's disease. MSCs are pluripotent cells with immunosuppressive properties. Recent data suggest that resting MSCs do not have significant immunomodulatory activity, but that the immunosuppressive function of MSCs has to be elicited by interferon-gamma (IFN-γ). In this article, we assessed the effects of IFN-γ prestimulation of MSCs (IMSCs) on their immunosuppressive properties in vitro and in vivo. To this end, we pretreated MSCs with IFN-γ and assessed their therapeutic effects in dextran sodium sulfate (DSS)- and trinitrobenzene sulfonate (TNBS)- induced colitis in mice. We found that mice treated with IMSCs (but not MSCs) showed a significantly attenuated development of DSS-induced colitis. Furthermore, IMSCs alleviated symptoms of TNBS-induced colitis. IMSC-treated mice displayed an increase in body weight, lower colitis scores, and better survival rates compared to untreated mice. In addition, serum amyloid A protein levels and local proinflammatory cytokine levels in colonic tissues were significantly suppressed after administration of IMSC. We also observed that IMSCs showed greater migration potential than unstimulated MSCs to sites within the inflamed intestine. In conclusion, we show that prestimulation of MSCs with IFN-γ enhances their capacity to inhibit Th1 inflammatory responses, resulting in diminished mucosal damage in experimental colitis. These data demonstrate that IFN-γ activation of MSCs increases their immunosuppresive capacities and importantly, their therapeutic efficacy in vivo.

INTRODUCTION

Mesenchymal stromal cells (MSCs) are multipotent cells present in virtually all tissues of the body.[1] In addition to their function as progenitor cells, MSCs have been found to have significant immunosuppressive capacities.[2-4] Recent data suggest that resting MSCs do not have immunomodulatory activity, but when activated by IFN-γ, MSCs develop their full immunosuppressive potential.[5, 6] IFN-γ-activated MSCs secrete large amounts of chemokines to attract T-cells and inhibit T-cell proliferation and proinflammatory cytokine production.[6] Different mechanisms have been proposed that describe how this suppression of T-cell activity might be mediated. In humans, IFN-γ-induced indoleamine 2,3-dioxygenase (IDO) expression appears to be important,[7] whereas the expression of inducible nitric oxide synthase (iNOS) may be more crucial in mice. MSCs lacking the IFN-γ receptor or iNOS are incapable of inhibiting disease progression in a mouse model of graft-versus-host disease (GvHD).[8, 9] However, pretreatment of MSCs with IFN-γ was found to prevent the development of murine GvHD more efficiently than unstimulated MSCs.[9]

Due to their immunomodulatory properties, much interest has focused on investigating MSCs as a potential treatment for inflammatory disorders.[10] In fact, transplantation of MSCs has already been successfully used for treating GvHD.[11-15] We recently demonstrated that MSC administration is safe and feasible in patients with refractory Crohn's disease,[16] and various ongoing studies are aimed at evaluating the proper dose and mode of administration needed for effective results. Alternatively, potentiation of MSC effector mechanisms may improve the efficacy of anti-inflammatory treatment. Given the central role of IFN-γ activation on the immunosuppressive potential of MSCs, we examined the effect of IFN-γ pretreatment of MSCs in two mouse models of colitis.

MATERIAL AND METHODS

Mesenchymal stromal cell isolation

Human bone marrow

MSCs were expanded following a previously described protocol developed by the European Group for Blood and Bone Marrow Transplantation (EBMT).[14] In brief, bone marrow was harvested from patients undergoing orthopedic surgery. Bone marrow mononuclear cells (MNCs) were isolated by Ficoll density gradient centrifugation (density: 1.077 g/cm^3). Cells were then washed and resuspended in low-glucose Dulbecco's Modified Eagle Medium (Invitrogen, Paisley, UK) supplemented with penicillin and streptomycin (Lonza, Verviers, Belgium) and containing 10% fetal bovine serum (FBS, HyClone, Logan, UT) without any additional growth factors. MSCs were plated at a density of 160,000 cells per cm^2. All sampling procedures were performed in accordance with the Helsinki Declaration and were approved by the ethics committee of Leiden University Medical Center (LUMC). All patients provided informed consent.

Mouse bone marrow

MSCs were isolated from 8- to 10-week-old BALB/c mice (Harlan Laboratories, Horst, the Netherlands). Femoral heads and condyles were

removed and bone marrow was flushed. Cells were plated in alpha-minimum essential medium (alpha-MEM, Lonza) supplemented with penicillin and streptomycin (Lonza), 3 mM L-glutamine (Invitrogen) and 10% FBS (Invitrogen). After 24 hours, non-adherent cells were removed. Adherent cells were washed with phosphate-buffered saline (PBS), and medium was changed every 3-4 days. Animal procedures were carried out in compliance with the Institutional Standards for Humane Care and Use of Laboratory Animals. All experiments were approved by the Animal Care and Use Committee of the LUMC.

MSC expansion and preparation

Cultures were grown in 175 cm^2 flasks (Corning Life Sciences B.V., Schiphol-Rijk, the Netherlands) in a 37°C humidified incubator containing 5% CO_2. When the cultures reached >80% confluence, cells were trypsinized (trypsin/EDTA; Lonza) and replated at a density of 4000 cells per cm^2. MSCs were passaged no more than five times prior to use. To generate IFN-γ-prestimulated MSCs (hereafter referred to as IMSCs), cells were plated at the same density in media supplemented with either 500 U/mL recombinant human IFN-γ (Sigma-Aldrich, St Louis, Missouri, USA) or murine IFN-γ (PeproTech, Rocky Hill, NJ). Immunophenotyping of cultured MSCs was performed using flow cytometry for the following markers: MHCII, CD73, CD90, CD14, CD34, CD45, CD19 and CD105 (BD Biosciences, Franklin Lakes, NJ, USA). Samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences), and data were analyzed with FlowJo software (version 7.2.5., Treestar, San Carlos, CA, USA).

In vitro differentiation

MSCs were plated at a density of 5×10^4 cells/cm^2 in 24-well culture plates and kept in complete medium until cells reached 80-90% confluency. 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 then stained for alkaline phosphatase with Fast Blue (Sigma-Aldrich). 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 (Sigma-Aldrich). Lipid droplets were visualized by Oil Red O staining. For chondrogenic differentiation, MSCs were grown in serum-free, high-glucose Dulbecco's Modified Eagle Medium (Invitrogen) medium containing 50 μg/mL L-ascorbic-2-phosphate, 40 μg/mL L-proline and 100 μg/mL sodium pyruvate (Sigma-Aldrich) and supplemented with insulin-transferrin-selenium (ITS)+ culture supplement (BD Biosciences), 10 ng/mL transforming growth factor (TGF)-β1 (R&D systems, Abingdon, United Kingdom), 10^{-7} M dexamethasone (Sigma-Aldrich) and 500 ng/mL bone morphogenetic protein (BMP)-2 (R&D systems). Cells were stained with Toluidine blue to confirm chondrogenic differentiation.

RNA isolation and quantitative pcr

RNA was isolated using the RNAeasy mini kit (Qiagen, Hilden, Germany) and cDNA was generated using RevertAid reverse transcriptase (Fermentas, St Leon-Rot, Germany) and random hexamer primers

(Promega, Leiden, The Netherlands). Quantitative pcr reactions were carried out using Invitrogen primers for IDO (forward GATGAAG AAGTGGGCTTTGC, reverse CAGGCAGATGTTTAGCAATGA) and SybrGreen PCR Kit (Qiagen). For relative expression, data were normalized against expression of the household gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Measurement of inducible NOS (iNOS)

iNOS levels were measured in cell lysates using the PathScan Total iNOS Sandwich ELISA Kit (Cell Signaling Technology, Bioké, Leiden, the Netherlands) following the manufacturer's instructions.

MSC/peripheral blood mononuclear cell (PBMC) proliferation assay

Cultured human MSCs were plated in 96-well flat-bottom plates (Corning) and allowed to adhere overnight. Human PBMCs isolated from buffy coats were stimulated with anti-CD28/anti-CD3-coated Dynabeads (1 bead/5 cells, Invitrogen) and were seeded in Iscove's Modified Dulbecco's Media (IMDM, Invitrogen) supplemented with 5% human serum (Sanquin, the Netherlands), 5% FBS and 100 IU/mL interleukin (IL)-2 (LUMC Pharmacy, Leiden, the Netherlands). Transwell experiments were performed using 0.2μm membrane inserts (Nunc, Denmark). Cell proliferation was measured by ^{3}H-thymidine incorporation.

Induction of colitis and study design

Animals were housed in individually ventilated cages and supplied with drinking water and food ad libitum. Experiments were conducted using 6- to 8-week-old female mice. For dextran sulfate (DSS)-induced colitis, C57BL/6Jlco mice (Charles River, the Netherlands) were supplied with drinking water supplemented with 2.25% (w/v) DSS (MW 36 000-50 000 kDa; MP Biomedicals, Illkirch, France) for seven days. On day 0, mice were injected intraperitoneally with 0.5×10^{6} human MSCs diluted in 100 μL PBS, 0.5×10^{6} human IMSCs diluted in 100 μL PBS or a vehicle control (PBS alone). All mice were sacrificed on day 9 after the start of the experiment (Figure 1A).

Trinitrobenzene sulfonate (TNBS, Sigma-Aldrich) colitis was induced in BALB/c mice (Harlan Laboratories, Netherlands). To study the therapeutic effect of MSCs on established colitis, mice were presensitized through the skin on day 0. On day 7, 2.75 mg TNBS diluted in 40% ethanol was injected intrarectally (4 cm from the anus) using a 22 G catheter (Abbocath, Hospira Benelux, Brussels, Belgium). Six hours after the second TNBS instillation, mice were injected intraperitoneally with 1.0×10^{6} mouse MSCs, 1.0×10^{6} mouse IMSCs or PBS alone. All mice were sacrificed on day 9 (Figure 1B). For survival analysis, an additional cohort of BALB/c mice was treated intrarectally with 3.25 mg TNBS diluted in 40% ethanol on days 0 and 6. In this case, mice were injected intraperitoneally with 1.0×10^{6} mouse MSCs, 1.0×10^{6} mouse IMSCs or PBS alone 6 hrs after the first TNBS instillation (Figure 1C).

Assessment of inflammation

Body weights were recorded daily and wasting disease progression was calculated by percentage of weight loss from initial body weight. Animals

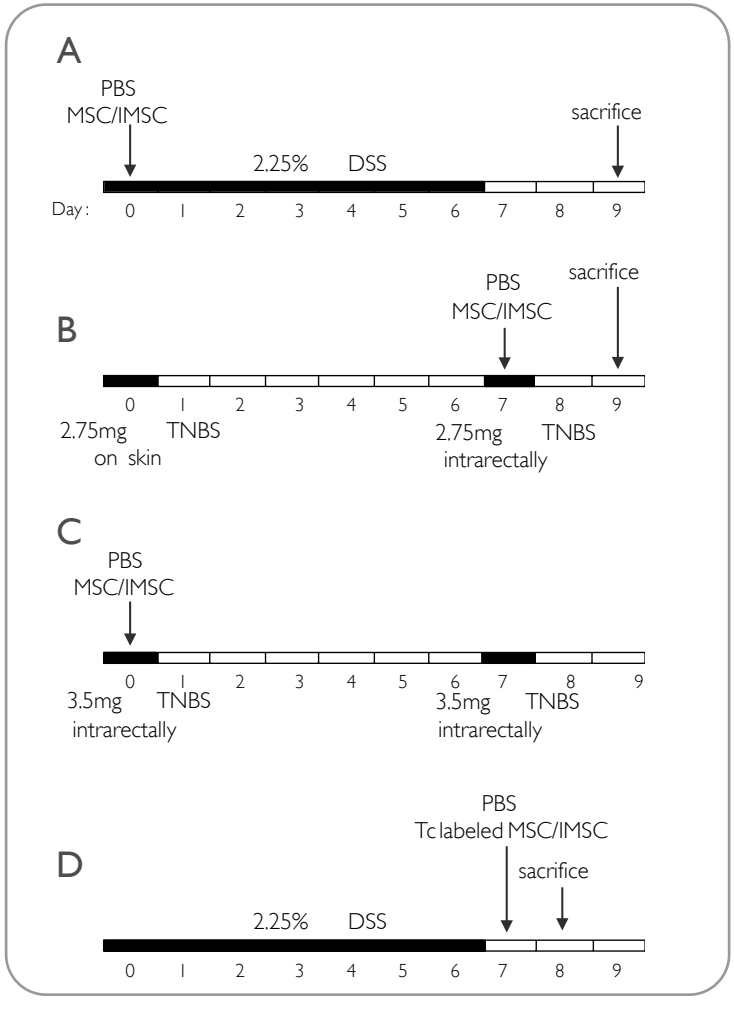


Figure 1. Schematic overview of experimental colitis experiments.

were withdrawn from the study when weight loss was >20% compared to that on day 0 or when weight loss was >10% within 24 hours. Blood samples were collected by cardiac puncture immediately upon sacrifice; serum was separated by centrifugation (10 000 x g for 10 min) from whole blood and stored at -20°C until use. Colons were removed and opened longitudinally. Two independent investigators blinded for treatment allocation scored the colons for the presence of loose stool, visible fecal blood, and macroscopic inflammation using a scale of 0 to 3 per item with a maximum score of 9, as previously described.[17] Tissue weights were recorded and used as an index of disease-related intestinal wall thickening. Colons were subsequently divided longitudinally into two parts: one part was immediately frozen in liquid nitrogen for protein extraction and cytokine determination, while the second part was stored in formalin and embedded in paraffin for (immuno)histological evaluation.

Histological analysis

Colonic segments were fixed in 10% neutral buffered formalin, serially dehydrated, cleared in xylene, and embedded in paraffin wax. Sections (4 µm thick) were collected on coded slides, stained with hematoxylin and eosin (H&E), and then independently and blindly scored by an experienced pathology resident. The histology damage score was calculated using the following criteria: (1) percentage of area involved, (2) number of follicle aggregates, (3) edema, (4) fibrosis, (5) erosion/ulceration, (6) crypt loss, and infiltration of (7) mononuclear and (8) polymorphonuclear cells. The percentage of area involved and crypt loss were scored on a scale of 0 to 4 (0, normal; 1, <10%; 2, 10%; 3, 10-50%; 4, >50%). Erosions were defined

as 0 if the epithelium was intact, 1 if the lamina propria was involved, 2 if ulcerations involved the submucosa, and 3 when ulcerations were transmural. The severity of the other parameters was scored on a scale of 0 to 3 (0, absent; 1, weak; 2, moderate; 3, severe). The histology damage score ranged from 0 points to a maximum of 26 points.[18]

Immunohistochemistry

T-cells were detected using an anti-CD3 antibody (1:1000, DakoCytomation BV, Eindhoven, the Netherlands). In brief, slides were incubated in 0.3% H_2O_2/methanol for 15 minutes at room temperature. After antigen retrieval, slides were blocked with Teng-T (10 mmol/L Tris, pH 8.0, 5 mmol/L EDTA, 0.15 mol/L NaCl, 0.25% gelatin, 0.05% (v/v) Tween-20) for 30 minutes and followed by overnight incubation at 4°C with primary antibody in PBS containing 0.1% Triton X-100 and 1% bovine serum albumin (BSA). Primary antibodies were detected using an avidin-biotin detection system. Secondary antibodies were obtained from DakoCytomation BV (1:200) (anti-rabbit, E0432), diluted in PBS containing .01% Triton-X and 1% BSA and incubated for 1 hour at room temperature. Slides then were incubated for 30 minutes with horseradish-peroxidase (HRP)-conjugated streptavidin-biotin (DakoCytomation BV). Antibodies were detected using the PowerVision Plus Poly-HRP detection system from Immunologic (DPVB + 110 HRP, Duiven, the Netherlands), and peroxidase activity was detected with Fast 3.3'-diaminobenzidine tablets (Sigma-Aldrich). Sections were counterstained with hematoxylin, dehydrated, and mounted in Pertex (Histolab Products AB, Göteborg, Sweden). Slides were analyzed and scored using ImageJ software (http://rsb.info.nih.gov/ij/).

Cytokine measurements

Cytokine levels were determined in colon mucosa. Colon homogenates were obtained using a Potter-Elvehjem glass homogenizer at 4°C in four volumes of Greenberger lysis buffer (150 mM NaCl, 15 mM Tris, pH 7.4, 1 mM $MgCl_2$, 1% Triton X-100) supplemented with protease inhibitors (Roche, Almere, the Netherlands). Samples were centrifuged at 30 000 × g for 10 minutes at 4°C and stored at −80°C until cytokine determination. Protein content was determined using the BCA Protein Assay (Thermo Scientific Pierce, Etten-Leur, the Netherlands), and cytokine levels in homogenates were measured using the Cytometric Bead Array System (BD Biosciences) following the manufacturer's instructions. Serum amyloid A (SAA) levels in mouse serum samples were measured using a commercial ELISA kit (Invitrogen).

Cell labeling with ^{99m}Tc-tropolon

To analyze the biodistribution and compare homing of MSCs and IMSCs, 1.0×10^6 human (I)MSCs were labeled with technetium-99m (^{99m}Tc) and intraperitoneally injected into different groups of animals on day 7 (n=3) (Figure 1D). In brief, human MSCs were washed twice with PBS by centrifugation in a two-step washing process (5 minutes at 150 × g). Cell labeling with technetium-99m was performed according to a leukocyte-labeling method that had been modified for stem cells.[19, 20] Ten milliliters of a 50 mg/mL 2-hydroxy-2,4,6-cycloheptatrienone solution (tropolone,

Aldrich Chemical Co., Milwaukee, WI, USA) was first dissolved in ethanol and then diluted in saline to 20% (v/v). A solution of $SnCl_2$ (950 mg/L $SnCl_2.2H_2O$ and 2 g/L sodium pyrophosphate.$10H_2O$) was obtained from a TechneScan® PYP® kit (Tyco Healthcare Mallinckrodt Medical BV, Petten, the Netherlands). KBH_4 (crystalline, Sigma, St. Louis, MO) was dissolved in 4 M NaOH and diluted with 0.1 M NaOH to a final concentration of 10 mg/ml. A lipophilic reactive complex was prepared by adding tropolone to a solution of $SnCl_2$ in a ratio of 2:1, which was then added to a solution of KBH_4 and ^{99m}Tc-sodium pertechnetate (200-500 MBq/mL, Technekow, Tyco Healthcare-Mallinckrodt Medical BV, Petten, the Netherlands). Two hundred microliters of the lipophilic reactive complex was immediately added to the cell pellet (containing $2x10^5$-$2x10^6$ cells) and gently shaken for 1 hour at 20°C. Labeled cell solutions were then centrifuged in a two-step washing process with PBS to remove non-cell-related (radioactive) reactants. To determine labeling efficiency, radioactivity in the final cell pellet and supernatant was measured using a dose calibrator (VDC 101, Veenstra Instruments, Joure, the Netherlands). Labeling yields ranged from 34-58%, and the stability of the complex was >90% for 24 hours in serum at 37°C. Animals were sacrificed 24 hours after injection of radiolabeled (I)MSCs in established colitis models, and relevant organs and tissues were excised, weighed, and measured for radioactivity using a dose calibrator. These values were calculated in relation to the percentage of the total dose of radioactivity administered (%ID/g tissue).

Microarray analysis

Human MSCs obtained from four individual subjects were treated with IFN-γ or left untreated for 6 days. RNA was isolated as described above, labeled using the Illumina TotalPrep RNA amplification kit (Ambion, Austin, TX) and hybridized to Ref12v3 Arrays. Data were quantile normalized and analyzed using Genome studio and Mayday software.[21]

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM) using two-sided t-tests and the Kruskal-Wallis test followed by Dunn's post hoc test for multiple comparisons. The Kaplan-Meier log-rank test was used to analyze survival curves. All analyses were performed using the SPSS statistical package (version 16.0, SPSS Inc., Chicago, IL) and GraphPad Prism software (version 5.01). $P < .05$ was considered statistically significant.

RESULTS

Pretreatment of MSCs with IFN-γ increases immunosuppression

Given the significant induction of various immune-related proteins by IFN-γ, we first examined whether MSCs continued to fulfill the minimal criteria for defining MSCs even after IFN-γ exposure.[22] Human MSCs cultured for 6 days in the presence of IFN-γ retained their capacity to adhere to plastic (data not shown). As previously described,[23-25] human MSCs increased the expression of major histocompatibility complex (MHC) class II genes in response to IFN-γ treatment (Figure 2A), whereas the expression of other

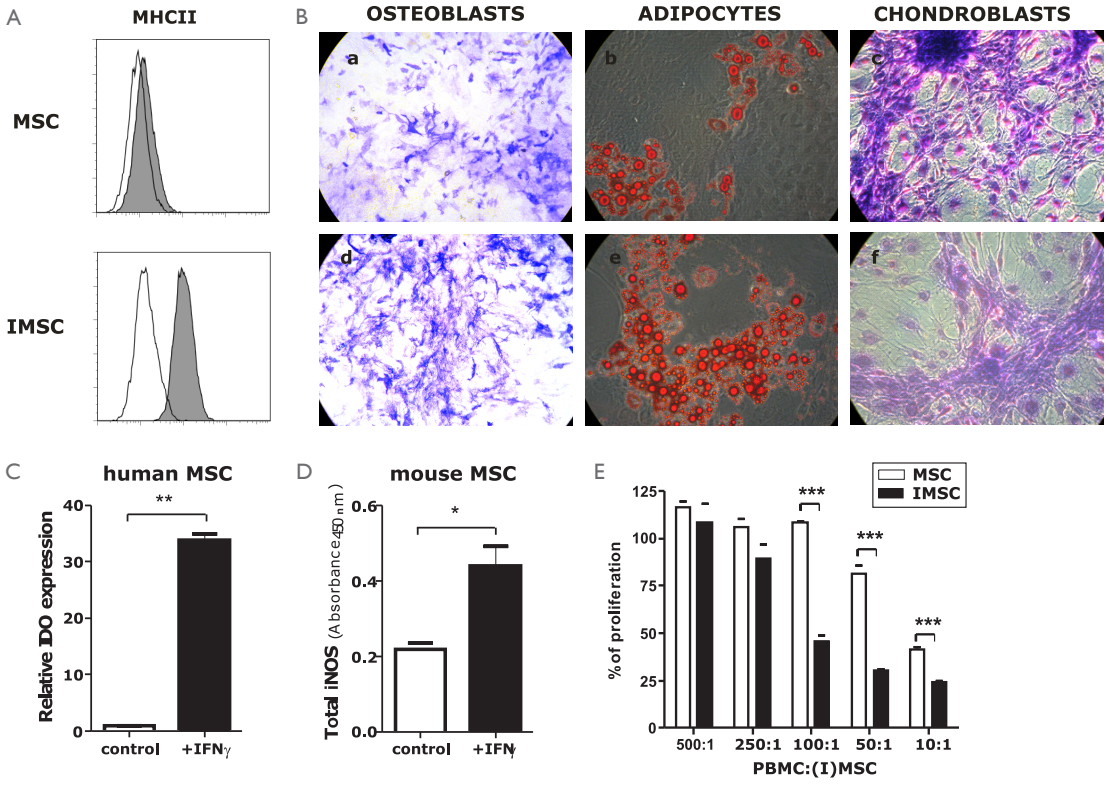


Figure 2. Effect of IFN-γ stimulation on MSC phenotype and function. A. Surface markers for MHC class II on unstimulated (upper panel) and IFN-γ-stimulated human MSCs (lower panel), as analyzed by flow cytometry. Markers are shown (solid histogram) compared to isotype control (line). B. Differentiation capacity of human MSCs (upper panels) and IMSCs (lower panels) into osteoblasts, adipocytes and chondroblasts. Osteogenic differentiation was detected with Fast Blue to show alkaline phosphatase activity (a, d). Lipid droplets in the cell cytoplasm were stained with Oil Red O (b, e). Toluidine blue staining corresponds with chondrogenesis (c, f). Magnifications, 10x (a, b, d, e); 32x (c, f). C. IFN-γ dependent relative IDO expression in human MSCs (left panel). Data are presented as means ± SEM; ** $P < .001$. D. IFN-γ treatment of mouse MSCs stimulates expression of iNOS (right panel). Data are presented as means ± SEM; * $P < .05$. E. Human IMSCs inhibit the proliferation of activated PBMCs in a dose-dependent fashion. PBMCs (100 000 cells) were stimulated with anti-CD3/CD28 beads and cocultured with MSCs (white columns) or IMSCs (black columns) in various ratios (PBMC:(I)MSC). Proliferation was measured by 3H-thymidine uptake in counts per minute and is expressed as a percentage of PBMC proliferation in the absence of MSCs. Data are presented as means ± SEM; *** $P < .001$.

markers in the MSC panel were unaffected (Figure 3). Furthermore, IMSCs maintained their in vitro differentiation capacity to form osteoblasts, adipocytes and chondroblasts (Figure 2B). In addition, we confirmed that IFN-γ stimulation leads to elevated IDO expression in human MSCs by RT-PCR (Figure 2C), and in increased iNOS levels in mouse MSC lysates (Figure 2D). At a functional level, human IMSCs significantly inhibited PBMC proliferation at lower PBMC:(I)MSC ratios compared to untreated MSCs, indicating that IMSCs have higher immunomodulatory capacities (Figure 2E).

IMSCs attenuate the development of DSS-induced colitis

Given the augmented immunosuppressive properties of IMSCs in vitro, we examined the anti-inflammatory effects of human IMSCs in vivo using a DSS-induced colitis model. Mice were intraperitoneally injected with human MSCs, IMSCs or PBS at the start of DSS induction (day 0). On day 9 (upon sacrifice), IMSC-treated mice showed significantly reduced weight loss compared to PBS-treated mice (Figure 4A). No differences in body weight were observed between MSC- and PBS-treated animals. Colons were assessed for the presence of loose stool, bleeding and macroscopic inflammation (Figure 4B). IMSC-treated (but not MSC-treated) mice had a significantly decreased disease score when compared with PBS-treated mice (Figure 4C). The PBS-treated group clearly exhibited a severe mucosal mononuclear cell infiltrate and disruption of crypt architecture (epithelial ulcerations and loss of goblet cells), whereas DSS-induced lesions were partially prevented in the IMSC-treated group (Figure 4D). These observations were also reflected by the histology scores (Figure 4E). These data consistently demonstrate that IMSCs are effective in the prevention of DSS-induced colitis.

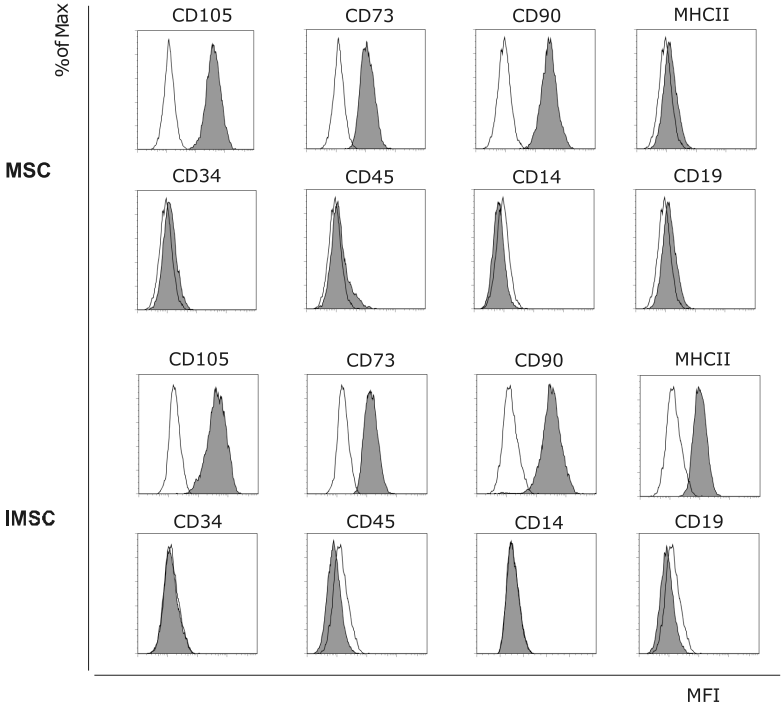


Figure 3. Effect of IFN-γ stimulation on MSC phenotype. Surface markers on unstimulated (upper panels) and IFN-γ-stimulated human MSCs (lower panels) were analyzed by flow cytometry. Markers are shown (solid histogram) compared to isotype control (line).

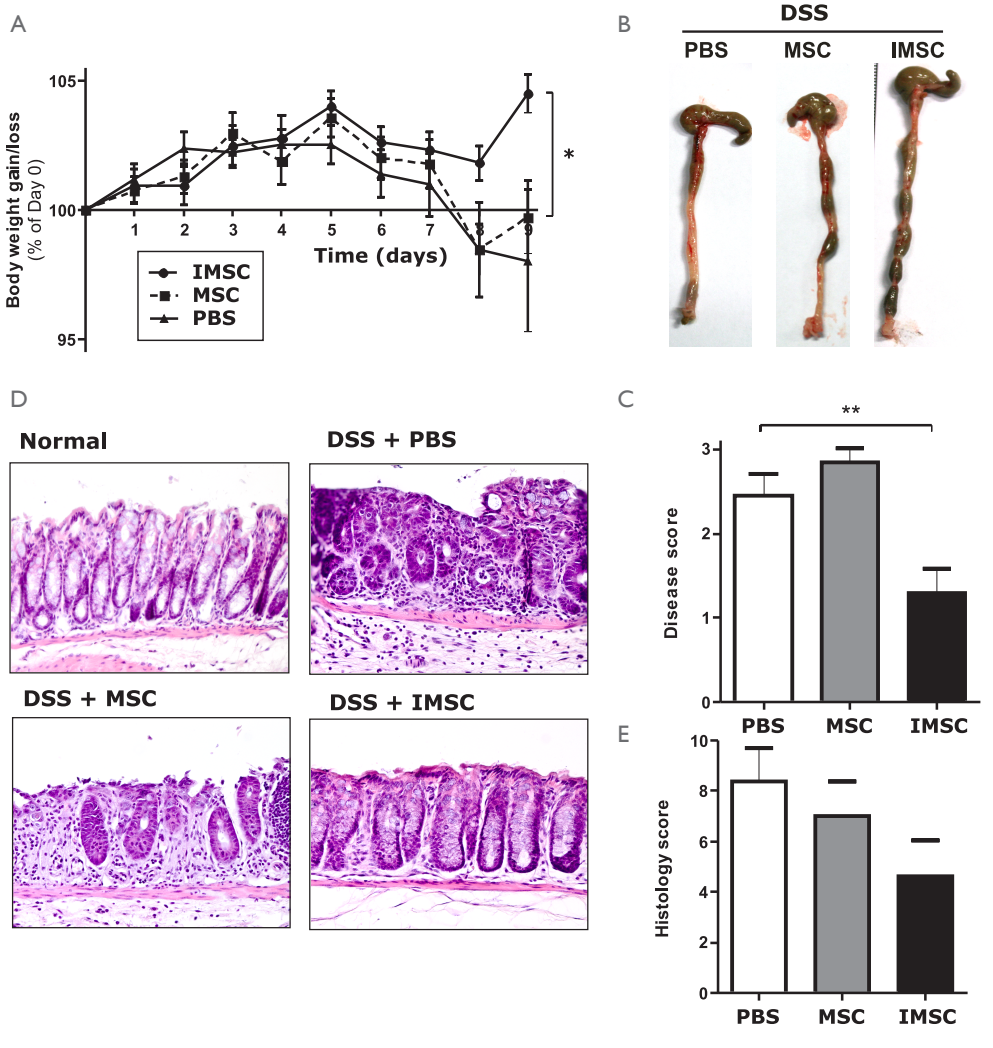


Figure 4. IMSCs prevent wasting disease progression in DSS-induced colitis. Mice received 2.25% DSS dissolved in drinking water for seven days. On day 0, mice were injected with human MSCs, IMSCs or PBS alone. Clinical evolution was monitored by body weight changes, macroscopic and microscopic scores. All mice were sacrificed on day 9. Data represent mean ± SEM; n=11 mice per group. A. Body weight changes during the course of the experiment. The values of body weight change are expressed as a percentage of initial body weight on day 0. *P < .05 compared to PBS treatment. B. Macroscopic images of representative mouse colons harvested on day 9. C. Macroscopic disease scores based on the presence of loose stool, bleeding and macroscopic inflammation determined on day 9 upon sacrifice. **P < .001. D. Histological evidence that IMSCs prevent DSS-induced pathology. Photomicrographs (20x magnification) of an H&E-stained paraffin section of a representative mouse colon from each treatment group. E. Histology scores were derived from microscopic analyses of longitudinal colon sections from each mouse.

IMSCs alleviate TNBS-induced colitis

We next examined the effects of IMSC administration on acute Th1-mediated colitis in mice. The therapeutic effect of mouse IMSCs was assessed after skin sensitization on day 0, followed by intrarectal TNBS challenge on day 7. Six hours after TNBS challenge, mice received an injection of mouse MSCs, IMSCs or PBS. Body weight, colon weight and length, as well as macroscopic signs indicative of colonic inflammation were determined upon sacrifice (Figure 5A, page 78). TNBS-induced colitis resulted in a body weight loss of 4.9% in PBS-treated animals. MSC-treated mice displayed an average decrease of 1.3% in body weight, whereas IMSC-treated mice had gained 2.5% (Figure 5B). This body weight increase was reflected in a lower disease score (Figure 5C) and a decreased colon weight-to-length ratio (Figure 5D). Histological grading showed that the IMSC group exhibited diminished severity of colitis (Figure 5E), primarily as a result of lower levels of inflammation, less crypt loss, and reduced number of granulocytes and mononuclear cells. Histochemistry demonstrated prominent infiltration of mononuclear cells into the affected colons, particularly in severely damaged regions. Immunohistochemistry showed that CD3+ cells (T-cells) were predominantly located in regions of inflammation (Figure 5F), and that IMSC treatment appeared to reduce the number of T-cells.

For survival analysis, a rectal enema of TNBS was applied to BALB/c mice on days 0 and 7. Mouse MSCs, IMSCs or PBS alone was injected intraperitoneally 6 hours after the first TNBS administration. Injection of either MSCs or IMSCs similarly increased the survival of BALB/c mice after TNBS-induced colitis (Figure 6).

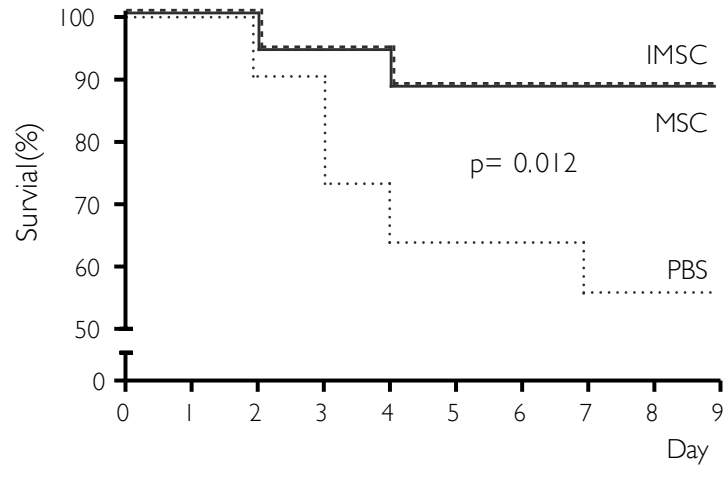


Figure 6. Treatment with mouse MSCs and IMSCs improves the survival of Balb/c mice with TNBS-induced colitis. Colitis was induced by intrarectal injection of TNBS in Balb/c mice. Mice were injected intraperitoneally with MSCs, IMSCs or PBS alone on day 0, starting 6 h after intrarectal TNBS administration. Survival (Kaplan-Meier analysis) was compared between (I)MSC- and PBS-treated animals. n=11 mice per group; *P = .012.

IMSCs reduce systemic markers of inflammation

We next examined the influence of IMSCs on serum amyloid A (SAA) levels. SAA is a systemic marker of inflammation similar to C-reactive protein (CRP) in humans. We detected high SAA levels in serum isolated from PBS-treated mice, which corresponded to the severe colitis seen in these animals. In contrast, treatment with mouse IMSCs resulted in significantly lower SAA serum levels, confirming the decreased inflammatory responses observed in these animals (Figure 7A).

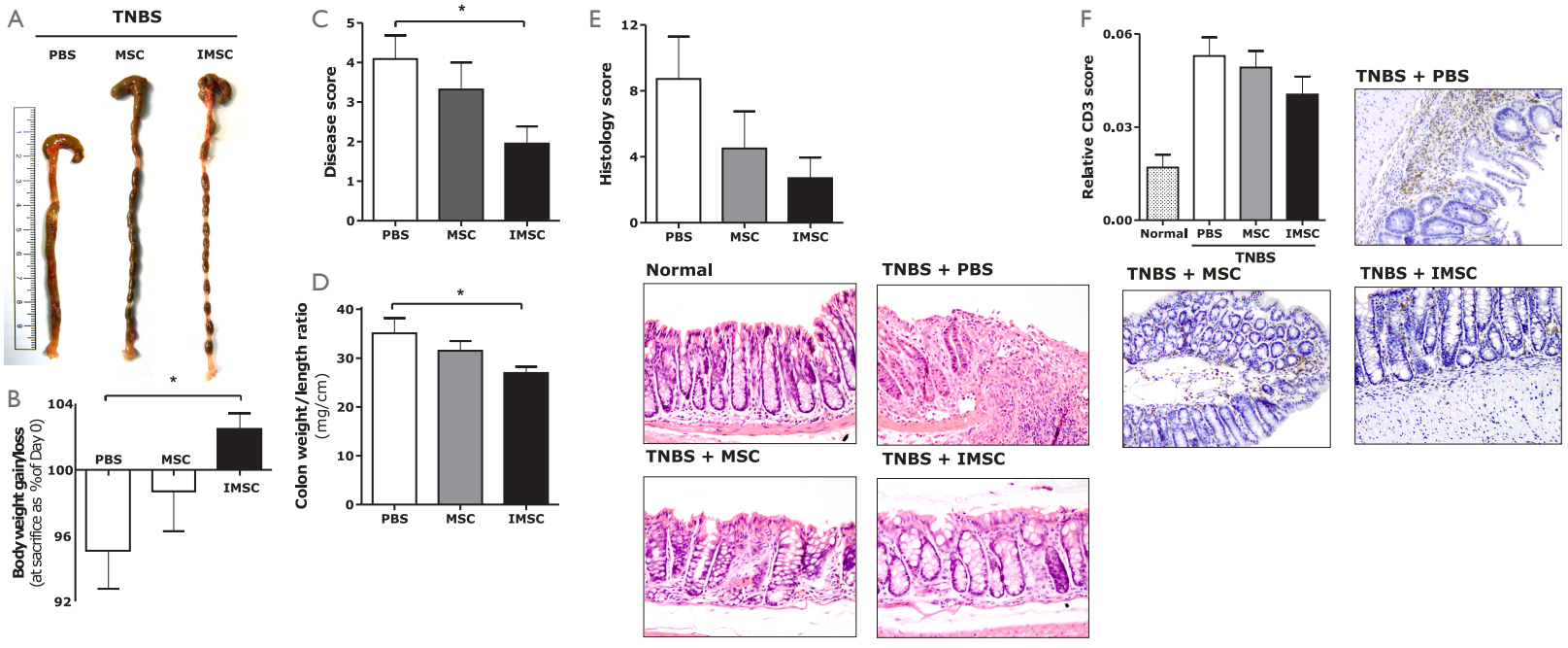


Figure 5. IMSCs alleviate TNBS-induced colitis. Mice were sensitized through the skin on day 0, challenged intrarectally on day 7, and received mouse MSC, IMSC or PBS injections 6 hrs after challenge. Disease progression was assessed by changes in daily body weight as well as macroscopic and microscopic scores on day 9 upon sacrifice. Data are presented as mean ± SEM; n=11 mice per group. A. Macroscopic images of representative mouse colons. B. Animal body weight change on day 9 upon sacrifice. The values of body weight are expressed as a percentage of initial body weight on day 0. *P < .05. C. Macroscopic disease score based on the presence of loose stool, bleeding and macroscopic inflammation determined on day 9 upon sacrifice. *P < .05. D. Assessment of colonic weight-to-length ratio upon sacrifice as an index of disease-related intestinal wall thickening. *P < .05. E. Representative histological sections of the mouse colon upon sacrifice and histological grading of colonic colitis scores. IMSCs decrease colitis activity in TNBS-treated mouse colons. F. CD3-positive T-cells in vivo. Representative colon sections stained with anti-CD3, revealing T lymphocytes. Magnification, 20x. CD3-positive T-cells expressed as the ratio of CD3 relative to hematoxylin counterstain.

IMSCs alter cytokine profiles in colon homogenates

To investigate the effect of (I)MSC treatment on immunomodulatory profiles, we analyzed cytokine concentrations in colon homogenates. As previously described, distinct cytokine patterns were observed in DSS- and TNBS-induced colitis models.[26] In both models, TNF-α and IL-6 levels were significantly elevated, reflecting ongoing general inflammation. Human and mouse IMSC treatment reduced TNF-α and IL-6 levels, while MSC treatment had no effect (Figures 7B and 7C). Acute DSS-induced colitis is mainly mediated by the innate immune system and results in a strong colonic induction of IFN-γ. Furthermore, IL-17A has been shown to be a pathogenic cytokine during DSS-induced colitis, as IL-17A knockout animals show an attenuated decrease in body weight as well as decreased disease activity.[27] Indeed, IFN-γ and IL-17A expression were both increased during induction of DSS colitis (Figures 7D and 7E, left panels). Human MSC administration did not alter IFN-γ and IL-17A levels. In contrast, IMSC administration reduced both IFN-γ and IL-17A expression essentially to the levels seen in control (non-induced) animals, consistent with the decrease in colonic inflammation observed in these mice.

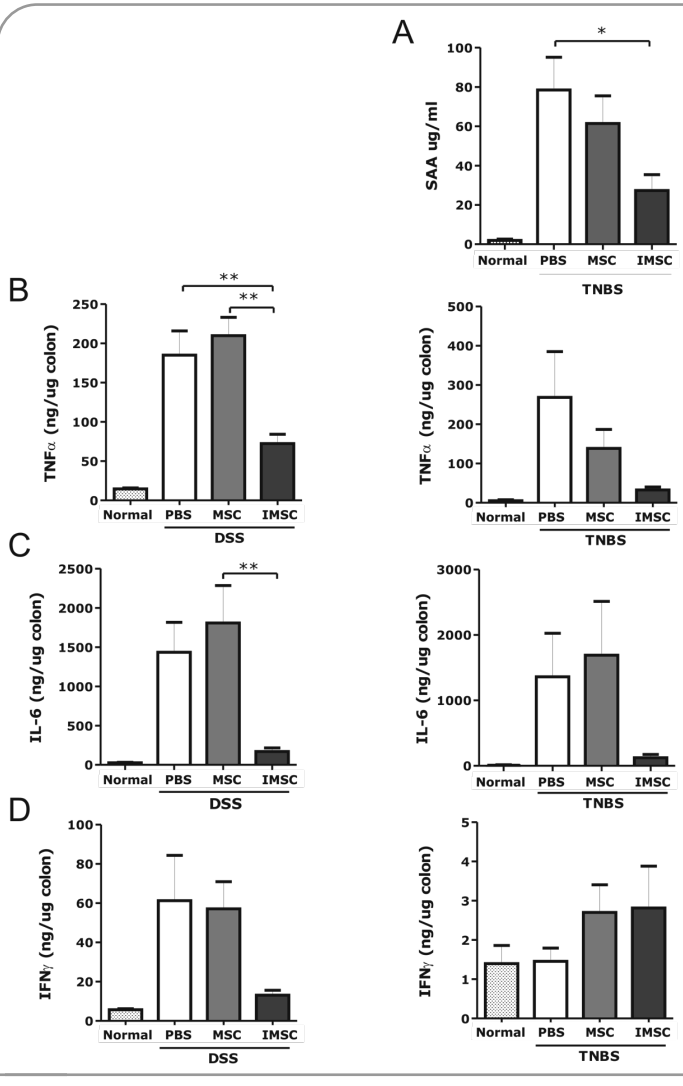


Figure 7. IMSCs decrease systemic and local inflammatory responses in experimental colitis models. Serum and protein extracts were obtained from the colons at the acute phase of disease (day 9), and cytokine levels were measured. Data are presented as mean ± SEM; n=11 mice per group; *P < .05, **P < .005. A. Serum amyloid A (SAA) levels were measured by ELISA and found to be reduced in mice with TNBS-induced colitis after IMSC treatment. B-G. Cytokine levels of TNF-α, IL-6, IFN-γ, IL-10, IL-17A and IL-4 in colon homogenates of DSS- (left) and TNBS-induced (right) mice.

TNBS-induced colitis is T-cell-mediated and mainly driven by a Th1 response.[28, 29] In this model, a lack of IL-17 signaling results in a more severe disease phenotype, likely due to uncontrolled induction of Th1 responses.[30] Interestingly, treatment of TNBS colitis with mouse IMSCs resulted in the induction of IL-17 (Figure 7E, right panel) as well as IL-4 (Figure 7G, right panel), indicating induction of Th2 and Th17 responses, respectively. These responses may inhibit Th1 skewing and thereby Th1-driven pathogenesis as well. In addition, an increase in colonic IL-10 levels was observed, suggesting the presence of an active immunoregulatory component (Figure 7F, right panel).

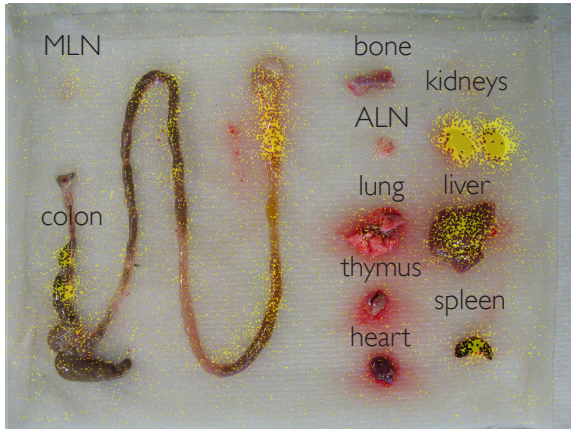
IMSCs migrate to sites within the diseased intestine

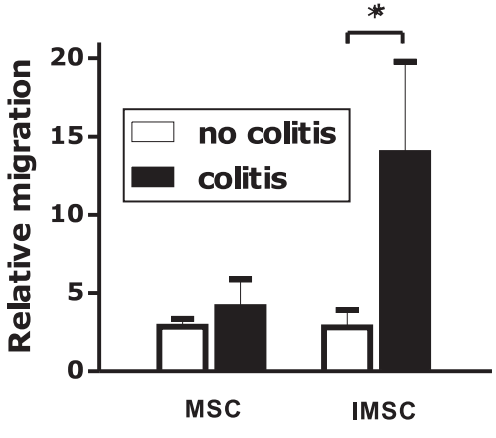
Previously, MSCs were thought to specifically migrate to sites of tissue damage or inflammation.[31] However, in these studies the focus was primarily on specific tissues of interest and the number of MSCs present at those sites in relation to the total number of MSCs administered was not correlated. We therefore administered human MSCs and IMSCs radiolabeled with technetium-99m to both control animals and animals with established colitis. The biodistribution data show that at 24 hours post-injection, the majority of radioactivity was present in the kidneys, urinary bladder, and a small portion in the liver (Table 1). Of note, we

	MSC				IMSC			
	No colitis		Colitis		No colitis		Colitis	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Colon	0.19	0.08	0.19	0.09	0.13	0.05	0.26	0.11
Coecum	0.15	0.06	0.1	0.05	0.1	0.05	0.19	0.09
Small intestine	1.24	0.51	1.58	0.71	0.61	0.25	2.48	1.01
MLN	0.12	0.05	0.19	0.09	0.13	0.06	0.1	0.05
Spleen	0.74	0.3	1.01	0.45	0.7	0.31	0.58	0.26
Kidney	8.22	3.35	10.09	4.51	5.13	2.09	7.63	3.11
Heart	0.09	0.01	0.03	0.02	0.01	0.01	0.04	0.02
Lungs	0.2	0.08	0.14	0.07	0.15	0.06	0.15	0.06
Liver	3.17	1.29	4.53	2.03	1.89	0.77	2.58	1.05
Femur	0.05	0.02	0.05	0.02	0.03	0.01	0.03	0.01

Table 1. Biodistribution of 99mTc-labelled human (I)MSCs 24 hrs after injection in healthy controls (no colitis) and DSS-treated (colitis) mice. All data are presented as the % (mean ± SEM) of the total injected dose (ID) per gram of excised organ.

found radioactivity across a wide array of tissues, arguing against a highly specific trafficking of MSCs (Figure 8A). Strikingly, when we compared cell trafficking under steady state conditions and during inflammation, we observed a clear difference between MSCs and IMSCs. IMSC migration to the intestine was significantly increased during colitis induction, whereas MSC distribution was unaffected, suggesting that IMSCs gain homing potential to sites of inflammation (Figure 8B).





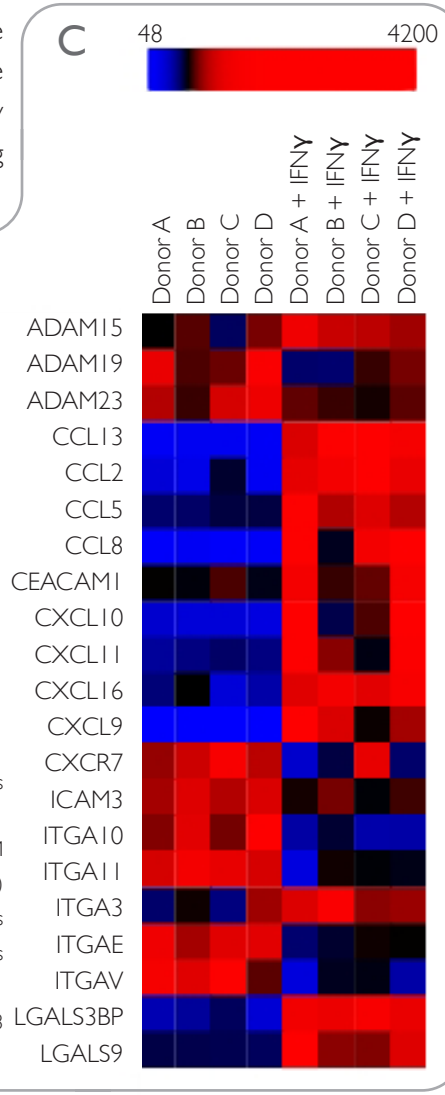


Figure 8. Human IMSCs migrate to the inflamed intestine. A. Overlay images of ^{99m}Tc scintigrams and macroscopic photos of various organs excised from diseased mice. ^{99m}Tc-labeled human IMSCs were injected intraperitoneally on day 7 of DSS experiments, and organs were excised, photographed and scanned 24 hrs after injection. B. Radioactivity distribution in various tissues was calculated as the mean ± SEM percentage of the total injected dose of radioactivity per gram of tissue (%ID/g). Data from two experiments (n=3 per group/experiment) were pooled and expressed as a ratio over MSC migration in control mice, which was set to 1. *P < .05, colitis (DSS) versus no colitis (healthy control). C. Changes in mRNA abundance after 6 days of MSC treatment with IFN-γ as determined by microarray analysis. This heat map shows 21 molecules involved in cell migration. Green indicates down-regulation and red indicates up-regulation. Each column represents a single microarray analysis. Column 1, 2, 3, and 4 represent unstimulated MSCs of donor A, B, C and D, whereas columns 5 -8 represents IFN-γ stimulated MSCs of the same donors A, B, C and D, respectively.

IFN-γ pretreatment alters expression of trafficking molecules

In order to examine more elaborately the mechanisms involved in the altered biodistribution of IMSC, gene expression profiles were generated from both MSC and IMSC. As expected, a large number of IFN-γ regulated genes was induced after treatment, including human leukocyte molecules and IDO (data not shown). We then focused on molecules associated with cell migration and adhesion. As has been described previously,[6] IFN-γ treatment of MSCs resulted in upregulation of a number of pro-inflammatory chemokines (Figure 8C). Chemokine receptor expression however, did not change dramatically after 6 days of treatment, with the exception of an increased level of CXCR7. This suggested that treatment with IFN-γ does not result in profound changes in chemokine responsiveness of the MSCs and that this may not be the underlying cause of the altered trafficking observed. In contrast, the expression pattern of adhesion molecules was strikingly altered in IMSCs compared with MSCs from the same individual. Expression of a number of integrins was decreased, including ITGA10, ITGA11, ITGAE and ITGAV. In contrast, lectins LGALS3BP and LGALS9 were increased, as was ADAM15, a matrix metalloproteinase also involved in adhesion of cells to epithelium.

DISCUSSION

MSCs can be used as a potential treatment for various immune disorders. Early phase I-II studies reported that MSCs are a safe and feasible treatment option in humans,[16,32] and trials are ongoing to demonstrate clinical efficacy. Exact treatment dose, timing and frequency of administration, as well as the optimal source of MSCs, are currently under investigation. In addition, many in vitro and animal studies are performed to further elucidate the mechanism of immune modulation by MSCs.

Recent studies demonstrated that the immunosuppressive ability of MSCs is not intrinsic but instead induced by proinflammatory cytokines.[6] Exposing MSCs to inflammatory signals was found to significantly potentiate the immunosuppressive effects of MSCs on T-cells, monocytes/macrophages and dendritic cells (DCs).[7, 9, 33, 34] These data were further validated in vivo using animal models, where IFN-γ pretreatment improved MSC efficacy for the treatment of GvHD.[9] Here, we demonstrate that IFN-γ stimulation potentiates MSCs to provide an effective treatment for both DSS- and TNBS-induced colitis, two experimental models for inflammatory bowel disease IBD.

Previous research revealed beneficial effects of MSCs on experimental colitis.[35, 36] In these model systems, the therapeutic effect was mediated, at least in part, by suppression of inflammatory cytokines/mediators, increased infiltration of regulatory T-cells, and expression of the anti-inflammatory cytokine IL-10.[37] Although the MSCs used in these studies were not pretreated with IFN-γ, it is possible that they were activated in vivo, as MSCs were administered after the onset of inflammation. The proinflammatory environment in experimental colitis is comprised of an influx of mononuclear cells and high levels of TNF-α and IL-6. Both TNF-α and IFN-γ treatment of MSCs induced IDO expression.[33] Since only IMSCs are efficacious in the prevention of DSS-induced colitis, one could

speculate that at time of MSC injection (on day 0), the negligible levels of proinflammatory cytokines were insufficient to activate MSCs and initiate MSC-induced suppression. In fact, the absence of a protective effect of MSCs derived from fat tissue injected one day or one week prior to DSS colitis induction was recently described.[35]

As IFN-γ is known to upregulate both MHC class I and II genes,[22-25] it would be important to determine whether this upregulation makes cells more susceptible to rejection in an immune-competent host, particularly in the case of allogeneic cells. However, we administered both human and mouse (I)MSCs to immunocompetent mice and did not observe any apparent induction of immunologic responses. In vitro, pre-treatment of MSCs by IFN-γ does not enhance T-cell proliferation. On the contrary, PBMC proliferation is significantly inhibited at lower PBMC:(I)MSC ratios compared to untreated MSCs. Furthermore, systemic levels of the acute phase protein SAA were not increased by administration of IMSC. Similarly, in previous studies,[35-39] positive treatment effects of human MSCs in experimental disease models was observed without apparent graft rejection, attributed to the inherent capacities of MSCs to reduce inflammatory cell infiltration, down-regulate inflammatory cytokine production, regulate immune tolerance by increasing the production of anti-inflammatory cytokines (e.g., IL-10) and promoting the generation/activation of Tregs. Nevertheless, dedicated studies into the immunogenic status of (I)MSC are required to formally exclude induction of unwanted immune responses and to determine potential for clinical application.

MSCs have been described to distribute broadly following systemic infusion (IV) and to be recovered from injured tissues such as skin, muscle and gut.[31, 40] In agreement with this, using ^{99m}Tc-labeling, we also found activity in almost all tissues after intraperitoneal administration of human (I)MSCs. Interestingly, when focusing on the intestines specifically during colitis, enhanced migration of IMSCs but not MSCs was seen. Recently, Ko et al. demonstrated that enhancing binding of MSCs to adhesion molecule VCAM-1 (vascular cell adhesion protein) enhances their migration to the inflamed colon. Strikingly, only under these conditions, MSCs had a curative effect.[41] These results are similar to those obtained by us in the present study as we show that MSC homing to the intestines is upregulated after IFN-γ stimulation and that only IMSCs were able to alleviate colitis symptoms. However, even under inflammatory conditions, only a small proportion of IMSC can be detected in the intestine, suggesting that additional mechanisms of immune suppression may be active.

Previous studies have shown that IFN-γ primed MSCs secrete pro-inflammatory chemokines, resulting in the recruitment of T-cells.[6] Our study corroborates these findings and also points out the need for MSC–T-cell proximity in order to achieve potent immunosuppression. Also in agreement with previous literature, we did not observe much alteration in chemokine receptor expression. However, a clear alteration was seen in the pattern of adhesion molecule expression. Most strinkingly, a number of integrins was downregulated, while various lectins were upregulated. In addition, ADAM15 expression was increased, which has been associated

with increased adhesion of fibroblast-like cells. The molecule forms homotypic cell-cell interactions and is expressed in human intestinal epithelium, thereby being a possible targeting molecule to this site. In summary, the complete balance between adhesion molecule expressions is strongly altered by IFN-γ treatment. As this balance influences the likelihood of a cell trafficking to a certain location, these alterations may underlie the altered trafficking observed after IFN-γ stimulation. However, further studies will be required to evaluate the individual contributions of all differentially expressed adhesion molecules.

CONCLUSION

We conclude that IFN-γ potentiates MSCs to suppress inflammatory responses in experimental colitis models. We show that IMSCs inhibit Th1 inflammatory responses, leading to reduced T-cell activation and less proinflammatory cytokine secretion, resulting in diminished mucosal damage in two pre-clinical colitis models. We hypothesize that preactivation of MSCs with IFN-γ could lead to a more rapid clinical response and a dose reduction of cells, which could have profound effects on current treatment development programs.

ACKNOWLEDGMENTS

The authors thank Dr. Izak Biemond for technical assistance, Jorge M. Perez Galarza for providing mouse MSCs, Johanna M. van der Zon for preparation of histological sections and the staff of the Central Animal Facility of the LUMC for animal care.

GRANT SUPPORT

The authors are grateful for the generous support from the Dutch Digestive Foundation (MLDS, W07-17) and the Digest Science Foundation.

REFERENCES

1. da Silva ML, Chagastelles PC, Nardi NB. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. J Cell Sci 2006;119:2204-2213.
2. Bartholomew A, Sturgeon C, Siatskas M, Ferrer K, McIntosh K, Patil S, Hardy W, Devine S, Ucker D, Deans R, Moseley A, Hoffman R. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. Exp Hematol 2002;30:42-48.
3. Glennie S, Soeiro I, Dyson PJ, Lam EW, Dazzi F. Bone marrow mesenchymal stem cells induce division arrest anergy of activated T cells. Blood 2005;105:2821-2827.
4. Krampera M, Glennie S, Dyson J, Scott D, Laylor R, Simpson E, Dazzi F. Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. Blood 2003;101:3722-3729.
5. Krampera M, Cosmi L, Angeli R, Pasini A, Liotta F, Andreini A, Santarlasci V, Mazzinghi B, Pizzolo G, Vinante F, Romagnani P, Maggi E, Romagnani S, Annunziato F. Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells. Stem Cells 2006;24:386-398.
6. Ren G, Zhang L, Zhao X, Xu G, Zhang Y, Roberts AI, Zhao RC, Shi Y. Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. Cell Stem Cell 2008;2:141-150.
7. Ryan JM, Barry F, Murphy JM, Mahon BP. Interferon-gamma does not break, but promotes the immunosuppressive capacity of adult human mesenchymal stem cells. Clin Exp Immunol 2007;149:353-363.
8. Meisel R, Zibert A, Laryea M, Gobel U, Daubener W, Dilloo D. Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. Blood 2004;103:4619-4621.
9. Polchert D, Sobinsky J, Douglas G, Kidd M, Moadsiri A, Reina E, Genrich K, Mehrotra S, Setty S, Smith B, Bartholomew A. IFN-gamma activation of mesenchymal stem cells for treatment and prevention of graft versus host disease. Eur J Immunol 2008;38:1745-1755.
10. Garcia-Olmo D, Garcia-Arranz M, Herreros D. Expanded adipose-derived stem cells for the treatment of complex perianal fistula including Crohn's disease. Expert Opin Biol Ther 2008;8:1417-1423.
11. Ball LM, Bernardo ME, Roelofs H, Lankester A, Cometa A, Egeler RM, Locatelli F, Fibbe WE. Co-transplantation of ex vivo expanded mesenchymal stem cells accelerates lymphocyte recovery and may reduce the risk of graft failure in haploidentical hematopoietic stem cell transplantation. Blood 2007 Oct 1;110(7):2764-7.
12. Kebriaei P, Isola L, Bahceci E, Holland K, Rowley S, McGuirk J, Devetten M, Jansen J, Herzig R, Schuster M, Monroy R, Uberti J. Adult human mesenchymal stem cells added to corticosteroid therapy for the treatment of acute graft-versus-host disease. Biol Blood Marrow Transplant 2009;15:804-811.
13. Le Blanc K., Rasmusson I, Sundberg B, Gotherstrom C, Hassan M, Uzunel M, Ringden O. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. Lancet 2004;363:1439-1441.
14. Le Blanc K, Frassoni F, Ball L, Lanino E, Sundberg B, Lonnies H, Roelofs H, Dini G, Bacigalupo A, Locatelli F, Fibbe WF, Ringden O. Mesenchymal Stem Cells for Treatment of Severe Acute Graft-Versus-Host Disease. ASH Annual Meeting Abstracts 2006;108:2918.
15. Ringden O, Uzunel M, Rasmusson I, Remberger M, Sundberg B, Lonnies H, Marschall HU, Dlugosz A, Szakos A, Hassan Z, Omazic B, Aschan J, Barkholt L, Le BK. Mesenchymal stem cells for treatment of therapy-resistant graft-versus-host disease. Transplantation 2006;81:1390-1397.
16. Duijvestein M, Vos AC, Roelofs H, Wildenberg ME, Wendrich BB, Verspaget HW, Kooy-Winkelaar EM, Koning F, Zwaginga JJ, Fidder HH, Verhaar AP, Fibbe WE, van den Brink GR, Hommes DW. Autologous bone marrow-derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: results of a phase I study. Gut 2010;59:1662-1669.
17. Melgar S, Karlsson A, Michaelsson E. Acute colitis induced by dextran sulfate sodium progresses to chronicity in C57BL/6 but not in BALB/c mice: correlation between symptoms and inflammation. Am J Physiol Gastrointest Liver Physiol 2005;288:G1328-G1338.

18. Camoglio L, te Velde AA, de BA, ten Kate FJ, Kopf M, Van Deventer SJ. Hapten-induced colitis associated with maintained Th1 and inflammatory responses in IFN-gamma receptor-deficient mice. Eur J Immunol 2000;30:1486-1495.
19. van Hensbergen Y., Schipper LF, Brand A, Slot MC, Welling M, Nauta AJ, Fibbe WE. Ex vivo culture of human CD34+ cord blood cells with thrombopoietin (TPO) accelerates platelet engraftment in a NOD/SCID mouse model. Exp Hematol 2006;34:943-950.
20. Welling M, Feitsma HI, Blok D, Calame W, Ensing GJ, Goedemans W, Pauwels EK. A new 99mTc labelling method for leucocytes: in vitro and in vivo comparison with 99mTc-HMPAO. Q J Nucl Med 1995;39:89-98.
21. Battke F, Symons S, Nieselt K. Mayday--integrative analytics for expression data. BMC Bioinformatics 2010;11:121.
22. Dominici M, Le BK, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;8:315-317.
23. Rameshwar P. IFNgamma and B7-H1 in the immunology of mesenchymal stem cells. Cell Res 2008;18:805-806.
24. Romieu-Mourez R, Francois M, Boivin MN, Stagg J, Galipeau J. Regulation of MHC class II expression and antigen processing in murine and human mesenchymal stromal cells by IFN-gamma, TGF-beta, and cell density. J Immunol 2007;179:1549-1558.
25. Stagg J, Pommey S, Eliopoulos N, Galipeau J. Interferon-gamma-stimulated marrow stromal cells: a new type of nonhematopoietic antigen-presenting cell. Blood 2006;107:2570-2577.
26. Alex P, Zachos NC, Nguyen T, Gonzales L, Chen TE, Conklin LS, Centola M, Li X. Distinct cytokine patterns identified from multiplex profiles of murine DSS and TNBS-induced colitis. Inflamm Bowel Dis 2009;15:341-352.
27. Ito R, Kita M, Shin-Ya M, Kishida T, Urano A, Takada R, Sakagami J, Imanishi J, Iwakura Y, Okanoue T, Yoshikawa T, Kataoka K, Mazda O. Involvement of IL-17A in the pathogenesis of DSS-induced colitis in mice. Biochem Biophys Res Commun 2008;377:12-16.
28. Neurath M, Fuss I, Strober W. TNBS-colitis. Int Rev Immunol 2000;19:51-62.
29. Scheiffele F, Fuss IJ. Induction of TNBS colitis in mice. Curr Protoc Immunol 2002;Chapter 15:Unit.
30. Zhang Z, Zheng M, Bindas J, Schwarzenberger P, Kolls JK. Critical role of IL-17 receptor signaling in acute TNBS-induced colitis. Inflamm Bowel Dis 2006;12:382-388.
31. Chapel A, Bertho JM, Bensidhoum M, Fouillard L, Young RG, Frick J, Demarquay C, Cuvelier F, Mathieu E, Trompier F, Dudoignon N, Germain C, Mazurier C, Aigueperse J, Borneman J, Gorin NC, Gourmelon P, Thierry D. Mesenchymal stem cells home to injured tissues when co-infused with hematopoietic cells to treat a radiation-induced multi-organ failure syndrome. J Gene Med 2003;5:1028-1038.
32. Garcia-Olmo D, Garcia-Arranz M, Herreros D, Pascual I, Peiro C, Rodriguez-Montes JA. A phase I clinical trial of the treatment of Crohn's fistula by adipose mesenchymal stem cell transplantation. Dis Colon Rectum 2005;48:1416-1423.
33. English K, Barry FP, Field-Corbett CP, Mahon BP. IFN-gamma and TNF-alpha differentially regulate immunomodulation by murine mesenchymal stem cells. Immunol Lett 2007;110:91-100.
34. Opitz CA, Litzenburger UM, Lutz C, Lanz TV, Tritschler I, Koppel A, Tolosa E, Hoberg M, Anderl J, Aicher WK, Weller M, Wick W, Platten M. Toll-like receptor engagement enhances the immunosuppressive properties of human bone marrow-derived mesenchymal stem cells by inducing indoleamine-2,3-dioxygenase-1 via interferon-beta and protein kinase R. Stem Cells 2009;27:909-919.
35. Gonzalez-Rey E, Anderson P, Gonzalez MA, Rico L, Buscher D, Delgado M. Human adult stem cells derived from adipose tissue protect against experimental colitis and sepsis. Gut 2009;58:929-939.
36. Gonzalez MA, Gonzalez-Rey E, Rico L, Buscher D, Delgado M. Adipose-derived mesenchymal stem cells alleviate experimental colitis by inhibiting inflammatory and autoimmune responses. Gastroenterology 2009;136:978-989.
37. Zhang Q, Shi S, Liu Y, Uyanne J, Shi Y, Shi S, Le AD. Mesenchymal stem cells derived from human gingiva are capable of immunomodulatory functions and ameliorate inflammation-related tissue destruction in experimental colitis. J Immunol 2009;183:7787-7798.
38. Gonzalez MA, Gonzalez-Rey E, Rico L, Buscher D, Delgado M. Treatment of experimental arthritis by inducing immune tolerance with human adipose-derived mesenchymal stem cells. Arthritis Rheum 2009;60:1006-1019.
39. Zhang J, Li Y, Chen J, Cui Y, Lu M, Elias SB, Mitchell JB, Hammill L, Vanguri P, Chopp M. Human bone marrow stromal cell treatment improves neurological functional recovery in EAE mice. Exp Neurol 2005;195:16-26.
40. Devine SM, Cobbs C, Jennings M, Bartholomew A, Hoffman R. Mesenchymal stem cells distribute to a wide range of tissues following systemic infusion into nonhuman primates. Blood 2003;101:2999-3001.
41. Ko IK, Kim BG, Awadallah A, Mikulan J, Lin P, Letterio JJ, Dennis JE. Targeting improves MSC treatment of inflammatory bowel disease. Mol Ther 2010;18:1365-1372.

CHAPTER 6

Summary, discussion, and future perspectives

SUMMARY

Hematopoietic stem cell transplantation and mesenchymal stromal (MSC) cell therapy are currently under investigation as novel therapies for inflammatory bowel diseases (IBD). Hematopoietic stem cells are thought to repopulate the immune system and reset the immunological response to luminal antigens. MSCs have the capacity to differentiate into a wide variety of distinct cell lineages and to suppress immune responses in vitro and in vivo. Recent results from animal models and early human experience in graft-versus-host disease (GvHD) and also Crohn's disease (CD) suggest that ex vivo expanded MSCs may have clinically useful immunomodulatory effects. The main goal of this thesis was to study the safety, feasibility, and applicability of stem cell therapy in IBD.

Chapter 1 gives an overview on inflammatory bowel diseases and stem cell therapy. This introduction highlights the present knowledge on hematopoietic stem cells and mesenchymal stromal cells in IBD treatment. The focus is on the immunomodulatory characteristics of stem cells and application of these cells in experimental colitis models. Furthermore, an overview on clinical trials with stem cells in IBD is provided.

In **Chapter 2** the long-term (4 to 6 years) outcome of hematopoietic stem cell therapy (HSCT) is presented. Three patients (two male, one female) with active severe Crohn's disease intolerant or refractory to conventional therapies, including anti-TNF-α antibodies were planned to undergo autologous HSCT. All three patients successfully completed stem cell mobilization and two of them subsequently underwent conditioning and autologous HSCT with CD34+ cell selection. Treatment was well tolerated, with acceptable toxicity. Five and six years post-transplantation, these patients are in remission under treatment. The third patient went into remission after mobilization and therefore she decided not to undergo conditioning and HSCT transplantation. After a successful pregnancy she relapsed two years later. Since then, she suffers from refractory Crohn's disease for which we are now reconsidering conditioning and transplantation. These observations suggest that autologous HSCT appears to be safe and can be an alternative strategy for Crohn's disease patients with severe and therapy resistant disease.

The aim of the clinical trial in **Chapter 3** was to determine the safety and feasibility of autologous bone marrow derived MSC therapy in patients with refractory Crohn's disease. To this extent ten adult patients with refractory Crohn's disease (eight females/two males) underwent bone marrow aspiration under local anesthesia. Bone marrow MSCs were isolated and expanded and MSCs were tested for phenotype and functionality in vitro. Nine patients received 2 doses of $1\text{-}2\times10^6$ cells/kg bodyweight, intravenously, 7 days apart. MSCs isolated from Crohn's disease patients showed similar morphology, phenotype and growth potential compared to MSCs from healthy donors. Importantly,

immunomodulatory capacity was intact, as these 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. Three patients showed clinical response 6 weeks post treatment, conversely three patients required surgery due to disease worsening. Our data imply that MSCs isolated from Crohn's disease patients have similar characteristics compared to MSCs from healthy donors and that administration of autologous bone marrow derived MSCs appears to be safe and feasible in the treatment of refractory Crohn's disease as no serious adverse events were detected during bone marrow harvesting and administration.

Previous studies showed that immunosuppressive drugs can be harmful to hematopoietic stem cells or endothelial progenitor cell proliferation, thereby affecting their functional capacities. Likewise, immunosuppressive agents used in the treatment of IBD might have an effect on MSC function and could, change the outcome of MSC therapy and affect safety. In **Chapter 4,** we therefore investigated the interaction between MSCs and immunosuppressive drugs frequently used in the treatment of IBD and concluded that in vitro, MSC phenotype and function are not affected by therapeutic concentrations of drugs commonly used in the treatment of IBD. These findings are important for the potential clinical use of MSCs in combination with immunomodulating drugs and anti-TNF-α therapy.

Recent data suggest that resting MSCs do not have significant immunomodulatory activity, but that the immunosuppressive function of MSCs has to be elicited by interferon-gamma (IFN-γ). In **Chapter 5,** we assessed the effects of IFN-γ prestimulation of MSCs (IMSCs) on their immunosuppressive properties in vitro and in vivo. To this end, we pretreated MSCs with IFN-γ and assessed their therapeutic effect in two experimental colitis models in mice. We found that mice treated with IMSCs (but not MSCs) showed a significantly attenuated development of induced colitis. IMSC-treated mice displayed an increase in body weight, lower colitis scores and better survival rates compared to untreated mice. In addition, serum amyloid A protein levels and local proinflammatory cytokine levels in colonic tissues were significantly suppressed after administration of IMSC. We also observed that IMSCs showed greater migration potential than unstimulated MSCs to sites within the inflamed intestine. In conclusion, we show that prestimulation of MSCs with IFN-γ enhances their capacity to inhibit inflammatory responses, resulting in diminished mucosal damage in experimental colitis. These data show that IFN-γ activation of MSCs increases their immunosuppresive capacities and importantly, their therapeutic efficacy in vivo.

DISCUSSION

Despite the improvement in medical therapy of inflammatory bowel disease with the introduction of anti-TNF-α compounds, disease control remains hard to achieve in many patients. Adult stem cells are currently under investigation for a variety of inflammatory disorders. HSCT may be an effective treatment for IBD and can be successfully used as a last resort in an attempt to control debilitating disease. However, it is associated with significant morbidity and mortality related to chemotherapy. The use of

MSCs derived from either bone marrow or adipose tissue could be an alternative approach. If effective, the big advantage of the use of MSCs is the fact that this treatment does not involve conditioning chemotherapy. In the limited number of patients treated with MSCs in the last decade, few adverse events have been attributed to MSC administration. Although acute toxicity appears low, little is known about long-term unwanted side effects. Potential hazards include the possibility of malignant transformation, ectopic tissue formation, and the possible xenogenic transmission of disease and antibody formation when fetal bovine serum (FBS) is added to the culture medium. Furthermore, questions remain to be addressed about the mechanism underlying the immunomodulating properties of MSCs and their in vivo survival after exogenous administration. Discrepancies in MSC isolation, source, and culture protocols, as well as experimental conditions and timing of analysis can explain variation in obtained results and possibly will complicate the interpretation of future trial outcomes.

FUTURE PERSPECTIVES

A multicenter, prospective, randomized phase III study has been initiated by the European Crohn's and Colitis Organisation (ECCO) in collaboration with the European Group for Blood and Marrow Transplantation (EBMT) to evaluate the efficacy of HSC mobilization followed by high dose immune ablation and autologous stem cell transplantation versus HSC mobilization only. In the case of MSC therapy, the Royal Perth Hospital, Australia, has just launched a multicenter phase II trial in 20 patients to evaluate the safety and efficacy of weekly intravenous infusion for 4 weeks with allogeneic bmMSCs. The University Hospital La Paz in Madrid is performing a phase I/IIa trial on allogeneic MSCs derived from fat tissue in the local treatment of recto-vaginal fistula in CD. Finally, the LUMC is currently investigating the safety and preliminary efficacy of allogeneic bmMSCs in the induction of response for active fistulizing CD in a dose escalation study.

HOOFDSTUK 6

Samenvatting, discussie en toekomstperspectieven

SAMENVATTING

INFLAMMATOIRE DARMZIEKTEN

De ziekte van Crohn en colitis ulcerosa zijn beide chronische inflammatoire (ontstekingsachtige) darmziekten. In het Engels wordt hiervoor de term IBD (Inflammatory Bowel Disease) gebruikt. Beide ziektes kunnen op elke leeftijd voorkomen, eerste uitingen van de ziekte wordt meestal vastgesteld tussen het 15e en 30e levensjaar. Er zijn verschillende factoren die van invloed kunnen zijn op het ontstaan inflammatoire darmziekten. Factoren die een rol spelen, zijn onder andere: het immuunsysteem, erfelijkheid, bacteriën in de darm, stress en roken. Ons immuunsysteem (afweersysteem) beschermt ons lichaam tegen schadelijke indringers, zoals bacteriën en virussen. Bij inflammatoire darmziekten is het immuunsysteem overactief. Het valt onschuldig weefsel aan, met als gevolg is dat er ontstekingen ontstaan in de darm.

Bij de ziekte van Crohn en bij colitis ulcerosa komen een aantal dezelfde klachten en symptomen voor, namelijk: buikpijn, diarree vaak met bloed en slijm, soms verstopping, bloedarmoede, vermoeidheid en gewichtsverlies. Het zijn chronische ziektes die periodes van rust (remissie) en ziekte (opvlamming) kent. Helaas zijn er nog geen medicijnen die de ziekte van Crohn of colitis ulcerosa kunnen genezen. Wel is het mogelijk om de ontstekingen te remmen en de klachten met behulp van medicijnen te onderdrukken. Ook moeten deze medicijnen voorkomen dat er nieuwe ontstekingen ontstaan. Soms is een operatie nodig, bijvoorbeeld als medicijnen niet voldoende helpen of als er een vernauwing of afsluiting van de darm ontstaat.

STAMCELLEN

Stamceltherapie wordt momenteel onderzocht als nieuwe therapie voor inflammatoire darmziekten. In dit proefschrift worden twee verschillende stamcellen besproken, namelijk hematopoietische stamcellen (HSC) en mesenchymale stromale cellen (MSCs).

Hematopoietische stamcellen HSC therapie wordt al veelvuldig toegepast om verschillende vormen van kanker te behandelen. Van HSC is bekend dat transplantatie van de cellen naar de patiënt het immuunsysteem als ware 'resetten' en hiermee het overactieve immuunsysteem remmen.

Mesenchymale stamcellen Sinds een aantal jaar wordt er steeds meer onderzoek gedaan naar de mogelijkheid om verschillende ziekten te behandelen met MSCs. Uit onderzoek in het laboratorium is namelijk gebleken dat MSCs uit kunnen groeien tot verschillende weefseltypen en in diermodellen van immuunziekten (zoals IBD) ontsteking remmen.

SAMENVATTING

Het belangrijkste doel van dit proefschrift was om de veiligheid, haalbaarheid en toepasbaarheid van stamceltherapie voor IBD te bestuderen.

Hoofdstuk 1 geeft een samenvatting van wat er al bekend is over HSC en MSC in de behandeling van IBD. De focus ligt op de immunomodulerende eigenschappen van stamcellen en geeft uitleg over de bevindingen in muismodellen van IBD. Ook wordt een overzicht gegeven van klinische studies die zich richten op stamcellen voor de behandeling van IBD.

In **Hoofdstuk 2** worden de lange termijn (4 tot 6 jaar) resultaten van hematopoietische stamcellen therapie (HSCT) gepresenteerd. Drie patiënten (twee mannen, één vrouw) met actieve ernstige refractaire ziekte van Crohn, dat wil zeggen niet te behandelen met bestaande therapieën, kregen de mogelijkheid aangeboden een autologe HSCT te ondergaan. Bij een autologe stamceltransplantatie worden stamcellen uit het bloed van de patiënt zelf gebruikt. Dat gebeurt na een chemotherapiekuur gecombineerd met de toediening van een groeifactor. De goede stamcellen worden ingevroren terwijl de patiënt een intensieve behandeling met een hoge dosering chemobehandeling ondergaat om zieke cellen uit het lichaam te verwijderen. Na de behandeling worden de goede stamcellen weer teruggegeven om het lichaam te laten herstellen. Bij alle drie de patiënten werden met succes stamcellen uit het bloed verkregen. Twee van de drie patiënten ondergingen nadien de intensieve, hoge dosering chemobehandeling. De behandeling werd goed verdragen, met aanvaardbare bijwerkingen. Vijf en zes jaar na transplantatie, zijn deze patiënten in remissie onder medicijnen. De derde patiënt ging in remissie nadat stamcellen werden verkregen middels de chemotherapiekuur gecombineerd met de toediening van een groeifactor. Omdat zij klachtenvrij was, werd besloten in dit geval niet ook de hoge dosering chemobehandeling te geven. Na een succesvolle zwangerschap is twee jaar later de ziekte van Crohn weer opgevlamd en wordt de intensieve, hoge dosering chemobehandeling en autologe HSC-transplantatie alsnog overwogen. Deze resultaten suggereren dat autologe HSCT veilig is en een alternatieve strategie zou kunnen zijn voor patiënten met ernstige therapieresistente ziekte.

Het doel van het klinische onderzoek in **Hoofdstuk 3** was om de veiligheid en de haalbaarheid van autologe mesenchymale stromale celtherapie bij patiënten met refractaire ziekte van Crohn te bepalen. In deze studie ondergingen tien volwassen patiënten (acht vrouwen / twee mannen) een beenmergpunctie onder lokale verdoving. Uit het beenmerg werden MSCs geïsoleerd en opgekweekt. Deze MSCs werden getest op uiterlijke kenmerken en hun functionaliteit met behulp van proeven in het laboratorium. Negen patiënten kregen twee doseringen van 1-2 miljoen cellen per kilo lichaamsgewicht, intraveneus toegediend, zeven dagen na elkaar. Uit het onderzoek is gebleken dat MSCs geïsoleerd uit het beenmerg van patiënten met de ziekte van Crohn vergelijkbaar zijn qua groeisnelheid en uiterlijke kenmerken met MSCs van gezonde donoren. Een belangrijk resultaat was dat de MSCs van de patiënten ook ontstekingsremmende eigenschappen hebben. MSC-transplantatie gaf geen

bijwerkingen, behoudens een licht allergische reactie op het invriesmiddel DMSO in één patiënt. Drie patiënten vertoonden klinische verbetering zes weken na de behandeling, echter drie andere patiënten hadden een operatie nodig omdat bij hun de ziekte activiteit verergerden. Onze gegevens laten zien dat MSCs van patiënten met de ziekte van Crohn dezelfde kenmerken hebben als MSCs van gezonde donoren. Daarnaast laat deze studie zien dat het toedienen van autologe MSCs veilig en haalbaar is in de behandeling van de ziekte Crohn; er zijn geen ernstige bijwerkingen geconstateerd tijdens het oogsten van de cellen uit het beenmerg en bij het intraveneus toedienen van de cellen bij de patiënt.

In **Hoofdstuk 4** hebben we onderzoek gedaan naar de interactie tussen MSC en de ontstekingsremmende medicijnen die veelvuldig in de behandeling van IBD worden gebruikt. Eerdere studies toonden aan dat bepaalde geneesmiddelen schadelijk kunnen zijn voor HSC en andere voorloper cellen. Onze hypothese was dat ontstekingsremmende medicijnen die gebruikt worden in de behandeling van IBD een effect zouden kunnen hebben op de functie van MSCs en daarmee de uitkomst van MSC-therapie zouden kunnen beïnvloeden. Uit het onderzoek is gebleken dat de eigenschappen en functie van MSCs niet worden beïnvloed door therapeutische concentraties van geneesmiddelen die veel worden gebruikt in de behandeling van IBD. Deze bevindingen zijn van belang voor de potentiële klinische toepassing van MSCs in combinatie met geneesmiddelen die worden toegepast in de behandeling van de ziekte van Crohn.

Recent gepubliceerde data laten zien dat MSCs in rust geen significante immunomodulerende activiteit hebben, maar dat de ontstekingsremmende werking van MSCs moet worden uitgelokt door interferon-gamma (IFN-γ). IFN-γ is een signaalstof en speelt een rol in de verdediging tegen virussen. In **Hoofdstuk 5** onderzochten we het effect van voorstimulatie van MSCs met IFN-γ (de zogenaamde IMSCs) op de eigenschappen van MSCs. Daartoe hebben we MSCs voorbehandeld met IFN-γ en hun functie beoordeeld in twee experimentele muismodellen van darmontsteking (colitis). Wij vonden dat muizen behandeld met IMSCs (maar niet de muizen behandeld met ongestimuleerde MSCs) minder darmontsteking ontwikkelden. De muizen behandeld met IMSC toonden een toename in lichaamsgewicht, minder ziekte activiteit en een betere overleving in vergelijking met onbehandelde muizen. Daarnaast kwamen er minder ontstekingsstofjes vrij en hebben we geconstateerd dat IMSCs beter migreren dan niet-gestimuleerde MSC naar de ontstoken darm. Tot slot laten we zien dat voorstimulatie van MSCs met IFN-γ hun vermogen vergroot om ontstekingsreacties te remmen, wat heeft geresulteerd in verminderde mucosale schade in experimentele colitis. Dit hoofdstuk laat zien dat IFN-γ activatie van MSCs hun ontstekingsremmende capaciteiten verhoogd en daarmee hun therapeutische werkzaamheid in vivo versterkt.

DISCUSSIE

Ondanks de verbetering in de medische behandeling van inflammatoire darmziekte met de introductie van nieuwe medicijnen (bijvoorbeeld anti-TNF-α) blijft het moeilijk de ziekte onder controle te houden bij veel patiënten. Volwassen stamcellen worden momenteel onderzocht voor een verscheidenheid van inflammatoire aandoeningen. HSCT kan een effectieve behandeling zijn van IBD. Echter, deze behandeling gaat gepaard met een aanzienlijke morbiditeit en mogelijk sterfte ten gevolge van chemotherapie. Het gebruik van MSCs zou een alternatieve strategie kunnen zijn. Het grote voordeel van het gebruik van MSCs, in tegenstelling tot HSCT, is dat voor het verkrijgen van deze cellen geen chemotherapie hoeft te worden gebruikt. Uit een beperkt aantal studies blijkt dat er weinig bijwerkingen zijn toegeschreven aan MSC transplantatie. Hoewel de acute toxiciteit laag lijkt, is er weinig bekend over de lange termijn bijwerkingen. Potentiële risico's omvatten de mogelijkheid van maligne transformatie, het uitgroeien van weefsel op ongewenste plaatsten en mogelijke xenogene overdracht van de virussen of het vormen van antilichamen door het gebruik van kalf serum (foetaal bovine serum: FBS), dat wordt toegevoegd aan het kweekmedium. Bovendien moeten er nog veel vragen worden beantwoord over hoe de cellen werken en waar zij blijven in het lichaam nadat zij getransplanteerd zijn. Verschillen in de manier van MSC-isolatie en opkweekprotocollen, maar ook omstandigheden waarin experimenten worden gedaan kunnen variatie tussen verkregen resultaten van verschillende onderzoeksgroepen verklaren en de interpretatie van verkregen resultaten bemoeilijken.

TOEKOMSTPERSPECTIEVEN

Momenteel vindt er een multicenter, prospectieve, gerandomiseerde fase III studie plaats geïnitieerd door de European Crohn's and Colitis Organisation (ECCO) in samenwerking met de European Group for Blood and Marrow Transplantation (EBMT) naar de werkzaamheid van HSCT in de ziekte van Crohn. In het geval van MSC-therapie is het Royal Perth Hospital, in Australië, onlangs gestart met een multicenter fase II studie bij 20 patiënten om de veiligheid en werkzaamheid van wekelijkse intraveneuze infusies met allogene MSCs (MSCs afkomstig van een donor) te evalueren gedurende vier weken. Het Universitaire Ziekenhuis van La Paz in Madrid bezig met het uitvoeren van een fase I / IIa studie met allogene MSCs uit vetweefsel in de lokale behandeling van recto-vaginale fistels bij patiënten met de ziekten van Crohn. Tot slot wordt in het Leids Universitair Medisch Centrum (LUMC) momenteel de veiligheid en werkzaamheid van allogene MSCs voor de lokale behandeling van fistels in een dosis-escalatie studie geëvalueerd.

CHAPTER 7

LIST OF PUBLICATIONS

CURRICULUM VITAE

ACKNOWLEDGMENTS

ABBREVIATIONS

LIST OF PUBLICATIONS

Geerts BF en **Duijvestein M.** Betere artsen door betere selectie? *NTVG-Studenten Editie*. 2002; 5(1); 3.

Hogewoning AA, **Duijvestein M**, Boakye D, Amoah AS, Obeng BB, van der Raaij-Helmer EM, Staats CC, Bouwes Bavinck JN, Yazdanbakhsh M, Lavrijsen AP. Prevalence of symptomatic tinea capitis and associated causative organisms in the Greater Accra Region, Ghana. *Br J Dermatol*. 2006 Apr;154(4):784-6.

Duijvestein M and Borgstein J. The Bradford grommet trainer. *Clin Otolaryngol.* 2006 Apr;31(2):163.

Hartgers FC, Obeng BB, Kruize YC, **Duijvestein M**, de Breij A, Amoah A, Larbi IA, van Ree R, Wilson MD, Rodrigues LC, Boakye DA, Yazdanbakhsh M. Lower expression of TLR2 and SOCS-3 is associated with Schistosoma haematobium infection and with lower risk for allergic reactivity in children living in a rural area in Ghana. *PLoS Negl Trop Dis*. 2008 Apr 16;2(4):e227.

Duijvestein M, van den Brink GR, Hommes DW. Stem cells as potential novel therapeutic strategy for inflammatory bowel disease. *J Crohns Colitis.* 2008 Jun;2(2):99-106.

Vos AC and **Duijvestein M**. Report from United European Gastroenterology Week/World Congress of Gastroenterology (Gastro 2009). *Inflamm Bowel Dis Monit.* 2010;11(3);106-109

van der Marel S, **Duijvestein M**, Hardwick JC, van den Brink GR, Veenendaal R, Hommes DW, Fidder HH. Quality of web-based information on inflammatory bowel diseases. *Inflamm Bowel Dis.* 2009 Dec;15(12):1891-6.

Duijvestein M, Vos AC, Roelofs H, Wildenberg ME, Wendrich BB, Verspaget HW, Kooy-Winkelaar EM, Koning F, Zwaginga JJ, Fidder HH, Verhaar AP, Fibbe WE, van den Brink GR, Hommes DW. Autologous bone marrow-derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: results of a phase I study. *Gut.* 2010 Dec;59(12):1662-9.

Vos AC and **Duijvestein M**. Report from United European Gastroenterology Week Barcelona 2010. *Inflamm Bowel Dis Monit*. 2011;10(3);134-137.

Vos AC, Wildenberg ME, **Duijvestein M**, Verhaar AP, van den Brink GR, Hommes DW. Anti-tumor necrosis factor-α antibodies induce regulatory macrophages in an Fc region-dependent manner. *Gastroenterology.* 2011 Jan;140(1):221-30.

Welling MM, **Duijvestein M**, Signore A, van der Weerd L. In vivo biodistribution of stem cells using molecular nuclear medicine imaging. *J Cell Physiol*. 2011 Jun;226(6):1444-52.

Duijvestein M, Molendijk I, Roelofs H, Vos AC, Verhaar AP, Reinders ME, Fibbe WE, Verspaget HW, van den Brink GR, Wildenberg ME, Hommes DW. Mesenchymal stromal cell function is not affected by drugs used in the treatment of inflammatory bowel disease. *Cytotherapy.* 2011 Oct;13(9):1066-73.

Duijvestein M, Wildenberg ME, Welling MM, Hennink S, Molendijk I, van Zuylen VL, Bosse T, Vos AC, de Jonge-Muller ES, Roelofs H, van der Weerd L, Verspaget HW, Fibbe WE, Te Velde AA, van den Brink GR, Hommes DW. Pretreatment with Interferon-γ Enhances the Therapeutic Activity of Mesenchymal Stromal Cells in Animal Models of Colitis. *Stem Cells.* 2011 Oct;29(10):1549-58

Vos AC, Wildenberg ME, Arijs I, **Duijvestein M**, Verhaar AP, de Hertogh G, Vermeire S, Rutgeerts P, van den Brink GR, Hommes DW. Regulatory macrophages induced by infliximab are involved in healing in vivo and in vitro. *Inflamm Bowel Dis*. Epub 2011 Sep 20

Duijvestein M and Hommes DW. Mesenchymal Stromal Cells in Crohn's Disease. *Inflamm Bowel Dis Monit.* 2011;11(2).

Hommes DW, **Duijvestein M**, Zelinkova Z, Stokkers PC, Ley MH, Stoker J, Voermans C, van Oers MH, Kersten MJ. Long-term follow-up of autologous hematopoietic stem cell transplantation for severe refractory Crohn's disease. *J Crohns Colitis.* 2011 Dec;5(6):543-9.

Wildenberg ME, Vos AC, Wolfkamp SC, **Duijvestein M**, Verhaar AP, Te Velde AA, van den Brink GR, Hommes DW. Dendritic cell autophagy attenuates the adaptive immune response by destabilizing the immunological synapse. Manuscript in preparation.

Strisciuglio C, **Duijvestein M**, Verhaar AP, Vos AC, van den Brink GR, Hommes DW, Wildenberg ME. Autophagy contributes to the refractory state of intestinal DC by regulation of interactions between DC and epithelial cells in the intestine. Manuscript in preparation.

Molendijk I and **Duijvestein M**. Stromal cell therapy in Crohn's disease. In: Mesenchymal Stem Cell Therapy. *Springer text book.* Submitted.

CURRICULUM VITAE

Marjolijn Duijvestein was born on February 10th 1982, in Tegucigalpa, Honduras. She started her secondary education at the Collegiate Middle School in Richmond, (Virginia) in the United States, which she completed at het Sint Vitus College, in Bussum, the Netherlands. She went on to study medicine at Leiden University in the Netherlands. She chose to spend her fourth year abroad at the faculty of medicine of the University of Oviedo in Spain as part of the ERASMUS Programme.

Marjolijn's first research experience was at the department of Parasitology in Leiden, where she worked under the supervision of prof. dr. M. Yazdanbakhsh and dr. F.C. Hartgers. She had several research projects, one of which was 'Detection of Markers of the Innate Immune System in Ghanaian School Children', for which she won the Dick Held Junior Prize.

Her interest in gastroenterology grew during her clinical rotations. After completing her gastroenterology rotation and graduating medical school in 2007, Marjolijn received a grant from the Maag Lever Darm Stichting (MLDS) to initiate and then develop a research project on stem cell therapy for inflammatory bowel disease under supervision of prof. dr. D.W. Hommes. This work is also the subject of her thesis. Obtained data has been presented at several (inter)national conferences on gastroenterology and stem cell therapy and has been published in renowned peer-reviewed medical journals. Furthermore, Marjolijn was the co-author of the ZonMW Translational Adult Stem Cell research grant 'Promotion of Repair by Mesenchymal Stem Cells in Immune Diseases Characterized by Tissue Injury' and the DigestScience grant entitled 'Mesenchymal Stromal Cell Therapy for Crohn' s Disease'. In addition, she is a committee member of the Young European Crohn's and Colitis Organisation (Y-ECCO) Committee.

Marjolijn started her specialty training in gastroenterology in March 2011 (supervisor dr. R.A. Veenendaal, LUMC) and is currently working at the department of internal medicine in the Onze Lieve Vrouwe Gasthuis (OLVG) in Amsterdam under the supervision of dr. P.H.J. Frissen en dr. Y.F.C. Smets.

ACKNOWLEDGMENTS

'As so often happens, the thing left undone tires you most of all, you only feel rested when it has been accomplished'

José Saramago

Onderzoek doe je nooit alleen. Dat blijkt uit alle mensen die *one way or another* hebben bijgedragen aan dit proefschrift:

Prof. dr. Hommes, beste Daan. Toen we elkaar in de lift voor het eerst spraken over promoveren had ik niet verwacht dat het zo'n project zou worden. Zonder dalen bestaan er geen pieken. We hebben ze beiden meegemaakt. Gelukkig bleef jij altijd positief. Dank voor de ruimte die en het vertrouwen dat je me gegeven hebt. Op naar nieuwe ideeën en projecten.

Gijs, ook jij stond in diezelfde lift; met je trolley in de hand op weg naar Zwitserland, vanuit waar je ons het eerste jaar hebt aangestuurd. Jouw continue nieuwsgierigheid ('heb je nog nieuwe data?' terwijl er al door je labjournaal wordt gebladerd), en onuitputtelijke energie (tijdens labbesprekingen, op de piste, in de kroeg) hebben zeker bijgedragen aan het tot stand komen van dit proefschrift. Dank daarvoor!

Helene. Ik weet nog goed dat ik je voor het eerst sprak over MSCs. Er moest een IMPD komen, en ik had geen idee. Dank voor je geduld en bijdrage aan 'ons' MSC/Crohn project. Fijn dat de lijntjes zo kort zijn, en dat jij mijn co-promotor wilde zijn.

Mijn collega's op de MDL. Hein, jouw kritische vragen hielden me altijd scherp. Maar ook dank voor jouw zorg dat de zaak draaiende bleef. Izak en Eveline, dank voor jullie bijdrage aan de muizenproeven. Annie, Marij, Wim en Johan, dank voor jullie ondersteuning op het lab! Daarnaast wil ik het secretariaat (Lokke en Maritza), het trialbureau (Marthe en Maartje) en alle dames en heren van de scopieafdeling bedanken. Veel waardering

gaat uit naar mijn directe IBD collega's: Auke en Christine. Maar ook zonder Willemijn, Rutger, Nikè, Bert-Jan, Patty, Pim, Eva, Philip, Sanne, Thijs, Jarom, Jessica, Lianne, Bart, Liudmila en Vanesa waren de afgelopen vier jaar een stuk minder leerzaam en bovendien vreselijk saai geweest.

Zonder het stamcellab was dit proefschrift nooit van de grond gekomen: dank aan prof. dr. Fibbe, JJ, Sarah en Vanessa. Melissa y Jorge, *¡muchas gracias a vosotros*! En natuurlijk dank aan alle analisten van het stamcellab die met veel zorg ons patiëntenmateriaal hebben opgewerkt voor transplantatie.

Daarnaast zijn er nog enkele personen die ik apart wil bedanken. Anje, de IBD muizenkoning van Nederland, fijn dat je me vanaf het begin af aan hebt willen helpen met het bedenken en ontwerpen van mijn pre-klinische studies. Mick, *only a genius can master chaos*, wat een samenwerking! Tjalling, dank voor het beoordelen van de coupes. Yvonne, dank voor het opwerken van onze biopten, jouw werk heeft toch enkele mooie grafieken opgeleverd. Lynne, mijn boekje is lang niet zo dik als die van jou, hoe dan ook: *I also believe in MSCs*. En last but not least, Marlies: we hebben het toch mooi voor elkaar gekregen om een aantal ton binnen te slepen.

Tenslotte nog dank aan mijn studenten die veel werk hebben verzet: Barbara, Simone en Anne. Ilse en Welmoed, mijn getalenteerde opvolgers, heel veel succes met jullie boekjes – ik heb er alle vertrouwen in!

Dan natuurlijk nog een speciaal woord aan mijn paranimfen. Manon, soms zie ik jou als een soort badmeester die een oogje in het zeil houdt om verdrinken te voorkomen. Heel veel dank voor je bijdrage, geniale ideeën en onuitputtelijke support! Frederike, toch wel mijn langste vriendinnetje, wat toevallig dat wij nu allebei in opleiding zijn tot MDL-arts. Mooi dat we ooit begonnen zijn met een fietsvakantie en we uiteindelijk de route 66 hebben gereden, zonder twijfel een van de vele hoogtepunten van mijn promotietijd.

Vervolgens zijn er nog mensen die niet direct hebben bijgedragen aan deze promotie, maar die mij zeker wel hebben bijgestaan de afgelopen jaren: mijn vriend(inn)en, hockeyteam en collega's van het OLVG.

Drie mensen dienen apart genoemd te worden. Zij hebben wel degelijk bijgedragen aan dit boekje, zonder dat ze ook maar iets medisch hebben. Anne en Suzanne dank voor jullie tekstuele bijdrage aan de resp. Engelse en Nederlandse teksten. Pepijn, jij hebt stiekem een rotklus op je genomen. Heel veel dank aan het vormgeven van dit boekje, het was jouw idee om de format liggend te doen, ik ben zeer tevreden met het resultaat en ben erg benieuwd naar wat anderen ervan denken.

Eén ding is zeker, zonder mijn ouders was dit proefschrift er nooit geweest. Dank voor alle mogelijkheden die jullie mij gegeven hebben. Lieve Peter, oom en buurman, jij neemt een speciale plek in in mijn leven, dank voor wie en hoe je (er) bent! En natuurlijk mijn lieve Oma, ik vind het bijzonder dat we ondanks het leeftijdsverschil nog zo goed contact hebben. Lieve Klaas, zoals ik al eerder zei: promoveren doe je niet alleen. Dank voor je steun en toeverlaat. Volgend jaar kom ik weer appels plukken in Dussen… beloofd!

ABBREVIATIONS

5-ASA	5-aminosalicylic acid
6-MP	6-Mercaptopurine
atMSC	adipose tissue MSC
ATG	antithymocyte globulin
bmMSC	bone marrow MSC
CCMO	Central Committee on Research involving Human Subject
CD	Crohn's disease
CDAI	Crohn's disease activity index
CDEIS	Crohn's disease endoscopic index of severity
CFU	colony forming-units
CRP	C-reactive protein
DC	dendritic cell
DMSO	dimethyl sulfoxide
DSS	dextran sulfate sodium
EBMT	European Group for Blood and Marrow Transplantation
ECCO	European Crohn's and Colitis Organisation
FBS	fetal bovine serum
gMSC	gingival MSC
G-CSF	granulocyte colony-stimulating factor
GvHD	graft-versus-host disease
HGF	hepatocyte growth factor
HLA	human leukocyte antigen
HSC	hematopoietic stem cell
HSCT	hematopoietic stem cell transplantation
IBD	inflammatory bowel disease
IDO	indoleamine 2,3-dioxygenase
IFN-γ	interferon gamma
IL	interleukin
IMSC	IFNγ-stimulated mesenchymal stromal cell
iNOS	inducible nitric oxidase synthase
i.p.	intraperitoneally
LUMC	Leiden University Medical Center
MHC	major histocompability complex
MNC	mononuclear cell
MRI	magnetic resonance imaging
MSC	mesenchymal stromal cell
PBMC	peripheral blood mononuclear cell
PGE2	prostaglandin E2
TGF	transforming growth factor
TNBS	trinitrobenzene sulfuric acid
TNF-α	tumor necrosis factor-α
TSG-6	tumor necrosis factor-inducible gene 6
UC	ulcerative colitis
RBC	red blood cells
TGFβ-1	transforming growth factor beta 1