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

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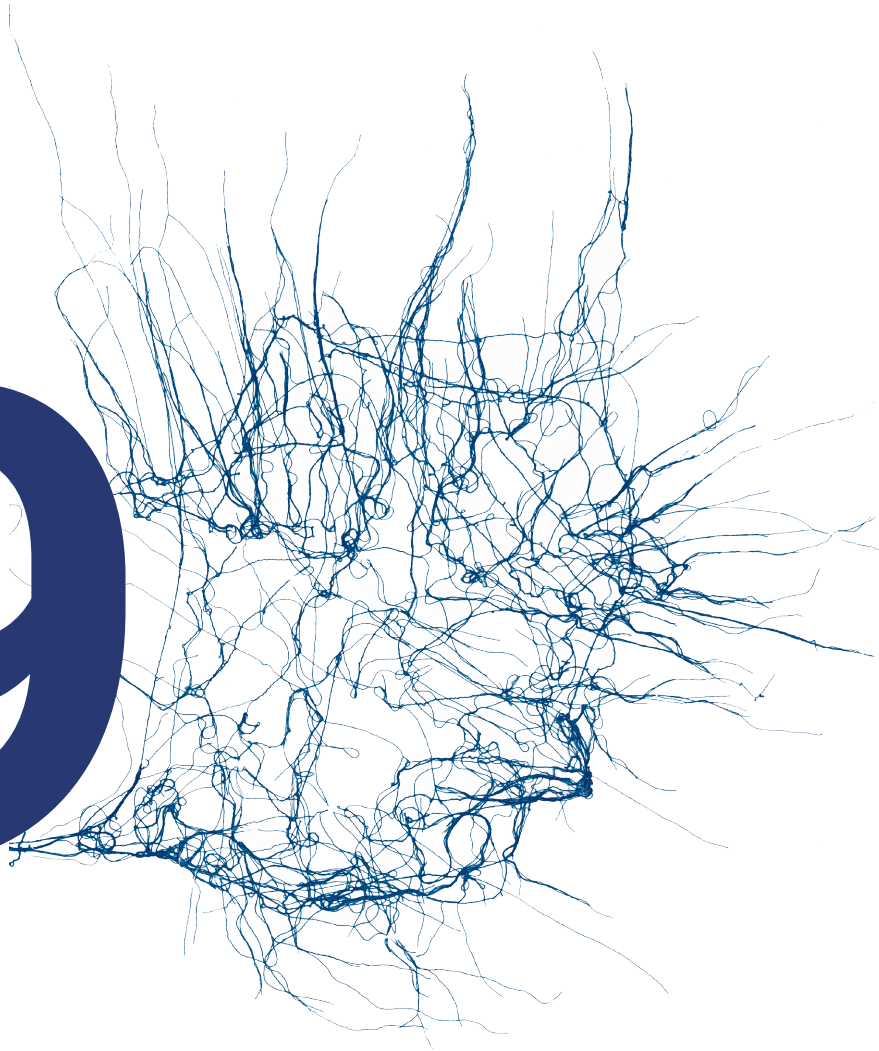

# THE LOCAL CYTOKINE MILIEU IN PATIENTS WITH INFLAMMATORY BOWEL DISEASE IMPACTS PHENOTYPE AND FUNCTION OF MESENCHYMAL STROMAL CELLS

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## ABSTRACT

Locally applied mesenchymal stromal cells (MSCs) have the capacity to promote healing of refractory perianal fistulas in Crohn's disease (CD) and are under clinical development for treatment of refractory proctitis in ulcerative colitis (UC). Despite this clinical effect, the mechanism of action of local MSC-therapy in inflammatory bowel disease is largely unknown. We hypothesized that a disease-specific local proinflammatory environment promotes the immunomodulatory properties of MSCs. In this study we investigated *in vitro* the effect of local cytokines found in IBD patients on expression of immunomodulatory molecules and function by MSCs. Therefore cytokine levels in inflamed tissues obtained from CD and UC patients were analyzed. Next, MSCs were cultured in the presence of these cytokines and both the expression of molecules known to be involved in immunomodulation by MSCs and the subsequent capacity of MSCs to modulate T cell proliferation and dendritic cell (DC) differentiation was assessed. Four different cytokine combinations were identified, representing a patient groups' local cytokine milieu. Each combination induced a unique expression pattern of immunomodulatory surface markers, chemokines, enzymes and cytokines by MSCs, including cyclooxygenase-2 (COX-2), indoleamine 2,3-dioxygenase (IDO), transforming growth factor-β1 and programmed death ligand 1 (PD-L1). Interestingly, cytokine mixtures that enhanced the capacity of MSCs to inhibit T cell proliferation did not result in the highest inhibition of DC differentiation and vice versa. The level of COX-2 and granulocyte-colony stimulating factor (G-CSF) expression by MSCs upon stimulation with different cytokine mixtures correlated with the ability to inhibit differentiation of monocytes into DCs. On the other hand, the expression levels of human leukocyte antigen (HLA)-DR, PD-L1, CD54, IDO and chemokine ligand (CCL)-5 correlated with the inhibition of T cell proliferation. Lastly, we observed that MSCs from various donors showed variable levels of marker expression upon exposure to the cytokine mixtures. In conclusion, we found different local cytokine milieus in inflamed UC colonic or CD fistulas tissue and different induction of immunomodulatory molecules in MSCs by these cytokine milieus.

# INTRODUCTION

Local injection of mesenchymal stromal cells (MSCs) is a recently approved therapy for the treatment of perianal fistulas in Crohn's disease (CD)[1-3]. Significant improvement of fistula healing was shown in our phase II clinical trial in patients treated with bone marrow-derived (bm)MSCs compared with placebo treated patients, with a reduction in the number of draining fistulas up to 86%[3]. At 4-years follow-up, sustained fistula closure was observed after MSC-therapy in these patients[4]. Furthermore, a large multicenter trial showed fistula closure after allogeneic adipose-tissue-derived MSC injection (darvadstrocel/Cx601) in 50% of the 107 patients versus 34% in the 105 placebo treated patients[1]. Recently, we started a phase II clinical trial to study the safety and tolerability of endoscopically applied bmMSCs in patients with proctitis in ulcerative colitis (UC) (EudraCT: 2017-003524-75, Dutch trial register: NTR7205), after our observation that endoscopically injected MSCs are able to alleviate colitis in a mouse model[5] and the report that biopsy-induced ulcers healed faster after local MSC application[6].

Although the exact mechanism of action of local MSC-therapy is unknown, we hypothesize that MSCs gain their immunomodulatory features upon encountering a proinflammatory cytokine environment[7,8]. The effects of individual cytokines on MSC surface marker expression and function have been studied before. For example, interferon-γ (IFN-γ) stimulation, resulted in upregulation of idoleamine-2,3 (IDO), human leukocyte antigen (HLA)-DR, intercellular adhesion molecule (ICAM), chemokine ligand (CXCL)-9 and CXCL-11 in human MSCs[8, 9]. IFN-γ stimulated MSCs also showed an improved capability to alleviate experimental colitis compared with their non-stimulated counterparts[10]. This indicates that MSCs benefit from priming with IFN-γ to exert enhanced anti-inflammatory activities. However, it is yet unknown how the inflammatory cytokine milieu found in IBD tissue impacts the immunomodulatory properties of MSCs.

In the present study we investigated *in vitro* whether the local cytokine milieu present in IBD patients could affect the expression of immunomodulatory molecules and function of bmMSCs. Cytokine levels in CD perianal fistulas and UC colon samples were compared with control tissue. Based on previously published research, it was decided to measure cytokines produced by T helper (Th)1 cells, which are main contributors to inflammation in CD, Th2 cells, which are mainly thought to play a role in UC and Th17 cells, which are important in both forms of IBD[11, 12,13]. Furthermore, cytokines produced by innate immune cells in the intestines of patients with IBD, like interleukin (IL)-1β, IL-12, oncostatin M (OSM), tumor necrosis factor (TNF)-α and IL-6, were measured. From this analysis, four cytokine profiles were identified in IBD tissue. Stimulation of MSC with each of these four cytokine cocktails induced changes in expression of immunomodulatory molecules by MSCs.

To study the functional effects of these changes, T cell proliferation and dendritic cell (DC) differentiation were evaluated in cocultures of stimulated MSC with peripheral blood mononuclear cells (PBMCs) and monocytes. Lastly, different bmMSC clinical products were examined for their surface marker expression upon stimulation with the different cytokine mixtures, in order to study differences between MSCs derived from various donors. Finally, insights in these responses might help to improve patient and product selection for MSC-therapy and thereby enhancing efficacy rates.

## MATERIAL AND METHODS

**Human samples**

Informed consent was obtained before sample collection. Perianal fistula tissue scrapings (n=20), serum (n=22) and rectum biopsies (n=8) were obtained during surgery before application of MSCs in CD patients participating in our phase II clinical trial[3] (NCT01144962). Only patients without inflammation in the rectum were included in this trial. All other tissue samples were obtained from the LUMC-Gastroenterology Biobank (B20.004) and included matched macroscopically inflamed (n=18) and uninflamed (n=18) colonic tissue from UC patients and normal colonic tissues (n=20) from patients who underwent surgery for colorectal cancer (obtained > 10 cm from the primary tumor). Lastly, also serum samples from healthy individuals (n=7) and UC patients (n=8) were obtained from the LUMC-Gastroenterology Biobank.

**Cytokine measurements**

Perianal fistula scraping, CD biopsies, UC and healthy colon tissues were homogenised in RIPA buffer consisting of Tris 50mM, NaCL 250 mM, NonidentP40 2%, EDTA-Na 2,5 mM, SDS 0.1%, deoxycholate 0.5%, pH 7.2, using the Tissuelyser LT (Qiagen, Hilden, Germany). Total protein content was determined using a BCA protein assay (ThermoFisher Scientific, Waltham, MA, USA). Serum and tissue levels of IFN-γ, TNF-α, interleukin (IL)-1β, IL-4, IL-5, IL-6, IL-8, IL-12, IL-13 and IL-17 were simultaneously measured with U-PLEX multiplex assay (Mesoscale, Rockville, MD, USA). A commercially available ELISA Duoset (R&D systems, Minneapolis, MN, USA) was used to measure oncostatin M (OSM) levels. All cytokine levels in colon homogenates were corrected for total protein content and presented as pg cytokine per µg protein. Production of cytokines and chemokines by unstimulated and stimulated MSCs after 24 and 48 hours was determined in the supernatant using a Th17 Bio-Plex multiplex assay (Bio-Rad Laboraties, Veenendaal, The Netherlands). Antibodies against vascular endothelial growth factor (VEGF), hepatic growth factor (HGF), CCL5 (RANTES) and CXCL-1 (all Bio-Rad) were added to the Bio-plex/multiplex assay. TGF-β1 levels in the supernatant were determined using an ELISA Duoset as described before[14].

The levels of cytokines and chemokines in the MSC culture supernatant were corrected by subtracting the levels present in non-conditioned medium.

**MSC culture**

Human bmMSCs were isolated and characterized as described before[3] and cultured in complete culture medium consisting of Dulbecco's Modified Eagle Medium (DMEM) with Glutamax (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum (FCS, Sigma-Aldrich, Saint Louis, MO, USA) and penicillin/streptomycin (Gibco). MSCs were passaged when 80-90% confluency was reached. For passaging and flow cytometry analysis, cells were harvested by TrypLE Select (Gibco) incubation for 10 minutes. MSCs between passage 4 -7 were used in experiments.

**MSC cytokine stimulations**

For stimulation experiments, MSCs were counted using an automated cell counter (TC20 automated cell counter, Bio-rad) and seeded (100,000 MSCs/well, 6-wells plate, Gibco) in complete culture medium. The next day, subconfluent MSCs were stimulated with different cytokine mixtures (Table 1) for 24 or 48 hours (all Peprotech, Rocky Hill, NJ, USA, except for IL-1β, R&D systems). The cytokine concentrations used for the *in vitro* stimulations were based on concentrations that are regularly reported in literature. The culture supernatant was collected after 24 and 48 hours of culture, centrifuged to remove debris and stored at -80°C until further analysis.

**TABLE 1.** Cytokine mixtures.

| *Mix* | *Cytokines* |
|---|---|
| *1* | IL-17A (50ng/mL), IL-1β (1ng/mL), OSM (20ng/mL) |
| *2* | IL-17A (50ng/mL), IL-1β (1ng/mL), OSM (20ng/mL), IFN-γ (0.33ng/mL), TNF-α (1ng/mL), IL-13 (20ng/mL) |
| *3* | IL-17A (50ng/mL), IL-1β (1ng/mL), OSM (20ng/mL), IFN-γ (0.33ng/mL), TNF-α (1ng/mL), IL-13 (20ng/mL), IL-4 (20ng/mL) |
| *4* | IFN-γ (0.33ng/mL), TNF-α (1ng/mL), IL-13 (20ng/mL) |

**Flow cytometry analysis**

After stimulation for 24 or 48 hours, 45,000 MSCs were stained for flow cytometry analysis. Standardized flow cytometry was used for indicated experiments, in which the staining and analysis of the upregulation of intracellular and extracellular molecules were standardized using specific flow cytometer settings and a selected set of monoclonal antibodies[15,16]. In all flow cytometry experiments, first, MSCs were incubated for 30 minutes at $4^0$C with

antibodies against cell surface markers HLA-DR (APC-H7, BD Bioscience, San Jose, CA, USA), CD54 (PE, BD Bioscience), PD-L1 (APC, eBioscience, Santa Clara, CA, USA) and a live/death marker (Amcyan, Invitrogen, Carlsbad, CA, USA). For staining of intracellular markers the MSCs were fixed (fixation/permeabilization buffer and diluent, eBioscience) for 20 minutes at room temperature (RT) and thereafter permeabilized (permeabilization buffer, eBioscience). MSCs were stained for the intracellular marker IDO (PE-Cy7, eBioscience) or cyclo-oxygenase 2 (COX-2, PE, BD) for 25 minutes at RT. For the standardized flow cytometry, unlabeled CD54 (Biolegend, San Diego, CA, USA) was added to the surface staining mix to ensure saturating conditions. Furthermore, in these experiments the cells were stained separately, after washing, with the AmCyan live/death marker (Invitrogen, Carlsbad, CA, USA) for 30 minutes at $4^0$C. Compensation was performed with OneComp eBeads Compensation Beads (eBioscience) and live/death beads (ArC reactive beads, Invitrogen), which were prepared according to the manufacturers protocol using the antibodies described above. The MSCs were analysed by flow cytometry with the BD FACS™ CantoII cytometer (BD Biosciences). Analysis of the data was performed with FlowJo software version 8.7.1 (Tree Star Inc., Ashland, OR, USA). For single and alive cells the mean fluorescent intensity (MFI) was obtained and fold induction was calculated by dividing the MFI of stimulated MSCs by the MFI of unstimulated MSCs.

**qPCR analysis**

mRNA was isolated from unstimulated and stimulated MSCs using the NucleoSpin RNA Kit (Macherey Nagel, Düren, Germany) according to manufacturers' instructions. RNA concentrations were determined with the NanoDrop 1000 spectrophotometer (ThermoFisher Scientific). cDNA was generated using RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific). Quantitative PCR (qPCR) reactions were carried out using SYBR Green (Biorad, Hercules, CA, USA) and primers (Invitrogen) for genes listed in Table 2. cDNA samples were subjected to 40 cycles qPCR as previously described[14]. All values were normalized for cDNA content by expression of the house keeping gene Ornithine Decarboxylase Antizyme 1 (OAZ1). Fold induction was calculated by dividing the delta $C_t$ of stimulated MSCs by the delta $C_t$ of unstimulated MSCs.

**T-cell proliferation and DC differentiation assays**

PBMCs were obtained by Ficoll (LUMC, Leiden, The Netherlands) separation from a fresh buffy coat (Sanquin, Leiden, The Netherlands). For the T cell proliferation assay, isolated PBMCs, mainly containing T cells, were cocultured with either stimulated or unstimulated MSCs. 100,000 PBMCs/well were seeded with MSCs in a 1:6 (MSCs: PBMCs) ratio, together with anti-CD3/CD28 beads (Gibco) in RPMI (Gibco) supplemented with 10% FCS and penicillin/streptomycin in a 96-wells plate with V-bottom (Corning, Corning NY, USA).

After 24 hours 50µl 20µCi $^{3}H$ thymidine (PerkinElmer, Waltham, MA, USA) was added to each well and incubated for 16 hours. Thereafter cells were harvested and thymidine incorporation was measured using a Topcount NXT (Canberra Packard)[17]. For the DC differentiation assay, monocytes were isolated from the PBMCs using CD14 microbeads (MACS, Milteny Biotec). CD14 cell purity was verified with flow cytometry using CD45 (APC-H7, BD Bioscience), CD3 (PerCP, BD Bioscience) and CD14 (PE-Cy7, eBioscience) antibodies. Between 96-98% CD14 purity after isolation was found. Monocytes were seeded 300,000/well in a 48-wells plate (Corning) and MSC were added in a 1:100, 1:300 or 1:500 (MSCs:monocytes) ratio in RPMI supplemented with 10% FCS, granulocyte-macrophage colony-stimulating factor (GM-CSF) (800 U/ml, Invitrogen) and IL-4 (1500 U/ml, Invitrogen). MSCs and monocytes were cocultured for 6 days, with a medium change and addition of fresh GM-CSF and IL-4 at day 3. After 6 days, cells were harvested from the wells by pipetting up and down a few times and analyzed by flow cytometry using antibodies for CD14, CD1α (PE, BD Bioscience) and CD73 (BV421, BD Bioscience) in order to measure the percentage of DCs ($CD73^{neg}CD14^{neg}CD1\alpha^{pos}$) from the total amount of $CD73^{neg}$ cells.

**TABLE 2.** Primers

| *Gene name* | *Fw* | *Rv* |
|---|---|---|
| OAZ1 | GGATCCTCAATAGCCACTGC | TACAGCAGTGGAGGGAGACC |
| VEGF | CACACAGGATGGCTTGAAGA | AGGGCAGAATCATCACGAAG |
| TSG-6 | GGCTGGCAGATACAAGCTCA | TCAAATTCACATACGGCCTTGG |
| CXCL-1 | CAGAAGGGAGGAGGAAGCTC | CTCTGCAGCTGTGTCTCTCT |
| Galectin-1 | GGTCTGGTCGCCAGCAACCTGAAT | TGAGGCGGTTGGGGAACTTG |
| CCL5 | TACACCAGTGGCAAGTGCTC | TGTACTCCCGAACCCATTTC |
| CSF-1 | GTGAGATTCCCGTACCCCAA | GAGAGGAAGTTGCTGGGTCT |
| CXCL-2 | CGCCCAAACCGAAGTCATAG | AGCTTCCTCCTTCCTTCTGG |
| IL-6 | CCCTGACCCAACCACAAATG | CTACATTTGCCGAAGAGCCC |
| CXCL-12 | CTCCACATCCTCCACGTTCT | GCTTTGGTCCTGAGAGTCCT |
| COX-2 | GCTTTATGCTGAAGCCCTATGA | TCCAACTCTGCAGACATTTCC |
| TGF-β1 | CCCTGGACACCAACTATTGC | CTTCCAGCCGAGGTCCTT |

**Data analysis and statistics**

Data are presented as mean ± standard deviation. Microsoft Excel software (version 1803, Microsoft, Redmond, WA, USA) was used to visualize and to calculate correlations. All other analyses were performed using GraphPad Prism software (version 7, San Diego, CA, USA). Statistical analysis of two groups was performed with an unpaired T-tests. Statistical analysis of more than two groups was performed with ANOVA test, with a Tukey's post-

analysis. Correlations between two groups were analysed with Pearson correlation. P-values ≤ 0.05 were considered statistically significant.

## RESULTS

**Identification of different cytokine profiles are identified in perianal fistula and inflamed colonic tissue from IBD patients**

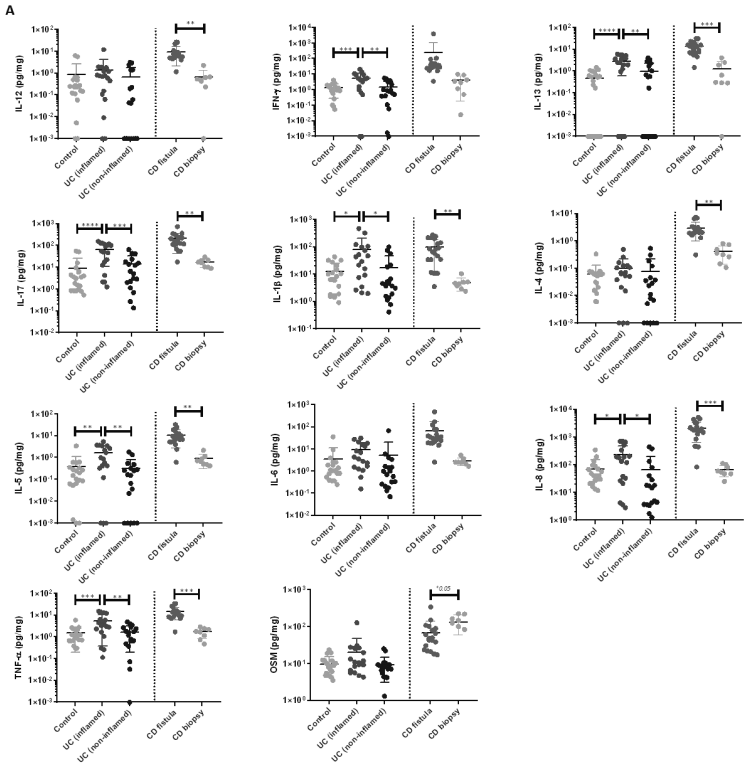
Upon local injection in IBD tissue, MSCs encounter a combination of proinflammatory cytokines. To analyze which cytokines are present in IBD tissue, we determined cytokine levels in perianal fistula tissue and rectum biopsies from CD patients and in the inflamed and uninflamed parts of the colon of UC patients. These levels were compared with the cytokine levels determined in normal colonic tissue. Analysis of cytokine levels in fistula tissue, rectum biopsies and colon samples showed the highest levels of most of the measured cytokines in the perianal fistula scrapings (Figure 1A). Interestingly, in contrast to other cytokines, OSM levels were found to be the highest in uninflamed rectum biopsies from patients with fistulizing CD and not in the perianal fistula tissue itself (Figure 1A). Furthermore, comparison of the different colon tissue samples showed significantly higher levels of IFN-γ, IL-13, IL-17, IL-1β, IL-5, IL-8 and TNF-α in UC inflamed tissue compared with UC uninflamed and normal colonic tissue (Figure 1A). To identify cytokine profiles in fistula and UC inflamed tissues, the most informative cut-off was selected and set at 0.001 pg/µg (explanation about the used cut-off can be found in Supplementary Figure 1). Using this cut-off, two cytokine profiles could be detected in the perianal fistulas, the first profile with high levels of IL-8, IL-17, IL-1β, OSM, IFN-γ, IL-6, TNF-α, IL-13, IL-5 and IL-12 (fistula Crohn's disease profile 1, FC01) and the second profile with high levels of the cytokines included in FC01 but with addition of IL-4 (fistula Crohn's disease profile 2, FC02) (Figure 1B). In inflamed UC colon biopsies also two cytokine profiles were identified. The first profile in inflamed colon tissue included high levels of IL-8, IL-17, IL-1β and OSM (ulcerative colitis profile 1, UC01), while the second profile showed additional high levels of IFN-γ, IL-6, TNF-α and IL-13 (ulcerative colitis profile 2, UC02) (Figure 1B). In conclusion, by analyzing tissues from different IBD patients, four cytokine profiles in IBD tissue were identified, two specific for CD perianal fistulas and two for colonic UC, that could be encountered by MSCs upon local injection.

When levels of local cytokines in IBD patients are reflected in the patients' serum, the local cytokine environment could be analyzed through measurement of a blood sample. To study this, the pro-inflammatory cytokines were measured in matched serum samples from CD fistula patients from which fistula scraping was already obtained. Most pro-inflammatory cytokines were found to be increased in the serum of CD fistula patients

compared with healthy controls, with significantly higher levels of IL-4 and IL-17 in CD patients (Supplementary Figure 2). Surprisingly, IL-13 levels were significantly lower in the serum of CD patients (Supplementary Figure 2). The measured cytokine levels in blood from CD patients did however not correlate with the cytokine levels observed in the perianal fistula tissues from the same patients (highest correlation for IL-17, correlation coefficient 0.34), indicating that the local fistula environment is not directly represented in the circulation (Figure 1C).

**Cytokine mixtures have specific effects on the expression of immunomodulatory molecules by MSCs**

To evaluate if MSCs express the receptors to respond to the measured local cytokines, available mRNA sequencing data sets (GSE115240 & GSM2154690)[18, 19] were used (Supplementary Figure 3A). Receptor expression below 100/20x10$^6$ reads was considered to be negligible. Human bmMSCs expressed mRNA transcripts of the receptors for IL-17A (IL-17RA/IL-17RC[20]), IL-13 (IL-4R/ IL-13Rα1[21]), OSM (gp130/LIFR/OSMR[22]), TNF-α (TNFR1/ 2[23] ), IFN-γ (INFR1/ 2[24]), IL-1β (IL-1R1/AP[25]) and IL-4 (IL-4R/ IL-13Rα1[21]). mRNA



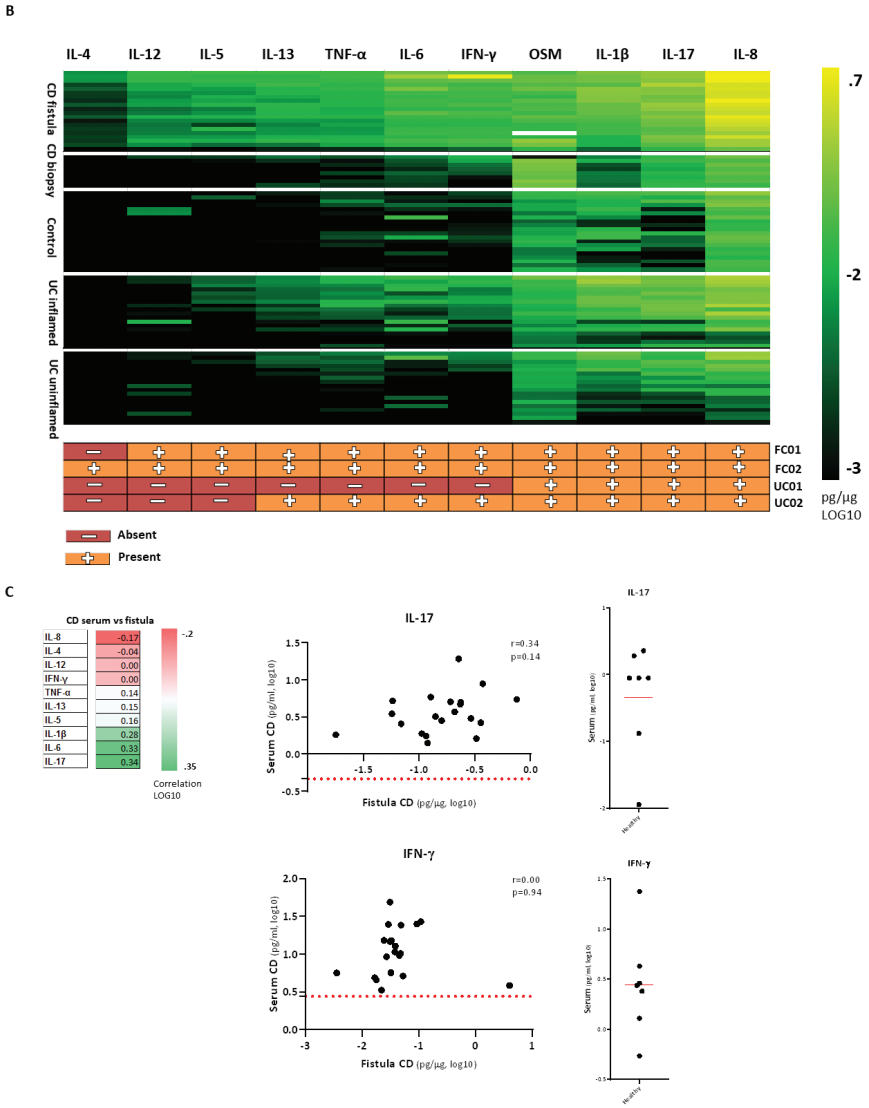



**FIGURE 1.** Four cytokine profiles are detected in fistula and colon tissue samples from IBD patients. A. Tissue cytokine levels in control colon, UC inflamed and UC uninflamed colon, CD fistula tissue and CD rectum biopsies (pg cytokine/µg protein). The significance level was calculated between colon tissue samples (ANOVA, Tukey's post-test) and between CD fistula and CD biopsy (T-test). Undetectable cytokine levels were transformed to 1x10^-3 for visualization in the graphs. B. Heat-map of the different cytokines measured in CD fistula, CD biopsy, control colon tissue and matched inflamed and uninflamed UC colon tissue samples (pg cytokine/µg protein, log10). Each row indicates one sample. FC01 - Perianal fistula cytokine profile 1, FC02 - Perianal fistula cytokine profile 2, UC01 - Ulcerative colitis cytokine profile 1, UC02 - Ulcerative colitis cytokine profile 2. C. Correlation coefficients between the levels of different cytokines in CD serum (pg/ml) and CD fistula tissue (pg/µg). Graphic illustration of the correlation of the levels of IL-17 and IFN-γ between fistula and serum samples, compared with the levels present in serum from healthy individuals. Red dotted line indicates mean cytokine levels in serum from healthy individuals. *p<0.05, **p<0.005, *** p<0.0005, ****p<0.0001.

transcripts for IL-5 (IL-5Rα/βcommon[21]), IL-8 (CXCR1/2[26]) and IL-12 (IL-12Rβ1/2[27]) receptors were below 100/20x10$^6$ reads. For the IL-6R[22] ~100/20x10$^6$ reads were found in human bmMSCs. However, stimulation of MSCs by IL-6 did not upregulate the IL-6 target gene cMYC (Supplementary Figure 3B). Based on the expression of mRNA transcripts for cytokine receptors, the four previously identified cytokine profiles were translated into three different cytokine mixtures representing the local cytokine profiles in IBD patients and to which MSCs are responsive (Supplementary Figure 3C, mix-1 [representing UC01]: IL-17, IL-1β, OSM; mix-2 [representing UC02/FC01]: IL-17, IL-1β, OSM, IFN-γ, TNF-α, IL-13; and mix-3 [representing FC02]: IL-17, IL-1β, OSM, IFN-γ, TNF-α, IL-13, IL-4; Table 1). A fourth cytokine mixture (Mix-4: IFN-γ, TNF-α, IL-13), not representing a local IBD cytokine profile, was also studied since this included the three cytokines that are added to mix-1 in order to obtain mix-2.

Next, factors involved in MSC-mediated immunoregulation, including among others programmed death ligand 1 (PD-L1), IDO, transforming growth factor (TGF)-β1 and tumor necrosis factor-inducible gene (TSG)-6 were measured after stimulation of MSCs with the four cytokine mixtures. MSCs derived from two donors were stimulated with the different cytokine mixtures for 24 hours to measure mRNA expression levels, using qPCR, and MSCs derived from three donors were stimulated for 48 hours to measure protein expression levels, using flow cytometry analysis (Figure 2, Supplementary Figure 4). The four cytokine mixtures induced different levels of immunomodulatory molecules, with remarkable variation in expression levels between MSCs stimulated with mix-1 (IL-17, IL-1β, OSM), -2 (IL-17, IL-1β, OSM, IFN-γ, TNF-α, IL-13) and -4 (IFN-γ, TNF-α, IL-13). For example, while stimulation with mix-2 and -3 showed the highest induction of TSG-6 and CXCL-2 mRNA in the MSCs, stimulation with mix-4 induced the highest upregulation of CCL-5 and CSF-1 transcripts (Figure 2A, Supplementary Figure 4B). Interestingly, TGF-β1 mRNA was decreased upon stimulation with mix-1 and -4 and even more after stimulation with mix-2 and -3, while CXCL-12 mRNA was downregulated upon stimulation with mix-4 and IFN-γ. For several molecules, like CCL-5 and CSF-1, the mRNA expression was highest after stimulation with mix-4 and addition of mix-1 to mix-4, represented in mix-2, resulted in downregulation of the mRNA expression. Following addition of mix-1 to mix-4 (Figure 2B, Supplementary figure 4B), protein expression data also showed downregulation of markers, like PD-L1, HLA-DR and IDO. These data are in agreement with mRNA expression data of these markers. PD-L1 and HLA-DR were highly expressed on MSCs stimulated with mix-4, while addition of IL-17, IL-1β and OSM to mix-4 (mix-2) lowered PD-L1 upregulation. IDO levels were highest in MSCs stimulated with IFN-γ only, while this upregulation was lower upon stimulation with mixtures including IFN-γ. This suggests an inhibitory effect on INF-γ-induced IDO expression by some of the (combinations of) additional cytokines.

On the other hand, upregulation of CD54 in response to cytokine stimulation was similar for all mixtures.

Thereafter, the levels of immunoregulatory soluble factors produced by MSCs after stimulation with the four cytokine mixtures for both 24 and 48 hours were measured in the supernatant. We evaluated a panel of soluble cytokines and chemokines, including amongst others VEGF, HGF, CCL5 and CXCL-1. In agreement with flow cytometry and qPCR data (Figure 2A and B), different levels of cytokines and chemokines were produced by MSCs after stimulation with the four cytokine mixtures (Figure 2C). High levels of G-CSF and CXCL-1 were found after stimulation with mix-1, -2 and -3. Highest levels of CCL-5 protein were secreted after stimulating MSCs with mix-4 (Supplementary Figure 5). GM-CSF was produced in small amounts by both unstimulated and stimulated MSCs, with the exception for MSCs stimulated with mix-3, which surprisingly resulted in lower GM-CSF level in the supernatant compared with the supernatant of unstimulated MSCs. In line with the RT-qPCR data for TGF-β1 mRNA, compared with unstimulated MSCs, less TGF-β1 protein was found in the supernatant of MSCs stimulated with the different mixtures, especially after stimulation with mix-3.

Altogether, these data showed that the four different cytokine mixtures, of which three resemble the local cytokine milieus in IBD patients, differentially regulated the expression of immunomodulatory molecules by MSCs.

**Not every cytokine mixture induces the same immunoregulatory functionality in MSCs**

To determine whether the cytokine mix-induced molecular differences also resulted in functional differences between the stimulated MSCs, the effects of pre-stimulation of MSCs with mix-1-4 on inhibition of T cell proliferation and DC differentiation were assessed (Figure 3A). In order to correct for potential donor variation in both immune cells and MSCs two approaches were used. First, MSCs, derived from one donor, were tested using three different PBMC donors (Figure 3B). Secondly, three different MSCs were tested, while using the same PBMC donor (Figure 3C). The addition of unstimulated MSCs to PBMCs resulted in slightly decreased T cell proliferation (Figure 3B). The inhibitory effect of MSCs on T cells was significantly increased if MSCs were pre-stimulated with cytokines. MSCs, stimulated with IFN-γ alone or mix-4, inhibited T cell proliferation to the highest extent (up to 71.7% inhibition). Stimulation with IL-17, IL-1β and OSM counteracted the effects of mix-4 (mix-2 and -3). In the DC differentiation assay, the culturing of $CD14^{pos}$ monocytes in the presence of IL-4 and GM-CSF for six days caused differentiation of almost all monocytes into DCs ($CD73^{neg}CD14^{neg}CD1\alpha^{pos}$) (Figure 3B). Addition of unstimulated MSCs to this co-culture reduced the differentiation into DCs for almost 50%. Interestingly, MSCs stimulated with

mix-1 (76% inhibition), but especially with mix-2 and -3 showed the highest inhibition of the differentiation of monocytes into DCs (96% and 94% inhibition, respectively). Thus, the three identified local cytokine profiles (reflected in mix-1, -2 and -3) in IBD tissue reduced T cell proliferation to the same extent, whereas MSCs stimulated with mix-2 or -3 were more effective inhibitors of DC differentiation. Analysis of three different MSC products in the two assays showed similar results (Figure 3C). Slight variation was observed in the level of T cell proliferation and DC differentiation between the different PBMC and MSC products.

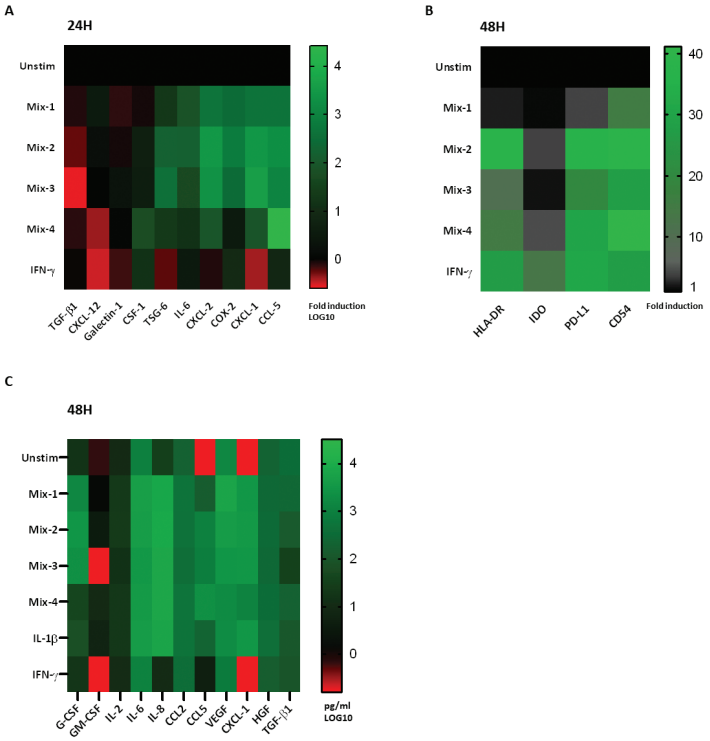



**FIGURE 2.** Cytokine mix-specific effects on the expression of immunomodulatory molecules by MSCs. A. Heatmap of fold induction (log10 transformation) of mean mRNA expression of different genes in two different MSCs after stimulating for 24 hours (24H) with mix-1, -2, -3, -4 or IFN-γ compared with mRNA expression in unstimulated MSCs. Mix-3 gene expression was measured in a separate experiment and compared with the unstimulated MSCs from the same experiment. B. Heatmap of fold induction of the mean fluorescent intensity (MFI) of HLA-DR, IDO, PD-L1 and CD54 from three different MSCs upon stimulation for 48 hours (48H) with mix-1, -2, -3, -4 or IFN-γ, compared with unstimulated MSCs. C. Heatmap of mean cytokine and chemokine levels (pg/ml, log10 transformation) measured in the supernatant of six different MSC products stimulated for 48H with mix-1, -2, -3, -4 or IFN-γ.

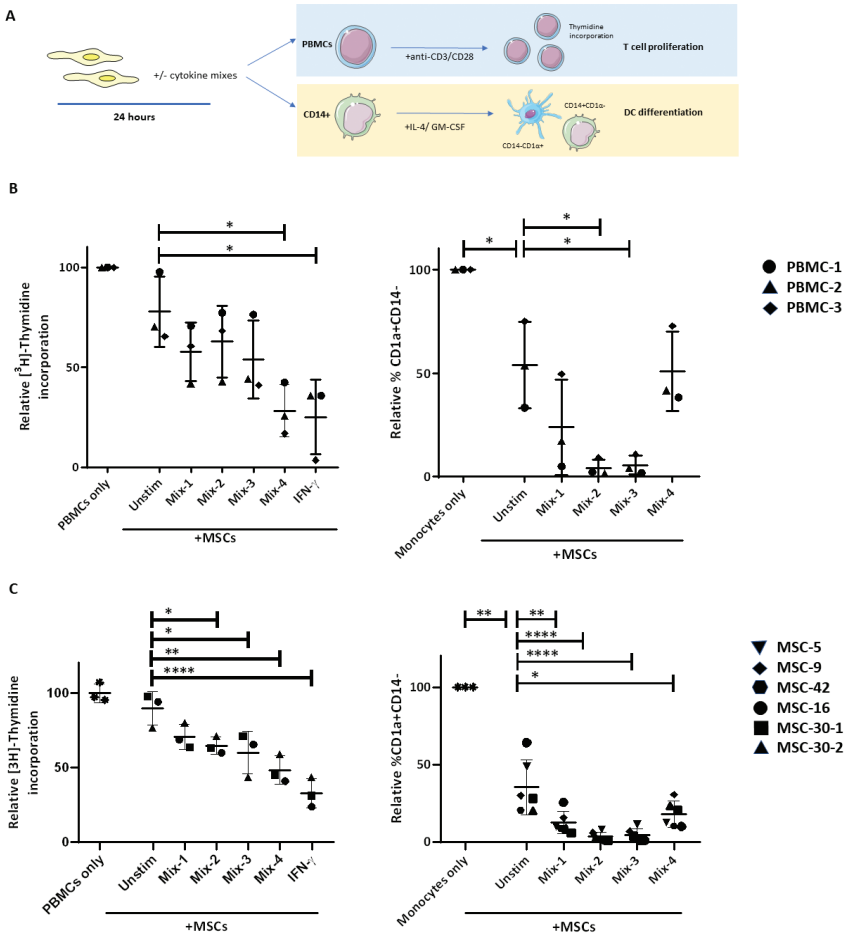



**FIGURE 3.** Cytokine-stimulated MSCs show different immunoregulatory properties. A. Schematic overview of the T cell proliferation and DC differentiation assay. B. T cell proliferation assay: relative $^{3}$H-thymidine incorporation in PBMCs cultured alone compared with PBMCs cocultured with unstimulated MSCs or MSCs stimulated with mix-1, -2, -3 or -4 or IFN-γ. Bars represent means of technical triplicates. DC differentiation assay: relative percentage of $CD73^{neg}CD1a^{pos}CD14^{neg}$ cells of all $CD73^{neg}$ cells after coculture with (un)stimulated MSCs (1:500, MSC:monocytes) for 6 days compared with monocytes cultured alone. PBMCs from three different donors were used. Significance levels were calculated between PBMCs/monocytes only and unstimulated (T-test), and between unstimulated MSCs and the different mixtures (Anova, post-test). C. Three different MSC products were used in the T cell proliferation assay as described in figure 3B. Six MSC products (different symbols) were used in the DC differentiation assay as described in figure 3B, while using two different PBMC products (open signs vs closed symbols) * $p<0.05$.

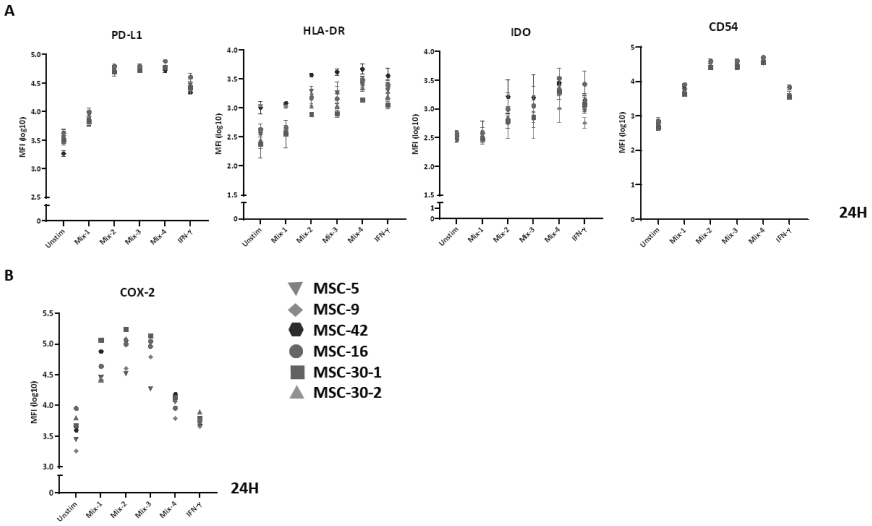



**FIGURE 4.** IBD cytokine mixtures induce donor-specific MSC responses. A. Mean fluorescent intensity (MFI) for different immunoregulatory markers in six MSC products (different colored symbols) after stimulation with different mixtures and IFN-γ after 24 hours (24H). Mean of two independent experiments, measured with standardized flow cytometry (log10 transformation of the data). B. COX-2 MFI of six MSC products after stimulation with different mixtures and IFN-γ after 24H (log10 transformation of the data).

### MSCs from different donors show variable levels of marker expression upon exposure to cytokine mixtures

After showing the impact of different cytokine mixtures on the expression of immunoregulatory molecules and the immunomodulatory function of MSCs, we wondered whether MSCs from various donors would differently respond to the cytokine milieus. Different MSC products (n=6) obtained from different MSCs donors (n=5) were stimulated for 24 hours with the different cytokine mixtures. From one donor, two MSC-products (MSC-30-1 and -30-2) were tested. Subtle differences in expression patterns of PD-L1, HLA-DR, IDO and CD54 were found between the MSC products upon stimulation with the different cytokines mixtures (Figure 4A). For example, MSC-16 expressed higher levels of PD-L1 and CD54 in both the unstimulated condition and after stimulation with the different cytokine mixtures compared with the other MSCs. Even bigger differences were observed between the HLA-DR expression by the various MSC products. MSC-42 consistently expressed the highest levels of HLA-DR, while MSC-30 (both products) showed the lowest levels. Intracellular IDO levels were higher in MSC-16 after stimulation with the different mixtures, and lower in MSC-9, although high variation was found between repeated measurements of the same MSC product.

COX-2 expression was also measured on protein level in different MSC products after cytokine stimulations. The same pattern of COX-2 induction was found on protein level compared with the previously observed mRNA level (Figure 2A), with an upregulation of COX-2 after stimulation with mix-1, -2 and -3 (Figure 4B). Comparison of the different MSC products showed that MSC-5 and MSC-9 expressed the lowest levels of COX-2 before and after stimulation with the cytokine mixtures, while MSC-16 and MSC-30 expressed the highest COX-2 levels. In conclusion, different levels of PD-L1, HLA-DR and COX-2 expression were observed between the different MSC products, both in unstimulated condition and after stimulation with cytokine mixtures. The functional consequences of these different expression levels are unknown.

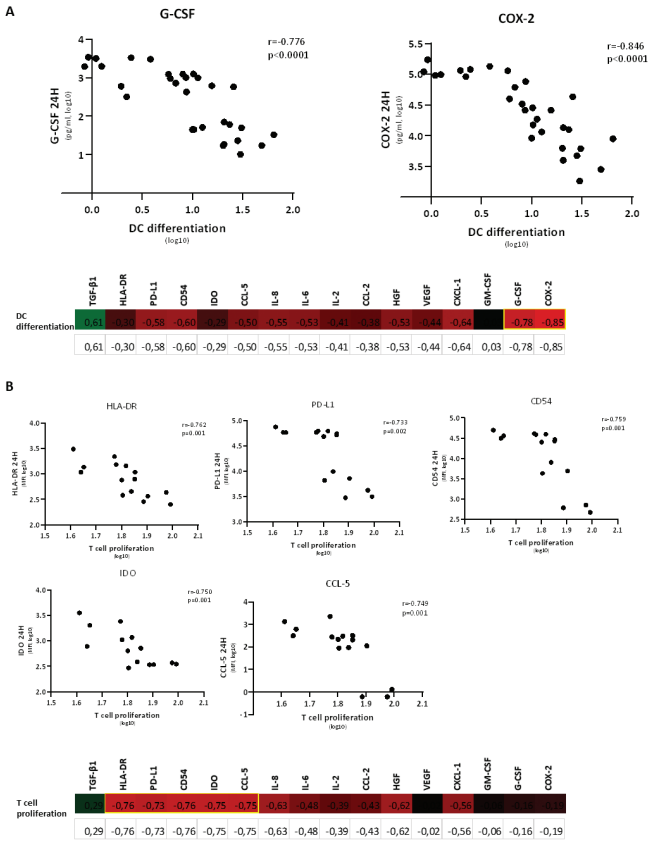



**FIGURE 5.** The inhibition of DC differentiation by MSCs correlates with MSCs' COX-2 and G-CSF expression. A. DC differentiation, figure 3C, correlates with COX-2 and G-CSF, figure 4B and 2C, expression (Pearson correlation, log10 data transformation). B. T cell proliferation, figure 3C, correlates with HLA-DR, PD-L1, CD54, IDO and CCL-5, figure 4A and 2C (Pearson correlation, log10 data transformation).

**The inhibition of DC differentiation by MSCs correlates with their COX-2 and G-CSF expression**

In order to identify which factors might be involved in the immunomodulatory function of MSCs, the expression levels of the different immunomodulatory molecules, after stimulation with the cytokine mixtures, were correlated to the MSC-mediated effects on T cell proliferation and DC differentiation (Figure 5). DC differentiation inversely correlated with the production of COX-2 and G-CSF. In other words, high COX-2 and G-CSF production by MSCs was significantly correlated with a lower percentage of DCs in the differentiation assay (Figure 5A). The levels of T cell proliferation were found to inversely correlate with HLA-DR, PD-L1, CD54, IDO and CCL-5 protein expression by MSCs (Figure 5B). Although it is unclear whether these molecules are directly involved in the immunomodulatory function of the MSCs, these factors could be used as surrogate marker to measure MSCs' immunomodulatory function *in vitro*.

# DISCUSSION

In the present study, we investigated whether the local cytokine milieu in IBD patients could have impacted the immunomodulatory characteristics and function of bmMSCs. Local MSC-therapy is now approved in Europe for the treatment of perianal fistulas in CD and studied in patients with ulcerative proctitis. Different disease-specific cytokine profiles were identified in both the inflamed gut and perianal fistulas from respectively UC and CD patients. Two cytokine profiles were identified in the inflamed colon of UC patients and two profiles in perianal fistula tissues from CD patients. MSCs diversely responded to mixtures of recombinant cytokines, representing these four local cytokine profiles in IBD patients, resulting in differential expression of several immunomodulatory molecules that have previously been implicated in the mode of action of MSCs[7]. Interestingly, we found that cytokine mixtures enhancing the capacity of MSCs to inhibit DC differentiation did not result in the highest inhibition of T cell proliferation. The extent of inhibition of DC differentiation by stimulated MSCs was inversely correlated with COX-2 and G-CSF expression, while the inhibition of T cell proliferation correlated with the expression of PD-L1, HLA-DR, IDO, CD54 and CCL-5, suggesting that these factors may be involved in immunomodulation. Lastly, we showed that different MSC donor products, used for the treatment of perianal fistulas in CD patients, showed subtle differences in the expression of HLA-DR, COX-2 and PD-L1 upon cytokine stimulations. Altogether, this study showed the potential impact of both the recipient cytokine milieu and the donor MSC product on the immunomodulatory characteristics of MSCs.

Several theories exist about the mechanisms of action of MSC-therapy. We and others showed that MSCs express immunosuppressive molecules, including IDO, PD-L1, TGF-β1, TSG-6 and COX-2 and are able to inhibit T cell proliferation and to affect DC differentiation *in vitro*[7, 28-31]. IFN-γ has been shown to enhance these immunosuppressive characteristics of MSCs[8, 10]. In the current study, the direct effects of different mixtures of cytokines, resembling the local IBD environments, on immunomodulating capacity of MSCs were shown. Using this model, only a selection of cytokines could be included, while MSCs upon local injection *in vivo* come across many more cytokines. Whether the detected cytokines were included in the mixtures that were used to stimulate the MSCs was determined by the expected responsiveness of MSCs towards these cytokines as based on mRNA expression of the cytokine receptors by unstimulated bmMSCs. As the expression of genes encoding for the receptors for IL-5, IL-12, IL-6 and IL-8 was below the threshold in unstimulated MSCs, these cytokines were not included.. However, the possibility of upregulation of receptors for IL-5, IL-12, IL-6 and IL-8 after stimulation with the other seven cytokines cannot be excluded. Two cytokine profiles were detected in the inflamed colon of UC patients, and stimulation of MSCs with one of them (UC02), reflected in cytokine mix-2, resulted in more inhibition of DC differentiation compared with MSCs stimulated with mix-1 (reflecting the other UC cytokine profile, UC01), while their inhibitory effects of MSCs on T cells did not differ for these two mixes. Surprisingly, stimulation with the two cytokine milieus present in perianal fistulas (represented in mix-2 and -3) made MSCs especially primed to inhibit DC differentiation and to a lesser extent for inhibiting T cell proliferation. Studying the exact contribution of each individual cytokine to these functional changes was beyond the scope of this study.

In the current study, an inverse correlation between monocyte differentiation into DCs and COX-2 production by MSCs was observed. The relation between COX-2 and redirecting the development of monocytes into $CD1\alpha^{pos}$ DCs has been described before[29, 32]. For the T cell proliferation assay, a correlation between PD-L1, CD54, IDO, HLA-DR and CCL-5 expression by MSCs and inhibition of T cell proliferation was found. This is in line with previous reports showing the importance of IDO, CD54 and PD-L1 in the inhibition of T cells[33-37]. In future experiments it would be interesting to use for the functional assays instead of PBMCs from healthy donors, purified T cells isolated from the local tissue of IBD patients and investigate whether immune cells from different patients would be differently modulated by stimulated MSCs.

Previously, MSCs are not considered to induce a strong immunogenic response, although they were known for their immunosuppressive capacities. This paradigm is based on low expression of HLA class I and II molecules and low levels of co-stimulatory molecules like CD40 and CD80 by MSCs[38, 39]. Currently it is known that HLA molecules are upregulated

by stimulation with certain cytokines, including IFN-γ[8]. Furthermore, in some patients transient donor-specific HLA antibodies are formed after local MSC-therapy for perianal fistulizing disease[1, 40], although no donor-specific HLA antibodies were detected in patients receiving local MSC-therapy in our clinical trial[4]. Interestingly, the current study showed that HLA-DR expression by MSCs is downregulated after stimulation with IL-17, IL-1β and OSM (mix-1) in addition to IFN-γ, TNF-α and IL-13 (mix-4), suggesting that the cytokine milieu could impact the level of HLA expression and thereby recognition by the host immune system. How the local cytokines determine the HLA I and II expression *in vivo* and whether this HLA expression correlates with the forming of anti-HLA antibodies in patients after injection with local MSCs remains unknown.

Since MSC-therapy is expensive and clinical outcomes are diverse, there is need to define patients' eligibility for local MSC-therapy on forehand[41]. In the current study, we showed the capacity of different cytokine mixtures, representing IBD cytokine milieus, to affect the immunomodulatory function of bmMSCs. MSCs stimulated with cytokine profile UC1, identified in UC colon, induced less inhibition of DC differentiation compared with MSCs stimulated with mix-2, representing UC02, the second profile identified in UC colon. Stimulation with mix-2 and -3, representing the two profiles identified in CD fistulas, showed no differences in DC differentiation. These data suggest that especially for the treatment of luminal UC the patients' cytokine milieu could be important for exerting the MSC regulatory function on DC differentiation. The present study showed that local cytokine levels in IBD are not reflected in circulating serum levels, which is in accordance with previous literature[42]. Therefore, in order to evocate patients' eligibility the individual local cytokine milieu might be measured in the local tissue and not in the serum before MSC-therapy. Patients with CD perianal fistulas that will be treated with MSC-therapy first undergo drainage of the fistula track and the placement of a seton[3], during which local fistula tissue could be easily collected. For patients with ulcerative proctitis, who will be endoscopically injected with MSCs, biopsies would have to be taken, to determine the local cytokine environment in the bowel.

In summary, disease-specific local cytokine milieus are found in IBD patients. Cytokine mixtures deducted from these profiles, affected the expression of immunomodulatory molecules and the in vitro immunomodulatory function of MSC. Our data suggest that the *in vivo* success of local MSC-therapy could be affected by the local cytokine milieu. Future research should reveal whether different clinical efficacy observed following local MSC-therapy in IBD is indeed determined by local cytokine expression.

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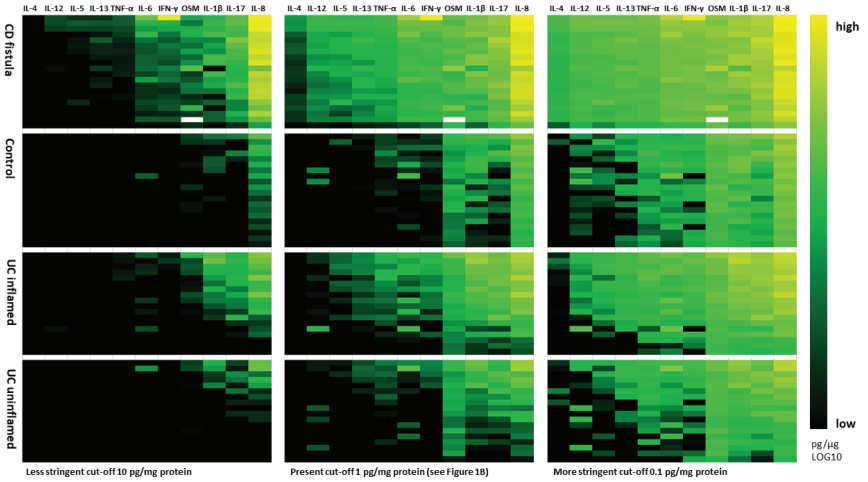
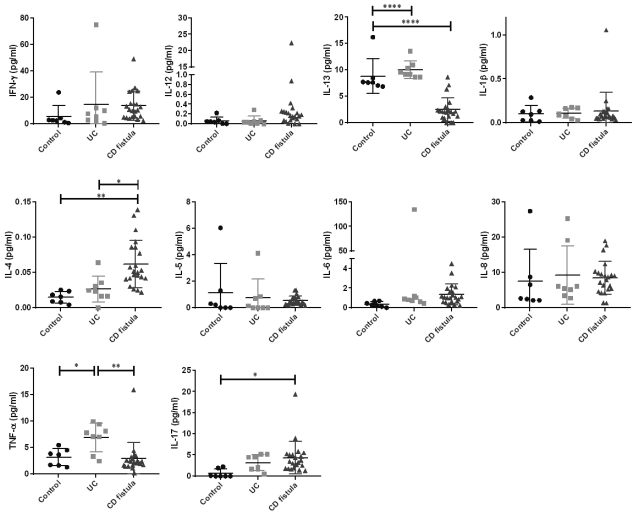
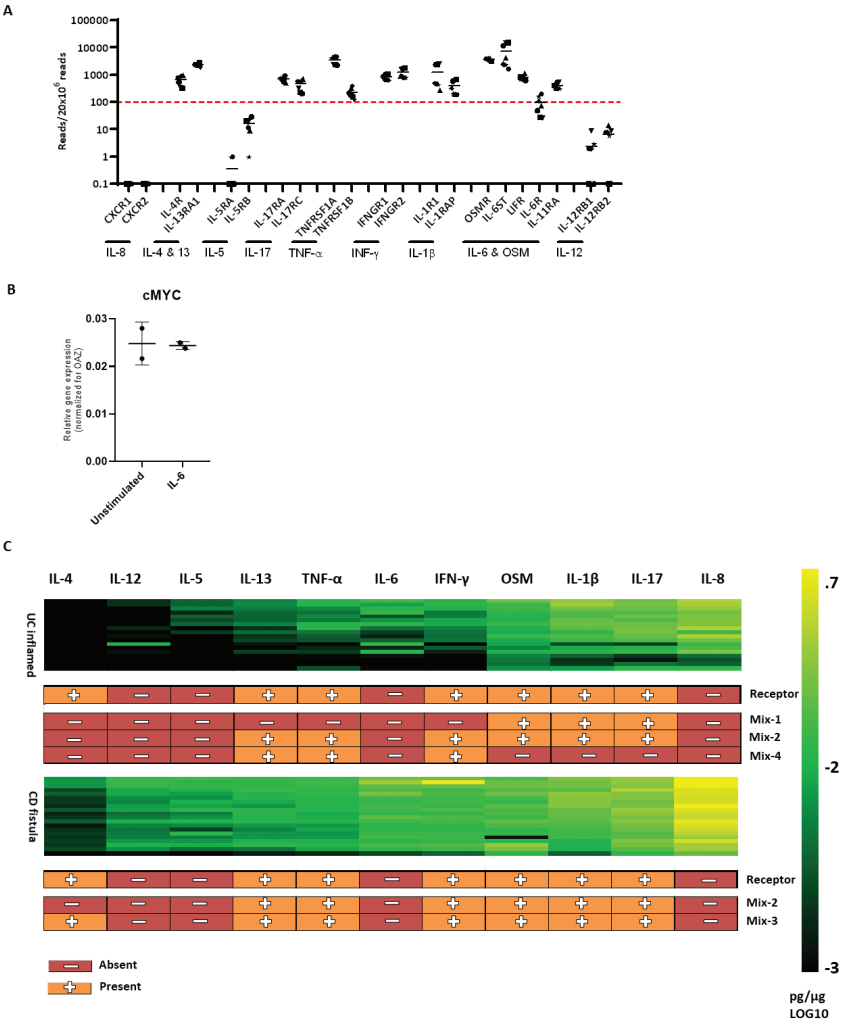


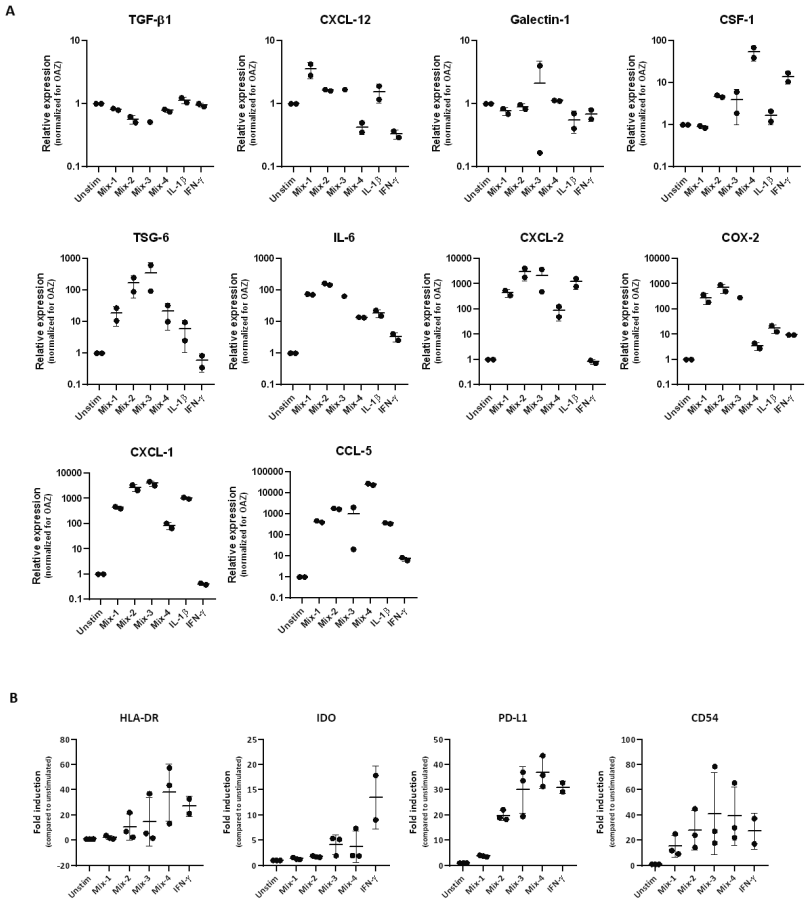

**Supplementary FIGURE 1.** Different cut-off's used to determine the cytokines present in local IBD tissue. Three different cut-off's were used before the arbitrary final cut off was set at 0.001 pg/µg protein for the different cytokines present in the local IBD tissues (pg cytokine/µg protein, log10 transformation of data). A less stringent cut-off at 0.01 pg/µg protein showed the absence of almost all cytokines except for IL-1β, IL-18 and IL-8 in the inflamed UC tissue (although we know that biologically relevant concentrations of for instance TNF-a are expected to be present in these tissues[12, 43]), while a more stringent cut-off at 0.0001 pg/µg protein showed the presence of all measured cytokines in the fistula and also the presence of all cytokines, except for IL-4 in the control colon tissue. Therefore, a cut-off at 0.001 pg/µg protein was used. In one fistula sample OSM measurement was not reliable and left out.



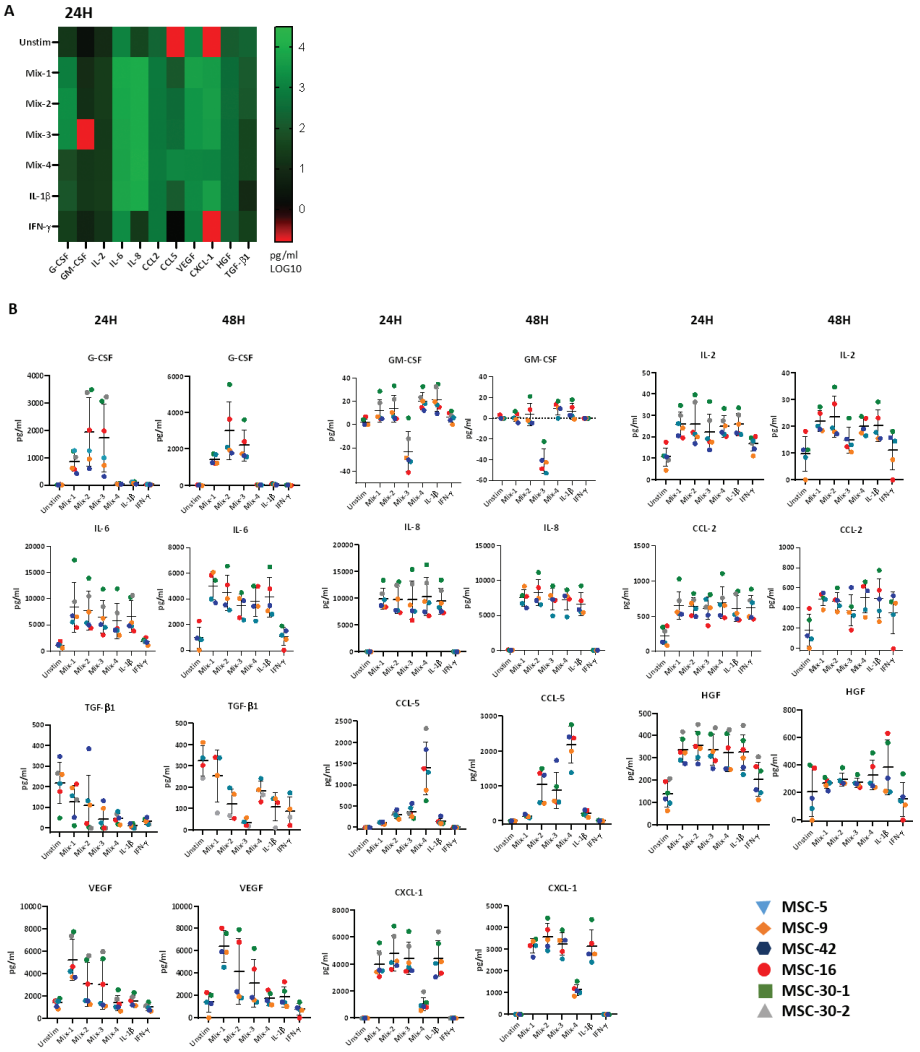

**Supplementary FIGURE 2.** Serum cytokine levels in healthy individuals, UC patients and CD fistula patients. Cytokine levels in serum of healthy individuals (controls), UC patients and CD fistula patients (pg cytokine/ml) (ANOVA, Tukey's post-test) *p<0.05, **p<0.005, *** p<0.0005, ****p<0.0001.




**Supplementary FIGURE 3.** From measured local cytokines towards cytokine mixtures for stimulation. A. mRNA reads/20x10$^6$ reads in six different MSCs (different symbols)[18, 19] for receptors for the measured cytokines measured. The different cytokines and corresponding receptors are indicated in the graph: IL-8 (CXCR1/2[26]), IL-4 (IL-4R/ IL-13Rα1[21]), IL-13 (IL-4R/ IL-13Rα1[21]), IL-5 (IL-5Rα/βcommon[21]), IL-17A (IL-17RA/IL-17RC[20]), TNF-α (TNFR1/ 2[23] ), IFN-γ (INFR1/ 2[24]), IL-1β (IL-1R1/AP[25]), OSM/IL-6 (gp130/LIFR/OSMR/IL-6R[22]) and IL-12 (IL-12Rβ1/2[27]). The red dotted line indicates the used threshold (100/20x10$^6$ reads) for relevant receptor expression. B. cMYC mRNA expression upon stimulation with IL-6. C. Heatmap of different cytokines present in CD fistula and UC inflamed colon as in figure 1B, accompanied by an overview of the presence of each cytokine receptor on MSCs and the cytokines finally included in each cytokine mix.




**Supplementary FIGURE 4.** A. mRNA expression of the indicated genes by two MSCs after stimulation with the different cytokine mixtures. Mean of two different MSCs, technical triplicate for every data point. B. Fold induction of mean fluorescent intensities of indicated proteins of stimulated MSCs compared with unstimulated MSCs. Mean of three different MSCs.




**Supplementary FIGURE 5.** A. Heatmaps represent the mean of measured cytokines and chemokines in the supernatant of 6 different MSC products stimulated for 24 hours (24H) with different mixtures (log10 transformation, pg/ml). B. Production of different chemokines and cytokines by the different MSC products after 24 hours (24H), summarized in the heatmap in figure 5A, and 48 hours (48H).