Stromal cells in inflammatory bowel disease : perspectives of local mesenchymal stromal cell therapy

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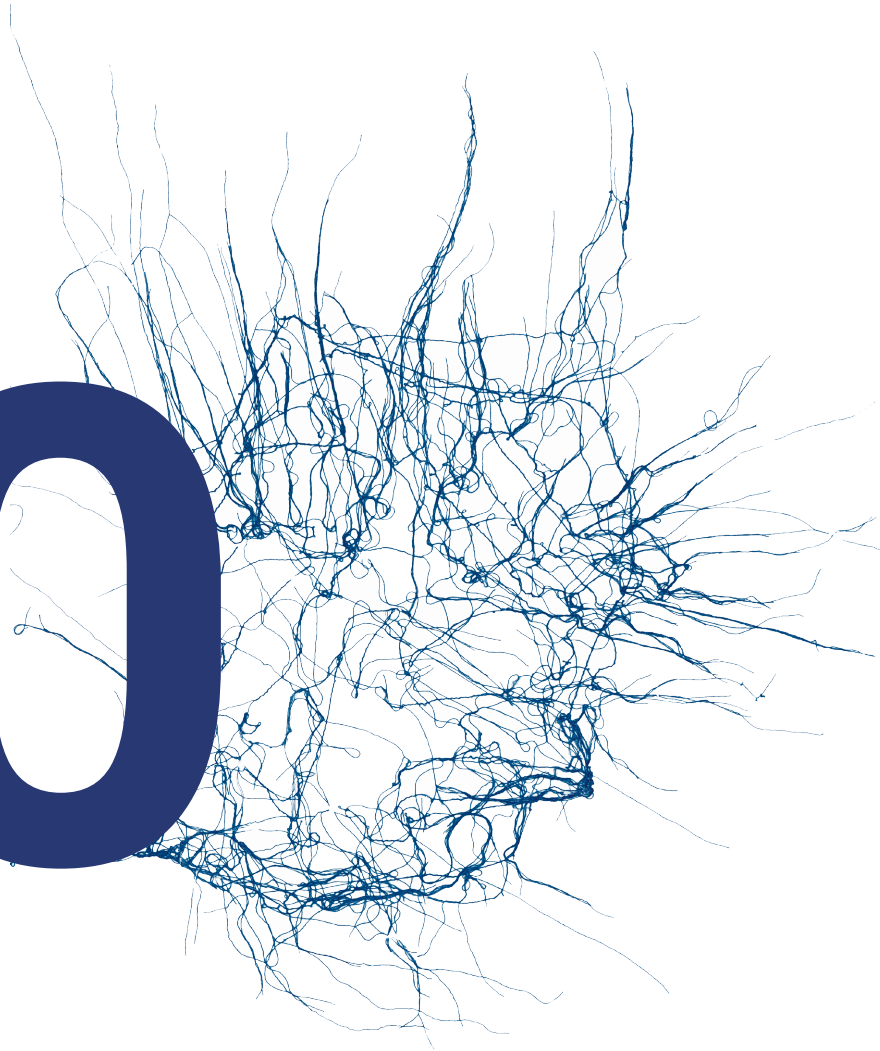

SUMMARIZING DISCUSSION

SUMMARY OF MAIN OBSERVATIONS

The role of stromal cells in inflammatory bowel disease (IBD) is the central theme of the studies described in this thesis, with focus on the therapeutic effects of locally applied mesenchymal stromal cells (MSCs). First, in **Chapter 2**, the current literature on stromal cells present in the inflamed bowel was reviewed, with special attention regarding the function of intestinal fibroblasts in epithelial and immune cell homeostasis. Secondly, the potential immunoregulatory function of fibroblasts derived from Crohn's disease (CD) fistulas and from non-CD associated fistulas was studied in **Chapter 3**. A high expression of cyclo-oxygenase 2 (COX-2) and Twist-related protein (Twist)-1 in the CD-associated fistula fibroblasts was observed. Furthermore, immunoregulatory characteristics of the fistula-derived fibroblasts seemed to correlate with the level of regulatory T cells within the fistula tract, indicating their role in immune cell regulation.

In the next chapters, focus of the studies was on the local application of MSCs to treat IBD. The evaluation of CD patients treated four years earlier with local MSC-therapy for perianal fistulas in **Chapter 4** revealed that MSCs were able to induce long-term fistula closure. One patient included in this long-term follow-up study developed an Epstein Barr virus (EBV)-related lymphoproliferative disease (LPD) in the rectum, without a direct relation with the previously applied MSC-therapy (**Chapter 5**). In **Chapter 6** the effectiveness of local MSC-therapy applied by enema to mice with experimental colitis was shown. Moreover, in **Chapter 7**, local injections of MSCs in the inflamed bowel during endoscopy were found to be effective in ameliorating experimental colitis in mice. MSC-derived exosomes were shown to be, at least partly, responsible for this effect in **Chapter 8** and these exosomes were also shown to support epithelial regeneration in *in vitro* models. Lastly, in **Chapter 9**, *in vitro* evidence was found illustrating that stimulation of human MSCs with various cytokine mixtures, representing the inflamed colonic tissue or CD fistulas milieus, differently induced both upregulation of immunomodulatory molecules in MSCs as well as the immunosuppressive function of MSCs. In conclusion, the studies in this thesis showed the efficacy of local MSC-therapy for the treatment of (experimental) IBD and revealed potential mechanisms through which they exert their efficacy in IBD.

The local environment in diseased tissue of IBD patients

Several studies in this thesis particularly focused on the use of local MSC-therapy, instead of systemic MSC-therapy, for the treatment of IBD. Two application routes for local MSC-therapy in mice with experimental colitis were investigated, first via an enema, as described in **Chapter 6**, and next through endoscopic injections, as described in **Chapter 7**. When MSCs are supplied locally, they will come in direct contact with the inflamed local environment. Two aspects of the local IBD milieu relevant for topical MSC efficacy were

investigated in more detail; the stromal cells that reside in the diseased tissue from IBD patients and the cytokines present in the same tissues. If and how resident stromal cells affect locally injected MSCs was not part of our research project, but the effect of the 'inflamed bowel' cytokines on MSCs was studied as reported in **Chapter 9**.

During inflammation in IBD there are significant changes in the function and composition of stromal cell populations in the intestine, as summarized in **Chapter 2**. Research on the role of stromal cells in IBD is rapidly evolving, highlighting the importance of stromal cells in the pathogenesis of IBD. Remarkable is the expansion of one subset of intestinal fibroblasts, involved in inflammatory processes, in ulcerative colitis (UC)[1,2]. Recently, the presence of a subset of activated fibroblasts was also shown in inflamed ileum tissue of CD patients[3]. This subset of fibroblasts expressed, amongst others, high levels of CD90, podoplanin (PDPN), interleukin (IL)-6, and C-X-C motif ligand (CXCL)-2. Interestingly, these activated stromal cells were part of an cellular signature (additionally including IgG-producing plasma cells, inflammatory mononuclear phagocytes and activated T cells), found in a subset of ileal CD patients, that correlated with unresponsiveness to anti-tumor necrosis factor (TNF)-therapy. This suggests that a subset of fibroblasts plays a role in the responsiveness to anti-TNF-therapy and thereby potentially to other immunomodulatory therapies used in IBD.

State-of-the-art techniques such as single cell RNA sequencing and (imaging) cytometry by time-of-flight (CyTOF) will allow researchers to perform detailed, in-depth characterization of the stromal compartment in IBD, while 'old techniques', as for example cell sorting and coculture experiments, are still needed to assess the function of the various stromal cell populations in IBD. These *in vitro* studies should be complemented with murine models for colitis, that are also useful in this quest. It was already shown that the changes in the stromal compartment upon colitis induction with dextran sodium sulphate (DSS) showed many similarities with the alterations observed in human UC[2]. It will be interesting to unravel if and how the stromal cell compartment changes in other murine models for colitis, such as the IL-10 knockout and T cell transfer model, and to evaluate if that mimics a specific feature observed in IBD patients. We hypothesize that these approaches will change our current view on stromal cells and their role in IBD pathogenesis, and will lead to describing the mucosal stromal system in such detail as the mucosal immune system. Besides focusing on the stromal cells present in the intestines, the stromal cells present in the draining lymph nodes could be of major importance in IBD pathogenesis as well. In the lymph nodes, fibroblastic reticular cells, that are immunologically specialized myofibroblasts, are thought to work immunosuppressively via T cell-suppressive mechanisms[4]. It was shown that inflammation induced by injection of lipopolysaccharide

and ovalbumin in mice causes transcriptional changes in the fibroblastic reticular cells, including upregulation of mRNA of inflammatory chemokines such as chemokine ligand (CCL)-5 and CXCL-9, and molecules of the MHC class II presentation pathway[5]. Keeping this in mind, it would be interesting to unravel potential changes in stromal cells, and in particular in these fibroblastic reticular cells, in the intestinal lymph nodes of IBD patients during disease exacerbation. Besides in lymph nodes, the role of stromal cells in tertiary lymphoid structures has also received more attention recently. Tertiary lymphoid structures are clusters of lymphocytes and stromal cells at sites of chronic inflammation[6]. Nayar and colleagues reported the differentiation of PDPN-positive immunoregulatory fibroblasts in tertiary lymphoid structures in Sjögren's syndrome[6]. They showed the importance of the IL-13 pathway in the priming of these fibroblasts and IL-22 for the proliferation of the fibroblasts thereafter. Immunohistochemistry and imaging CyTOF should reveal whether the identified inflamed/activated fibroblast that expand in IBD intestines[1-3] are located in these tertiary lymphoid structures.

The focus of our study reported in **Chapter 3** was on the fibroblasts derived from perianal fistulas from patients with CD. Only subtle differences in the gene expression levels were found between these fibroblasts and fibroblasts isolated from non-CD fistulas regarding genes involved in the regulation of inflammatory processes. More pronounced differences in both gene and protein expression were observed between CD fistula fibroblasts and histological normal colonic fibroblasts. These colon fibroblasts were isolated from normal tissue from patients with colon cancer, at least ten centimeter from the primary tumor. Future research, using fibroblasts derived from the rectosigmoid of CD patients, instead of colonic fibroblasts from patients with colon cancer, should reveal whether fistula fibroblasts differ from intestinal fibroblasts derived from inflamed tissue and if indeed fistula-specific fibroblasts exist or whether they represent a more general inflammation-induced fibroblast phenotype. Interestingly, correlations between the expression of certain immunoregulatory proteins on fistula-derived fibroblasts, as programmed death ligand (PD-L)1, PD-L2 and IL-6, and the number of Forkhead box P (FOXP)3^{pos} cells in the fistula tissue were reported (**Chapter 3**). In ongoing experiments, a potential direct relation between these fibroblasts and regulatory T cells is studied *in vitro*, using coculture experiments between fibroblasts and T cells. We hypothesize that specific fibroblasts (PD-L1^{low}PD-L2^{low}IL-6^{high}) are able to induce differentiation, proliferation or migration of FOXP3^{pos} cells. Although it is hypothesized that fibroblasts affect immune cells, the reverse could also be true, i.e., that FOXP3^{pos} cells determine the phenotype of the fibroblasts in the perianal fistulas. In that regard it is very interesting that a recently published single cell RNA-sequencing study showed that a subset of regulatory T cells is a major source of TNF-α production[1]. These cells are still enriched for regulatory T cell markers, such as FOXP3 and

IL-10. To determine the anti-inflammatory or pro-inflammatory character of the regulatory T cells identified in perianal fistulas, double immunostaining including both FOXP3 and TNF-α should be performed and the number of both the number of FOXP3^{pos}TNF-α^{neg} and FOXP3^{pos}TNF-α^{pos} cells could be correlated with fibroblast phenotypes. Furthermore, in coculture experiments, the cytokine profile of FOXP3^{pos} cells should be revealed after coculture with different fistula fibroblasts, but on the other side also the immunoregulatory phenotype of intestinal fibroblasts after coculture with various subsets of FOXP3^{pos} cells.

The effects of local cytokines, detected in the tissue from IBD patients, on MSC phenotypes were studied in more detail, as reported in **Chapter 9**. In perianal fistulas, two cytokine profiles were observed, both containing pro-inflammatory cytokines as TNF-α, IFN-γ, IL-13, IL-17, but only one including IL-4. In the high cytokine levels found in the inflamed colonic tissue from patients with UC also two profiles were identified, the first including oncostatin-M (OSM), IL-1β, IL-17 and IL-8, while the second profile included the cytokines of the first profile, and in addition IL-5, IL-13, TNF-α, IL-6 and interferon (IFN)-γ. Interestingly, the local cytokine profile in perianal fistulas was not directly reflected in the serum of these patients. Previously, Ruffolo and colleagues also found no relation between the level of IL-12, IL-1β, TNF-α and IL-6 in the rectum mucosa and serum of CD patients with fistula[7]. Furthermore, it was found that the addition of specific cytokines to selective cytokine sets could downregulate the observed upregulation of immunomodulatory markers on MSCs. For example, indoleamine 2,3-dioxygenase (IDO) and human leukocyte antigen (HLA)-DR expression were downregulated after the addition of IL-17, IL-1β and OSM to the mixture of IFN-γ, TNF-α and IL-13. These data indicate that stimulation experiments with single cytokines should be interpreted carefully since combinations of cytokines could have opposite effects. Furthermore, cytokine mixes that improved the inhibitory capacity of MSCs on T cell proliferation *in vitro* did not result in the highest inhibition of dendritic cell (DC) differentiation. These observations showed the importance of the local cytokine milieu, that differs among IBD patients, in the regulation of the MSC-function *in vitro*.

Mechanism of action of local MSC-therapy

The paradigm of how systemically applied MSCs exert their function, has changed considerably over the last years. Whereas a decade ago intravenously injected MSCs were thought to migrate to sites of injury and to differentiate *in vivo* into adult differentiated mesenchymal cells, recent studies show that most of the intravenously injected MSCs seem to accumulate in the lungs and do not survive for a long period of time, although they still show clinical effiacy[8,9]. Probably MSCs deploy a plurality of immunomodulatory and tissue regenerative properties, not connected by a single effector pathway.

Instead of the classical systemic MSC-therapy, studies in this thesis focused on local MSC-therapy as explored in experimental IBD models. The first approach, as described in **Chapter 6**, was by applying MSCs via an enema, succeeded by a study on direct injection of the MSCs into the inflamed bowel during endoscopy (**Chapter 7**). Both administration routes showed amelioration of DSS-induced colitis. In accordance, Manieri and colleagues[10] previously showed that endoscopically applied MSCs could promote intestinal repair and angiogenesis in intestinal wounds. Secretion of paracrine factors is recognized as one of the primary mechanisms by which MSCs adjust inflammation. The efficacy of MSCs aggregated into spheroids, to mimic the observed *in vivo* aggregatory behavior of MSCs after intraperitoneal or intravenous injection[11], was evaluated in both **Chapter 6** and **7**. IDO, transforming growth factor (TGF)-β1, hepatocyte growth factor (HGF), prostaglandin (PG) E2 and tumor necrosis factor inducible gene (TSG)-6 are produced by MSCs and thereby MSCs interfere with the amino acid metabolism in the inflammatory microenvironment[9]. *In vitro* upregulation of TGF-β1, CD200, HGF and vascular endothelial growth factor (VEGF) was found in MSCs aggregated in spheroids, while CCL2 and CXCL12/stromal cell derived factor 1 (SDF)-1 expression was downregulated compared with MSCs cultured in monolayer. This data suggested that MSC-spheroids can potentially display enhanced anti-inflammatory and tissue regenerative capacities. Although the effectiveness of MSC-spheroids given by an enema was shown, as reported in **Chapter 6**, direct comparison *in vivo*, however, revealed that both forms of MSCs, i.e., as single cells or in spheroids, had comparable efficacy in ameliorating colitis after endoscopic injections. The equally effects of MSCs and MSC-spheroids might be explained by the fact that single MSCs tend to form multicellular clusters in the bowel wall, or by effects of the local colon milieu on the injected MSCs/MSC-spheroids that transcend the differences observed after *in vitro* aggregation.

It is described in literature that intravenously administered MSCs accumulate in the lungs and do not survive for more than 24 hours, while the survival time of MSCs after local application is unknown[8,9]. In this thesis, MSCs could be detected up to 6 days after endoscopic injection in the inflamed bowel, as shown in **Chapter 7**. However, the possibility of apoptosis of at least a part of the MSCs after injection cannot be excluded. Interestingly, it was shown that after intracranial administration of allogeneic MSCs in macaques, MSCs were only detectable after 6 months in the brain of the macaques that received the lower dosages of MSCs and not in those administered the highest dosages[12]. This suggests that high MSC levels cause MSC apoptosis. Both our short- and long-term clinical results of the treatment of perianal fistulas with MSCs, as described in **Chapter 4**, showed lower efficacy rates in the cohort of patients that had received the highest dosage of MSCs. In combination these observations imply a relation between MSC dosage, MSC survival and efficacy in local MSC-therapy.

As illustrated in **Chapter 7**, both T cells and macrophages were found to surround the MSC clusters in the colon wall of mice with DSS-induced colitis. MSCs are known for their production of chemo-attractants, such as CXCL-12/SDF-1, that attract different immune cells. After chemoattraction, MSCs are thought to modify these immune cells. In **Chapter 9** our observations that MSCs are able to inhibit the differentiation of monocytes into dendritic cells next to inhibition of T cell proliferation are described. This data also showed that the local cytokine environment in which the MSCs are injected can affect the MSC effectiveness in inhibiting immune cell function activity. In **Chapter 9** only human MSCs were studied and although the response of murine MSCs on cytokines can differ from that of human MSCs, we hypothesize that the local murine cytokine milieu also optimizes the anti-inflammatory capacity of the endoscopically injected murine MSCs. Another explanation for the surrounding of MSC clusters by immune cells in the inflamed murine intestine is that the host immune cells recognize the MSCs as 'foreign' cells. While this would not prevent them from amelioration of experimental colitis, allogeneic MSCs may be recognized by the immune system through MHC I and II molecules. However, Galleu and colleagues showed that *in vitro* MSC apoptosis by $CD8^{pos}$ T cells was not affected by the presence of neutralizing antibodies for class I or class II human leukocyte antigen molecules[9]. Furthermore, they found that both $CD56^{pos}$ natural killer and $CD8^{pos}$ T cells were responsible for initiating MSC apoptosis *in vitro* and explained the apoptosis as a bystander role for cytotoxic granules released by activated cytotoxic T cells[9].

Next to T cells, there are also reports showing the importance of phagocytes as potential mediators in MSC-dependent immunomodulation. Phagocytes that engulfed apoptotic MSCs start producing IDO and IL-10, and thereby delivered MSC immunosuppressive activity[9,13,14,18]. It would be interesting to investigate the phenotype of the phagocytes surrounding the MSC-clusters in the bowel wall, as illustrated in **Chapter 7**, since engulfment of MSCs induced the anti-inflammatory CD163 and CD206 on monocytes[14]. Also, after intravenous injection of PKH26-labeled MSCs in a murine asthma model, double positive cells for phagocyte markers and PKH26 were identified in the lungs after 1 day, suggesting the uptake of (most of the) MSCs by phagocytes[15]. Phagocytized MSCs, as shown by $PKH26^{pos}$ macrophages, compared with $PHK26^{neg}$ macrophages expressed higher mRNA levels of the anti-inflammatory cytokines TGF-β and IL-10. Phagocytosis of foreign stromal cells, is also observed during pregnancy, in which apoptotic fetal stromal cells travel from the uterus to the maternal lung and leads to upregulation of anti-inflammatory cytokines, as IL-10 and IDO, due to phagocytosis by maternal macrophages[16,17]. Thereby, the fetal stromal cells are creating a more immune tolerogenic milieu in the mother. Although there is extensive literature on the apoptosis of MSCs after intravenous injection, it is unknown whether locally injected MSCs in an inflamed environment also undergo apoptosis and if

so, in which timespan. Our data in **Chapter 7** showed that some MSCs are detectable up to day 6 after local MSC injection. Furthermore, the previous citated literature on the survival of intracranial injected MSCs only after application of a low dosage in macaques[12] and the low fistula healing rates after application of the highest dosage of MSCs (**Chapter 4**), would suggest that in local MSC-therapy living MSCs are more important than apoptotic MSCs. Lastly, locally injected MSCs will always first encounter the pro-inflammatory milieu and directly inhibit inflammation through their surface markers and secreted factors, before potential apoptosis-mediated immunomodulation could take place.

To investigate the local effects of locally applied MSC-therapy, the colons of mice receiving MSC- and placebo-therapy were compared. Lower levels of IL-6 and IFN-γ were observed in the colon of mice after treatment with MSC-spheroids given via an enema (**Chapter 6**), while only significantly lower colonic IL-8 levels were found in mice treated with locally injected MSCs (**Chapter 7**). Strikingly, higher IL-10 and IFN-γ serum levels were observed in mice treated with endoscopically injected MSCs. Higher IDO levels were also found in the bowel of these mice. IDO has been described as one of the important factors regulating human MSC function, since it can inhibit T cell proliferation through the conversion of tryptophan into kynurenine[18,19]. The level of T cell inhibition by different sets of cytokine-stimulated MSCs was found to be correlated with their *in vitro* production of IDO, as shown in **Chapter 9**. In addition, as illustrated in **Chapter 5**, murine MSCs were also found to be able to produce IDO, especially after stimulation with TNF-α and IFN-γ. It is unknown whether the substantial levels of IDO found in the bowel are produced by MSCs directly, as proposed by Ren et al[20], or indirectly through their modification of macrophages.

Besides soluble factors, MSCs also produce exosomes, vesicles that contain several proteins, (micro-) RNAs and DNAs. Therefore, the regenerative capacity of MSC-exosomes on epithelial cells *in vitro* and in DSS-induced colitis *in vivo* was investigated, as described in **Chapter 8**. A high dose of MSC-exosomes was found to be effective in the stimulation of epithelial proliferation, wound closure and inhibition of epithelial cell apoptosis in two-dimensional and three-dimensional epithelial cell models. However, local MSC-derived exosome-therapy seemed to be less effective compared with MSC-therapy in the treatment of experimental colitis. MSC-derived exosomes can therefore only be effective as a cell-free therapy after optimization, for example by stimulating the MSCs with cytokines before harvesting the vesicles[21,22]. Besides the effects of MSC-exosomes on epithelial regeneration, it has also been shown that MSC-exosomes can inhibit macrophage activation by modulating Toll-like receptor signaling[23], increase the regulatory T cell/effector T cell ratio *in vitro*[24] and decrease the frequency of $CD14^{pos}CD16^{pos}$ inflammatory monocytes[22], illustrating their immunomodulatory capacity.

Next to the immunoregulatory and pro-regenerative effects of MSCs, they can also be recognized as a therapy that introduces 'healthy' stromal cells in diseased tissue. The comparison with IBD-derived fibroblasts seems then relevant, especially for perianal fistulas in CD, for which Cx601/darvadstrocel (containing adipose tissue-derived MSCs) is now approved by the European Medicines Agency. The immunoregulatory expression pattern of fistula-derived fibroblasts from CD patients was therefore compared with that of human MSCs, as described in **Chapter 3**. In comparison to MSCs, CD fistula fibroblasts showed high *in vitro* levels of the pathogenic fibroblast marker PDPN, but low levels of immunoregulatory CXCL-12, COX-2, OSM receptor, TGF-β1 and colony stimulating factor (CSF)-1. These differences could be due to the different tissues, fistula versus bone marrow, they were derived from and it would be interesting to determine the expression levels of these immunoregulatory proteins after the MSCs have been settled in the fistula environment. To get an impression of this local effect, the impact of the perianal fistula cytokine milieu was investigated on the characteristics and function of human MSCs (**Chapter 9**). TGF-β1 production by MSCs was downregulated after stimulation with the cytokine cocktail representing the fistula environment, more in accordance with the TGF-β1 production by fistula fibroblasts. However, CSF-1 and COX-2 expression was even further increased in MSCs upon stimulation, while CXCL-12 expression was not affected by the fistula cytokines. Previously, it was found that MSCs expressed more COX-2 after injection in an asthma mouse model[15]. These observations suggest that MSCs after injection in perianal fistulas might indeed generate higher levels of COX-2 compared with fistula fibroblasts. Interestingly, COX-2 production by MSCs strongly correlated with their level of inhibition of DC differentiation, as described in **Chapter 9**. This might imply that MSCs are indeed 'healthy' stromal cells that do not adopt the same phenotype as fistula fibroblast *in vitro* upon contact with the fistula milieu and are thereby better capable of exerting their immunoregulatory potential.

Local MSC-therapy translated to clinical practice

Allogeneic adipose tissue-derived MSCs (Cx601/darvadstrocel) were registered for the treatment of CD perianal fistulas in Europe in 2017. A breakthrough in MSC research, since it was the first marketing authorization for MSC-therapy in Europa, and also a step forward in the unsatisfactory treatment of refractory perianal fistulas in patients with CD. Conventional treatment of perianal fistulizing CD consists of antibiotics and drainage of the tract with loose setons, which in itself will not result in fistula healing. Therapy with anti-TNF is most effective in inducing fistula healing, either alone or in combination with other immunosuppressants[25]. Recently, it was shown in a multinational retrospective cohort study that 65% of the patients received a multi-modality approach with both medical treatment along with surgical approach and only 52% of these patients experienced

complete fistula healing[26]. MSC-therapy might increase these healing rates. Previously, we conducted a phase II clinical trial where patients with CD fistulas were treated with respectively 10, 30 or 90 million allogeneic bone marrow-derived MSCs[27]. The safety and effectiveness, of especially the lowest two dosages was shown after 24 weeks. Thereafter, a large phase III study was performed by Panes and colleagues that showed that adipose-derived MSCs (Cx601/darvadstrocel) induced complete fistula closure in 51% of the patients versus 36% of the placebo-treated patients, with 80% of the patients already using anti-TNF-therapy[28]. In addition to the short-term data, the long-term efficacy and safety of allogeneic bone marrow-derived MSCs for the treatment of perianal fistulas in CD of our cohort was also assessed, as reported in **Chapter 4**. Four years after MSC-therapy, fistula closure rates of 63%, 100% and 43% were found in respectively cohort 1 (lowest dose MSCs), 2 (middle dose MSCs) and 3 (highest dose MSCs). These positive long-term results suggest that MSC-therapy could be a definite therapy to close perianal fistulas in CD patients. In our cohort two patients developed cancer in the gastrointestinal tract. Next to a caecum adenocarcinoma, one of the patients developed an EBV-positive LPD in the rectum as detected during the long-term follow-up at endoscopy and discussed in **Chapter 5**. By thorough analyses it was excluded that the EBV-virus was transferred via the infused MSCs or by contaminating EBV-positive B cells present in the MSC product. Furthermore, no allogeneic MSCs or DNA specific for the MSC donor was detected in the lesion in the rectum. We rendered it highly unlikely that this LPD was related to MSC-therapy. In the clinical trial conducted with Cx601/darvadstrocel no systemic complications, including infections, malignancies or mortality were reported in the short-term[28,29]. Furthermore, a meta-analysis evaluating the 3 randomized controlled trials conducted with MSCs for the treatment of perianal fistulizing CD reported no serious adverse events directly related to MSC-therapy[30]. It is remarkable that they did not mention the adenocarcinoma in the caecum as reported in our clinical trial[27], while the study was included in their meta-analysis. Although the direct relation between local MSC-therapy for perianal CD and adenocarcinoma in the caecum is very unlikely, it has been described that MSCs are able to stimulate tumor growth *in vivo* by their effects on epithelial cell proliferation[31]. Therefore, the precise registration of adenocarcinomas and other types of cancers after MSC-therapy, is of great importance.

The results of clinical trials for MSC treatment of luminal IBD, as reviewed previously[32,33], have been rather variable with regard to the efficacy, with clinical remission in around 40% of the MSC-treated patients. In these trials, MSC-therapy was applied intravenously. In one placebo-controlled trial, using allogeneic placenta-derived MSCs, clinical remission was achieved in 14% of the patients treated with MSCs versus 0% in the placebo group, whereas serious adverse events were reported in 41% of MSC-treated patients[34]. Many of

these serious adverse events concerned thrombophlebitis. Trials using MSCs from other organ sources did not report this adverse events, suggesting thrombophlebitis is related to placenta-derived MSCs. Before definite conclusions can be drawn regarding the use of intravenous MSC-therapy for luminal disease in IBD more placebo-controlled trials with adipose tissue and bone marrow derived-MSCs are needed.

Another, possibly more effective, form of MSC administration is local MSC injection. In order to study whether local MSC-therapy, as used for the treatment of perianal CD fistulas and tested in experimental colitis, as described in **Chapter 4** and **7**, is also applicable and safe for the treatment of luminal disease in IBD patients, we recently started a clinical phase II trial on the safety and tolerability of endoscopically injected bone marrow-derived MSCs in the rectum of patients with ulcerative proctitis, the MSC-proctitis trial (EudraCT: 2017-003524-75, Dutch trial register: NTR7205). We hypothesize that local MSC-therapy in the bowel is safe, given the good safety profile in perianal fistulas, and, based on our experimental data, is able to induce suppression of activated mucosal immune responses and, in addition, to support regeneration of epithelial cells. In this and other future clinical trials with allogeneic MSCs a few things need to be addressed.

Firstly, for clinical MSC trials it would be of the outmost importance to investigate the mode of action of MSC-therapy, besides safety and efficacy. After almost twenty years of clinical trials, we have only limited knowledge on the workings mechanism of MSCs in patients. Therefore the aim of the MSC-proctitis trial is to study, next to primary endpoints like safety, tolerability and feasibility, also the effects of MSC-therapy on immune subsets and inflammatory cytokines in the inflamed rectum. In systemic lupus erythematosus, for example, it was recently discovered that the number of peripheral tolerogenic $CD11c^{pos}$ dendritic cells was significantly upregulated after intravenous injections with allogeneic MSCs[35]. Furthermore, fluorescent in-situ hybridization of Y chromosomes will be used to try to trace male MSCs in the rectum of female recipients. Hereby, the survival of MSCs in the inflamed colon could be monitored.

Secondly, the importance of immunologic compatibility between donor MSC and recipient should be unraveled. Allogenic MSC-therapy is a more attractive option than autologous MSC-therapy since it allows for immediate treatment with previously approved cells. The prevailing paradigm is that MSCs are not considered to induce strong immunogenic responses, while they do display immune modulatory capacities. This paradigm is based on low expression of HLA class I and II molecules and low levels of co-stimulatory molecules as CD40 and CD80 by MSCs. Therefore no HLA-matching is performed before applying allogeneic MSC-therapy. In macaques, cytotoxic peripheral blood leukocytes were found

capable of lysing donor MSCs after intracranial administration of MHC-mismatched MSCs, but not after receiving autologous MSCs[12]. Interestingly, in this study the magnitude of the immune response was correlated with the number of mismatches of MHC molecules. The Cx601 phase III clinical trial showed *de novo* generation of anti-HLA class I antibodies in 34% of the patients at week 12 after MSC-therapy[28]. Some of these *de novo* antibodies seemed transient, since in 35% of the patients clearance of the antibodies was seen at week 52[36]. No safety signals were associated with development of donor specific antibodies (DSAs) nor was the presence of DSAs associated with clinical response[28]. In our phase II study on bone marrow-derived MSC-therapy for perianal fistulas, described in **Chapter 4**, no de novo anti-HLA antibodies could be detected in any of the patients, 24 weeks after MSC-therapy. This could be due to the timing of antibody measurements or the origin (bone marrow or adipose tissue) of the MSC product. Anti-HLA antibodies are able to bind HLA and subsequently initiate antibody-dependent cytotoxicity through interaction with innate immune cells via Fc receptors or via complement activation[37]. Cells are able to escape complement dependent cytotoxicity by expression of membrane-bound complement regulatory proteins (mCRPs) as CD55, CD46 and CD59. Interestingly, the basal levels of these mCRPs were higher in adipose tissue-derived MSCs compared with bone marrow-derived MSCs, and could be further increased by stimulating with IFN-γ in adipose, but not in bone marrow-derived MSCs[36]. This suggests that bone marrow-derived MSCs are more prone to be killed by complement cytotoxicity compared with adipose tissue-derived MSCs. Whether these mCRPs, as CD55, CD46 and CD59 are induced upon encountering the local IBD pro-inflammatory environment is unknown, but upregulation of CD46 was shown after IFN-γ stimulation. In conclusion, there is a lack of knowledge on the consequences and mechanisms behind non-matched allogeneic MSC-therapy. Therefore, the results of our ongoing trial should reveal the existence of (transient) donor-specific HLA antibodies at several time points, including earlier timepoints, in relation to the number of HLA-mismatched genes and elucidating their functional relevance.

Thirdly, efforts should be made to find selection criteria for MSC products and patients receiving MSC-therapy. Predictive biomarkers for patient stratification could help clinicians to decide which patient should receive which MSC product. Galleu and colleagues showed that the *in vitro* cytotoxic activity of PBMCs against MSCs predicted clinical response to intravenously injected MSCs in graft-versus-host disease patients[9]. They suggested to use the ability of the recipient to induce apoptotic MSCs as a potential biomarker to stratify patients for MSC therapy in graft-versus-host disease. Chinnadurai and colleagues suggested that IDO induction in MSCs during coculture with PBMCs or after IFN-γ stimulation could be used as a readout for MSC functionality[38]. Besides IDO, they also found that IFN-α, IL-7, CXCL-9 and -10, VEGF and CCL-2 production during coculture

between MSCs and PBMCs correlated with T cell suppression. Our observations reported in **Chapter 9** indicate that the *in vitro* inhibition of T cell proliferation correlates with the expression of IDO, but also with PD-L1, HLA-DR and CD54 expression by MSCs. Chinnadurai and colleagues only focused on the suppression of T cell proliferation by MSCs and not on the effects of MSCs on other immune cells. Our study revealed that the inhibition of DC differentiation was correlated with COX-2 and G-CSF production of the MSCs. Furthermore, the relevance of different cytokine mixtures, representing different IBD patients' milieus, on the *in vitro* characteristics of MSCs and on MSC-dependent immunomodulation was clearly demonstrated. These observations substantiate the impression that besides the MSC donor product and the recipient immune cells also the local cytokine milieu determines the efficacy of MSC-therapy. The theory that changes upon IFN-γ stimulation could be used as a readout for MSC functionality[38] does not seem to be applicable for local MSC-therapy in IBD, since IFN-γ alone did not reflect the complex IBD cytokine milieu. Remarkable was the downregulation of different immunoregulatory markers by addition of IL-17, IL-1β and OSM, all present in local IBD tissue, to IFN-γ-stimulated MSCs. The registration of Cx601/darvadstrocel as a therapy in CD-associated perianal fistulas provides the excellent opportunity to study whether various cytokine milieus influence MSC therapy outcome *in vivo* in the coming years. The results from our clinical trial, described in **Chapter 4**, suggest that one MSC donor product is indeed less capable of fistula healing. However, some reservation for this interpretation has to be made due to the low number of MSC donor products analyzed and to the limited number of patients included. In **Chapter 9**, only small differences in the upregulation of immunomodulatory molecules in different MSC donor products on the stimulation with cytokine mixtures were found. Further research is indicated to assess whether these differences between MSC donor products have clinical importance and whether some MSCs are indeed 'better' than others. This could even lead to personalized medicine, in which the best MSC donor is selected for each patient, based on MSC responses toward the patients' cytokine milieu.

Fourthly, new clinical MSC trials should include long-term follow up of at least 5 years, but preferable up to 15 years, to define long-term safety and efficacy. Although the short-term safety profile of local MSC-therapy is good, the occurrence of LPD in the rectum of one of our MSC-treated patients, as reported in **Chapter 5**, shows that it is important to monitor potential long-term side effects. In our ongoing clinical trial, we included a 1, 3 and 5 years follow-up visit.

In most clinical trials testing MSC-therapy, patients were allowed to continue treatment with immunosuppressive drugs as azathioprine and biologicals as anti-TNF therapy, while little is known about the interaction of these therapies with MSCs and their functional

activity. Patients in the MSC-fistula trial were allowed to continue medical therapy. In our ongoing phase II clinical trial patients are requested to cease rectal therapy, although they are allowed to continue systemic therapy. Most medical therapies used in IBD block one or more inflammatory pathways, thereby potentially affecting the priming of MSCs by the local mucosal milieu into their immunomodulatory phenotype. Although immunomodulatory therapy might decrease local and circulating cytokine levels, in our study (**Chapter 9**) high levels of cytokines were still present in the perianal fistulas of patients treated with immunosuppressive therapies. *In vitro* model systems as well as murine experimental colitis models provide good pre-clinical opportunities to study the effects of co-medication on MSC-therapy in order to provide guidelines for clinicians how to combine MSC-therapy with other immunomodulatory therapies in IBD. This should reveal how immunomodulatory therapies influence MSC priming, MSC engraftment or MSC apoptosis and eventually therapeutic efficiency.

In conclusion, many studies report successful treatment of perianal fistulas with allogeneic MSCs. Yet, it remains difficult to implement MSC-therapy into daily clinical practice, mainly due to the cell-product requirements needed for cell-based therapies. Novel regenerative therapeutic approaches, including treatment with MSC-exosomes or with autologous adipose tissue, without *ex vivo* cellular expansion[39,40], are expected to be introduced into the clinic in the coming years. The introduction of (products derived from) various types of healthy stromal cells as well as the inhibition of pathogenic stromal cells, may very well be near-future avenues to heal the inflamed bowel in IBD patients.

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