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Original Research

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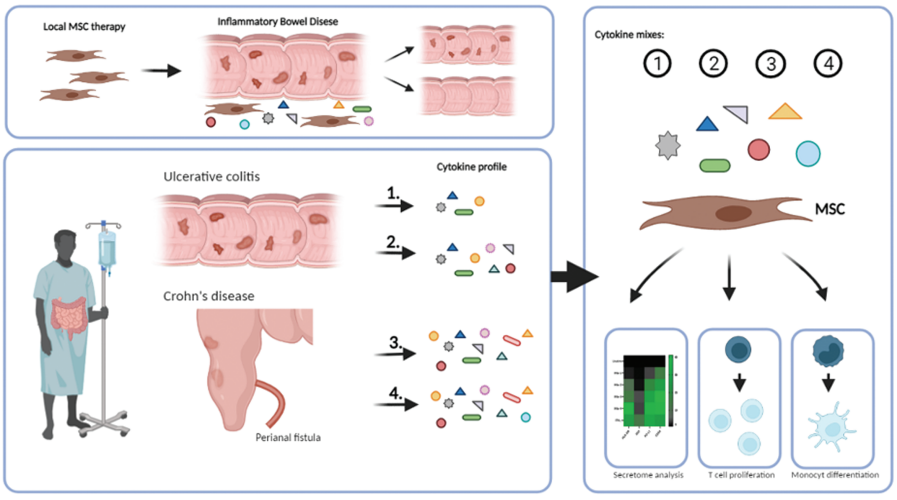
Abstract

Locally applied mesenchymal stromal cells (MSCs) have the capacity to promote the healing of perianal fistulas in Crohn's disease (CD) and are under clinical development for the treatment of proctitis in ulcerative colitis (UC). Despite these clinical advances, the mechanism of action of local MSC therapy in inflammatory bowel disease (IBD) is largely unknown. We hypothesized that the local cytokine environment in IBD patients affects the immunomodulatory properties of MSCs. To evaluate this, 11 cytokines were analyzed in inflamed tissues obtained from CD and UC patients. Based on the identified cytokine profiles 4 distinct cytokine mixtures that mimic various inflammatory IBD environments were established. Next, MSCs were cultured in the presence of either of these 4 cytokine mixtures after which the expression of immunomodulatory and tissue regenerative molecules and the capacity of MSCs to modulate T-cell proliferation and dendritic cell (DC) differentiation were assessed. Our data show that MSCs respond, in a cytokine-specific manner, by upregulation of immunomodulatory and tissue regenerative molecules, including cyclooxygenase-2, indoleamine 2,3-dioxygenase, and transforming growth factor-β1. Functional studies indicate that MSCs exposed to a cytokine profile mimicking one of the 2 UC cytokine milieus were less effective in inhibition of DC differentiation. In conclusion, our data indicate that cytokine mixes mimicking the local cytokine milieus of inflamed UC colonic or CD fistulas tissues can differentially affect the immunomodulatory and tissue regenerative characteristics of MSCs. These data support the hypothesis that the local intestinal cytokine milieu serves as a critical factor in the efficacy of local MSC treatment.

Key words: inflammatory bowel disease; Crohn's disease; ulcerative colitis; perianal fistulas; cytokines; MSC; mesenchymal stromal cell; T cell; dendritic cell.

Graphical Abstract



Impact of the local cytokine milieu in IBD patients on MSC phenotype and function Created with BioRender.com

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Significance Statement

Mesenchymal stromal cells (MSCs) are approved as a cellular therapy for the local treatment of perianal fistulas in Crohn's disease. It is unknown why some patients respond to this new therapy and others do not. In this study the impact of the inflammatory cytokine milieu in inflammatory bowel disease (IBD) on MSC phenotype and function was investigated. The authors found four different cytokine profiles in IBD patients. MSCs stimulated with one of the four identified cytokine profiles showed less capacity to inhibit dendritic cell differentiation, but more tissue regenerative properties, compared with the other profiles. This research could help to select eligible patients for MSC therapy.

Introduction

Mesenchymal stromal cells (MSCs) are multipotent cells with immunomodulatory and tissue regenerative features. The minimal criteria to define MSCs have been proposed by the society for cellular therapies and include adherence to plastic, expression of surface markers like CD105, CD90, and CD73, and absence of the hematopoietic markers CD45 and CD34. Furthermore, they should be able to differentiate in vitro into chondrocytes, osteoblasts, and adipocytes.[1] MSC therapy is currently under investigation/approved in several autoimmune-related diseases, including inflammatory bowel disease (IBD). IBD comprises ulcerative colitis (UC) and Crohn's disease (CD) and is a chronic inflammatory disease characterized by a relapsing inflammation of the intestines. Patients commonly present with abdominal pain, (bloody) diarrhea, and fatigue. The exact pathogenesis of IBD is unknown, but it is generally accepted that IBD is the result of an aberrant immune response against the intestinal microbiota in a genetically susceptible person.[2] No definite cure is available for IBD; therefore, the disease requires lifelong therapy and lifestyle changes to prevent disease progression and complications. Most of the currently available therapies for IBD target the immune system, like for example anti-tumor necrosis factor (TNF)-α and anti-α4β7-integrin therapy. Furthermore, patients with CD are also at risk to develop perianal fistulas,[3,4] which are abnormal tracts that connect the intestinal lumen with the perianal skin. CD-associated perianal fistulas are highly refractory since only 37% of the patients with complex perianal fistulas show fistula closure after a median follow-up of 10 years using combined medico-surgical therapies.[5] In IBD, both intravenous and local injections of MSCs have been investigated with variable clinical outcomes.[6] Significant improvement in fistula healing was shown in patients treated with bone marrow (bm)-derived MSCs, compared to placebo, with a reduction in the number of draining fistulas up to 86%.[7] After 4-years of follow-up, sustained fistula closure was observed in most of the patients after MSC treatment.[8] Furthermore, a large multicenter trial showed fistula closure after allogeneic adipose-tissue-derived MSC injection (Darvadstrocel/Cx601) in 50% of the patients versus 34% in the placebo group at 24 weeks.[9] Local injection of Darvadstrocel was approved by the European Medicine Agency for the treatment of perianal fistulas in CD.[9,10] More recently, we conducted 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 preclinical observations that endoscopically injected MSCs are able to alleviate colitis[11] and that biopsy-induced ulcers were reported by others to heal faster after local MSC application.[12]

Despite these clinical advances, the exact mechanism of action of local MSC therapy is unknown. In the past decades, it has become apparent that MSCs are able to modulate the immune system in various ways. First, MSCs produce different immunomodulating molecules, like transforming growth factor (TGF)-β and TNF-stimulated gene 6 (TSG)-6.[13,14] Second, the importance of phagocytes, like macrophages, as potential mediators of MSC-dependent immunomodulation has become clear. Macrophages that engulfed apoptotic MSCs may produce the immunomodulatory molecules indoleamine (IDO) and interleukin (IL)-10 in response.[15-17] Furthermore, MSCs can induce polarization of monocytes toward immature dendritic cells and immune-suppressive M2 macrophages. Third, MSCs can transform immune cells via cell–cell contact. For example, direct contact between MSCs and CD4+ T cells can induce regulatory T-cell differentiation.[18] Next to their capacity to modify immune responses, MSCs have also been found to stimulate tissue regeneration.[12]

The importance of local proinflammatory cytokines in determining MSC function has been suggested by different research groups. Previous work has shown that the immunosuppressive activity of MSCs is not contact-dependent and requires the presence of interferon (IFN)-γ produced by activated T-cells.[19] Furthermore, IFN-γ stimulated MSCs have been shown to display an enhanced capacity to alleviate experimental colitis compared to their non-stimulated counterparts.[20] Therefore, we hypothesized that the local proinflammatory environment in IBD patients may affect the immunomodulatory properties of MSC. To establish which cytokines are present in IBD tissues, 11 cytokines were measured in tissues from patients with fistulizing Crohn's disease (FC) or UC. Based on the evaluation of the corresponding cytokine receptors on MSCs, we designed 4 inflammatory cytokine mixes that mimic the inflammatory environments in IBD patients. Finally, MSCs were exposed to these cytokine combinations and the effects on MSC phenotype and function were studied in T-cell proliferation and dendritic cell (DC) differentiation assays. Our data show that the phenotype and function of MSCs is orchestrated by cytokine mixtures mimicking the local inflammatory environments and indicate that the local inflammatory milieu might serve as a critical factor for response to MSC treatment.

Material and Methods

Human Samples

All studies were approved by the local Medical Ethics Committee (METC Leiden-Den Haag-Delft) or Central Committee on Research involving Human Subjects (CCMO). Perianal fistula tissue scrapings (n = 20), serum (n = 22), and rectum biopsies (n = 8) were obtained from FC patients participating in our phase II clinical trial[7] (trial number:

NCT01144962) at the operation room just before application of MSCs. Only patients without endoscopic inflammation in the rectum were included in this trial. All other tissue samples were obtained from the LUMC-IBD biobank (protocol: B20.004) and included matched macroscopically inflamed (n = 18) and matched macroscopically uninflamed (n = 18) colonic tissue from UC patients. As a control normal colonic tissues (n = 20) from patients who underwent surgery for colorectal cancer (obtained > 10 cm from the primary tumor) were obtained. Serum samples from healthy individuals (n = 7) and UC patients (n = 8) were also obtained from the LUMC-IBD biobank.

Cytokine Measurements

Perianal fistula scraping, rectum biopsies, UC, and healthy colon tissues were homogenized in radioimmunoprecipitation (RIPA) buffer consisting of Tris 50 mM, NaCl 250 mM, NonidentP40 2%, Na-EDTA 2.5 mM, sodium dodecylsulphate-polyacrylamide (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/μg protein. Production of cytokines and chemokines by unstimulated and stimulated MSCs was determined in the supernatant using a Th17 Bio-Plex multiplex assay (Bio-Rad Laboratories, Veenendaal, The Netherlands) after 24 and 48 h. Antibodies against vascular endothelial growth factor (VEGF), hepatic growth factor (HGF), chemokine ligand (CCL)5 (RANTES), and C-X-C motif chemokine ligand (CXCL)1 (all Bio-Rad) were included in the Bio-plex/multiplex assay. TGF-β1 levels were determined using a human TGF-β1 duo-set as previously described (R&D Systems Europe, Abingdon, UK).[21] The levels of cytokines and chemokines in the MSC culture supernatant were corrected for levels observed in non-conditioned medium.

Cytokine-Receptor Combinations

To determine if MSCs could respond to the different inflammatory cytokines that were detected in the supernatants of the tissues, a literature study was conducted to identify which receptor(s) is/are supposed to bind to the cytokines. PubMed was searched for recent (published after 2000), peer-reviewed studies in English. Using this method, the following cytokine-receptor combinations were identified: IL-17; IL-17RA/IL-17RC,[22] IL-13; IL-4R/IL-13Rα1,[23] OSM; gp130/LIFR/OSMR,[24] TNF-α; TNFR1/2,[25] IFN-γ; INFR1/2,[26] IL-1β; IL-1R1/AP[27] and IL-4; IL-4R/IL-13Rα1,[23] IL-5; IL-5Rα/βcommon,[23] IL-8; CXCR1/2,[28] IL-6; IL-6R[24] and IL-12; IL-12Rβ1/2.[29]

MSC Culture

Human bmMSCs were isolated and characterized as described before[7] and cultured in the 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% confluence was reached. For passaging and flow cytometry analysis, cells were harvested by TrypLE Select (Gibco) incubation for 10 min. MSCs between the passage 4 and 7 were used in experiments.

MSC Cytokine Stimulation

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

Flow Cytometry Analysis

After stimulation for 24 or 48 h, 45 000 MSCs were stained for flow cytometry analysis. In all flow cytometry experiments, MSCs were incubated for 30 min at 4°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 live/death marker (Fixable Aqua Dead, Invitrogen, Carlsbad, CA, USA). For staining of intracellular markers, MSCs were fixed (fixation/permeabilization buffer and diluent, eBioscience) for 20 min 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 min at RT. For indicated experiments, standardized flow cytometry was used in which the staining and analysis of upregulation of intracellular and extracellular molecules were standardized using specific Euroflow protocols and a selected set of monoclonal antibodies.[30,31] For the standardized Euroflow cytometry, unlabeled CD54 (Biolegend, San Diego,

Table 1. Cytokine mixtures.

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

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 min at 4 °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 in combination with the antibodies described above. The MSCs were analyzed 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 stimulated MSCs, the mean fluorescent intensity (MFI) was obtained and fold induction was calculated relative to unstimulated MSCs.

qPCR Analysis

mRNA was isolated from 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 (Bio-Rad, Hercules, CA, USA) and primers (Invitrogen) listed in Supplementary Table 1. cDNA samples were subjected to 40 cycles of qPCR as previously described.[32] All values were normalized for cDNA content by expression of the house-keeping gene Ornithine Decarboxylase Antizyme 1 (OAZ1).

T-cell Proliferation and DC Differentiation Assays

PBMCs were obtained by Ficoll (LUMC, Leiden, The Netherlands) separated from a fresh buffy coat (Sanquin, Leiden, The Netherlands). For the T-cell proliferation assay, isolated PBMCs were cocultured with either stimulated or unstimulated MSCs. 100 000 PBMCs/well were seeded with MSCs in a 1:6 (MSCs: PBMCs) ratio, in the presence of anti-CD3/CD28 beads (Gibco) in RPMI (Gibco) supplemented with 10% FCS and penicillin/streptomycin in a V-bottom 96-well plate (Corning, NY, USA). After 24 h, 50 µL 20µCi ^{3}H thymidine (PerkinElmer, Waltham, MA, USA) was added to each well and incubated for 16 h. Thereafter, cells were harvested and thymidine incorporation was measured using a Topcount NXT (Canberra Packard). 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. In the monocyte isolates, between 96% and 98% of CD14-positive cells were found. Monocytes were seeded 300 000/well in a 48-well plate (Corning) and MSCs 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 the addition of fresh GM-CSF and IL-4 on day 3. After 6 days, cells were harvested and analyzed by flow cytometry using antibodies for CD14, CD1α (PE, BD Bioscience), and CD73 (BV421, BD Bioscience) to determine the percentage of DCs ($CD73^{neg}CD14^{neg}CD1\alpha^{pos}$) from the total number of $CD73^{neg}$ cells.

Data Analysis and Statistics

Data are presented as mean ± SD. Microsoft Excel software (version 1803, Microsoft, Redmond, WA, USA) was used to visualize and calculate correlations. All other analyses were performed using GraphPad Prism software (version 7, San Diego, CA, USA). Statistical analysis of 2 groups was performed with an unpaired *T*-tests. Statistical analysis of more than 2 groups was performed using the ANOVA test, and a Tukey's post-analysis. Correlations between 2 groups were analyzed with Pearson correlation. *P*-values ≤ .05 were considered statistically significant.

Results

Identification of Distinct Cytokine Profiles in Perianal Fistula and Inflamed Colonic IBD Tissue

To map the local inflammatory environment in IBD, we determined the levels of 11 cytokines in perianal fistulas and rectum biopsies from FC patients and in the (un)inflamed parts of the colon of UC patients. Cytokine levels were compared to healthy colonic tissue. Of all tissues evaluated, perianal fistula scrapings contained the highest levels for 10 out of 11 measured cytokines (Fig. 1A). Interestingly, OSM levels were found to be the highest in CD rectum (uninflamed), rather than in the perianal fistula tissues itself (Fig. 1A). Comparison of 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 to matched UC uninflamed and normal colonic tissue (Fig. 1A). To provide discrete cytokine profiles in fistulas and UC inflamed tissues, the cutoff was set at 1 pg/mg protein (Supplementary Fig. S1), resulting in 4 cytokine profiles identified in the different IBD-patient-derived tissues. The first cytokine profile, mainly observed in perianal fistulas, showed 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 (FC) disease profile 1, FC01). The cytokine IL-4 was detected in only a selection of the FC samples; therefore, FC02 was composed of the cytokines of FC01, including IL-4 (FC02, Fig. 1B). In inflamed UC biopsies, also 2 cytokine profiles were identified. The first profile included high levels of IL-8, IL-17, IL-1β, and OSM (ulcerative colitis profile 1, UC01). These cytokines were present in the healthy colon samples but were strongly increased in the inflamed UC tissue. The second profile (UC02) showed additional high levels of IFN-γ, IL-6, TNF-α, and IL-13. High levels of these cytokines were only present in a subset of inflamed UC tissues (Fig. 1B).

To study whether the increased local cytokine levels are reflected in the circulation of IBD patients, cytokines were also measured in serum samples from FC patients. Most pro-inflammatory cytokines were increased in the serum of FC patients compared to healthy controls, with significantly higher levels of IL-4 and IL-17 in FC patients (Supplementary Fig. S2). Surprisingly, IL-13 levels were significantly lower in the serum of FC patients compared to UC and control (Supplementary Fig. S2). Strikingly, serum cytokine levels from FC patients did not correlate with the levels in perianal fistula tissue from the same patient. This indicates that the cytokine levels in local fistulas are not reflected in the systemic circulation (Fig. 1C).

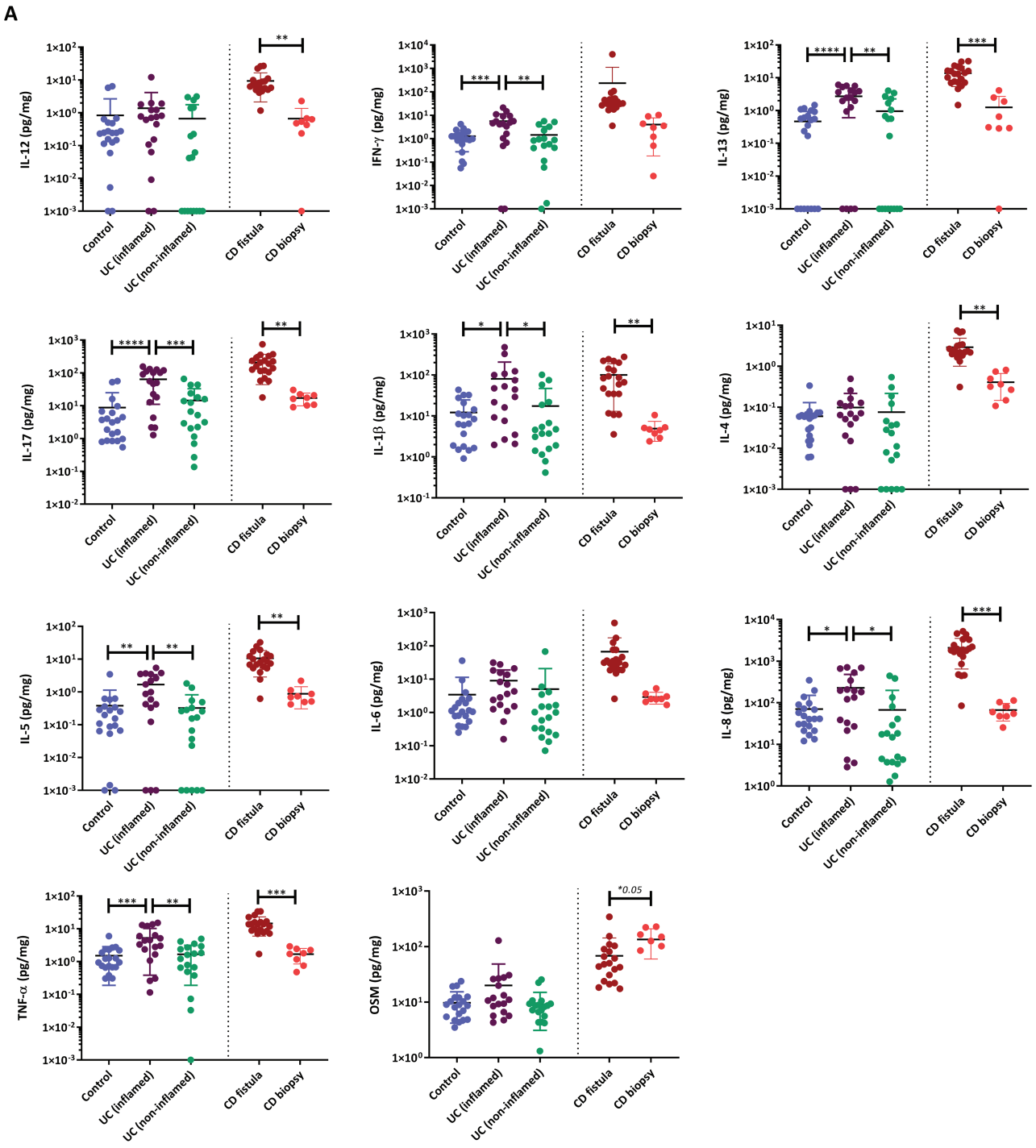

Figure 1. Continued

MSCs Exposed to Different Cytokine Mixes Show Distinct Immunomodulatory Phenotypes

To evaluate if MSCs express the receptors to respond to the (increased) cytokines that were observed in the tissues, 2 publicly available mRNA sequencing datasets of unstimulated human bmMSCs (GSE115240 & GSM2154690)[33,34] were used (Supplementary Fig.. S3A). Receptor expression below 100/20 × 10^6 reads was considered to be negligible. Human bmMSCs expressed mRNA transcripts of the receptors for IL-17A, IL-13, OSM, TNF-α, IFN-γ, IL-1β, and IL-4. mRNA transcripts for receptors for IL-5, IL-8, and IL-12 were negligible. For the IL-6 receptor, only around 100/20 × 10^6 reads were found in human bmMSCs. Given this low expression of the IL-6 receptor, we first established that MSCs stimulated with IL-6 did not show upregulation of the IL-6 target gene cMYC (Supplementary Fig. S3B) and only then decided to not include IL-6 in any of the follow-up experiments.

From the identified cytokine–receptor combinations, which mimic the local inflammatory spectrum in the IBD tissue, 4 cytokine mixtures were designed. Mix-1 consisted of IL-17, IL-1β, and OSM and mimics one of the cytokine profiles of UC01. Mix-2 was composed of IL-17, IL-1β, OSM, IFN-γ,

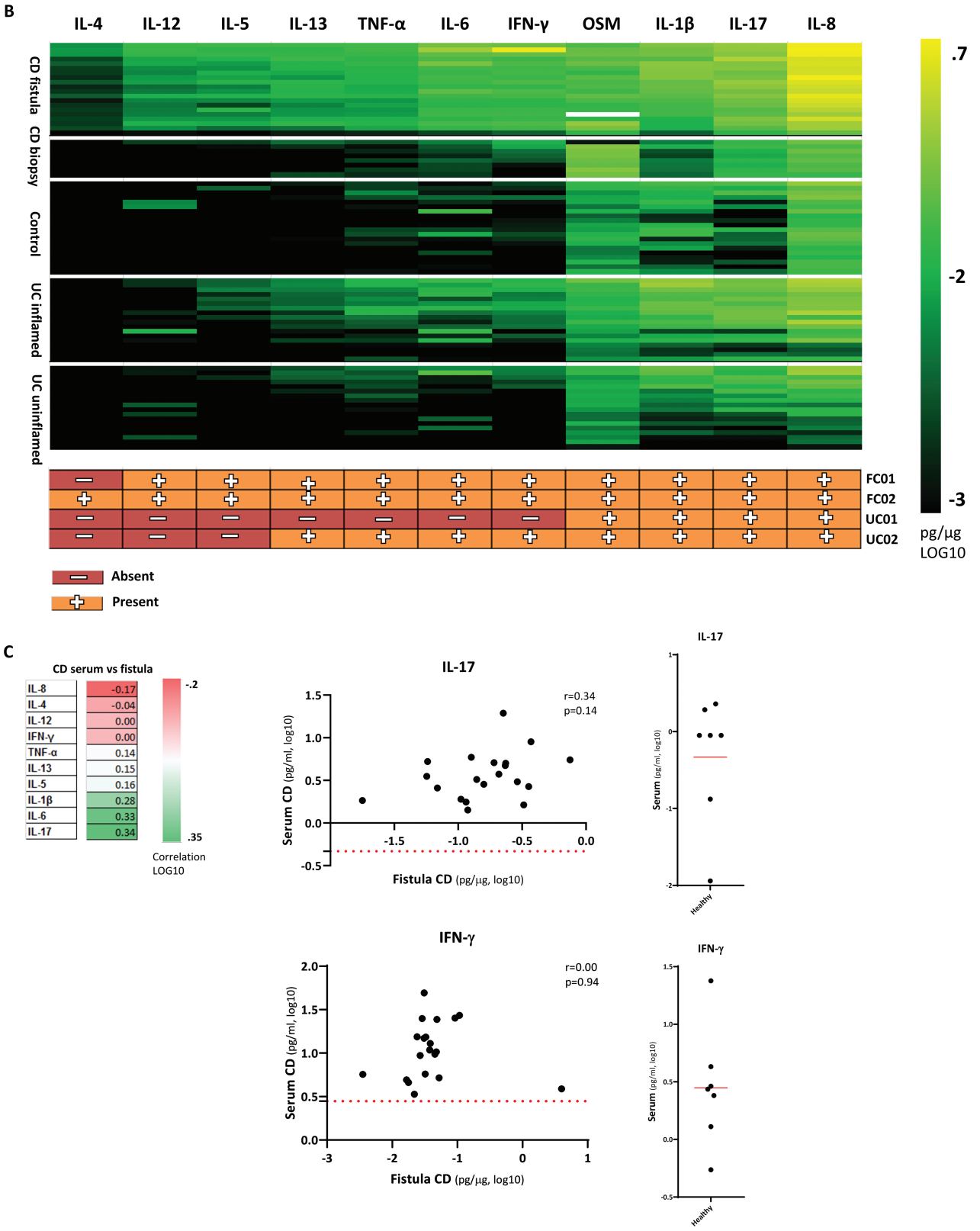

Figure 1. Four cytokine profiles are detected in fistula and colon tissue samples from IBD patients. (**A**) Tissue cytokine levels in control colon, UC inflamed and UC uninflamed colon, CD fistula tissue and CD rectum biopsies (pg cytokine/µg protein). The significance level was calculated between colon tissue samples (ANOVA, Tukey's post-test) and between CD fistula and CD biopsy (*T*-test). Undetectable cytokine levels were transformed to 1×10^{-3} for visualization in the graphs. (**B**) Heat-map of the different cytokines measured in CD fistula and matched 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 serum (pg/mL) and fistula tissue (pg/µg) from the same CD patient. Graphic illustration of the correlation of the levels of IL-17 and IFN-γ between fistula and serum samples, compared to the levels present in serum from healthy individuals. The red dotted line indicates the mean cytokine level in serum samples from healthy individuals. **P* < .05, ***P* < .005, ****P* < .0005, *****P* < .0001.

TNF-α, IL-13 and mimics both a cytokine profile found in the UC colon and FC (UC02/FC01, Table 1). Mix-3 [mimicking FC02] was based on a profile found in perianal fistulas and consisted of IL-17, IL-1β, OSM, IFN-γ, TNF-α, IL-13, and IL-4 (Supplementary Fig. S3C). Finally, Mix-4, containing IFN-γ, TNF-α, IL-13 served as a control for Mix-1 and -2 and did not mimic a local IBD cytokine profile.

To evaluate the effects of the different cytokine mixtures on MSC-induced immunoregulatory molecules, MSCs were stimulated with cytokine Mix 1-4 and mRNA as well as protein changes of various immunomodulatory and tissue regenerative molecules (such as PD-L1, IDO, TGF-β1, and TSG-6[13,35-38]) were analyzed. Each cytokine mixture induced distinct levels of mRNA expression (Fig. 2A, Supplementary Fig. S4A). Upregulation of CXCL12, TSG-6, IL-6, CXCL2, COX-2, CXCL1, and CCL5 mRNA was found upon stimulation with all mixtures representing the local IBD environment (Mix 1-3). CXCL12 was induced most after stimulation with Mix-1 which mimics one of the UC environments, while the other upregulated immunomodulatory molecules were especially elevated after stimulation with Mix-3 or -4. Interestingly, mRNA expression of TGF-β1 was decreased upon stimulation with the inflammatory cytokines representing the IBD environment, especially after stimulation with the 2 mixtures mimicking the fistula environment (Mix-2 and -3). At protein level, the highest expression of HLA-DR, IDO, and PD-L1 was found after stimulation with the mix of cytokines representing a subset of the perianal fistulas (Mix-3, Fig. 2B, Supplementary Fig. S4B). However, flow cytometric experiments performed in a more standardized and sensitive way based on Euroflow principles,[30] showed similar expression of HLA-DR, IDO, and PD-L1 in the 2 mixtures mimicking the fistula environment (Mix-2 and -3) (Supplementary Fig. S5). Furthermore, also COX-2 was equally elevated in Mix-2 and -3 (Supplementary Fig. S4C). The expression of CD54 was induced more than 10 times by all different mixtures, including Mix-1, mimicking the cytokine profile only found in UC. IDO levels showed the strongest increase in MSCs stimulated with IFN-γ only, while the presence of other cytokines next to IFN-γ (in Mix-2 and -3), reflecting the different IBD environments, inhibited IDO upregulation. This indicates that when analyzing the effects of the cytokine environment on MSCs, it is important to take into account the total cytokine environment.

Next to cell surface expression of immunoregulatory molecules, the levels of MSC-derived soluble factors known to be involved in chemotaxis, immune cell modulation, and tissue repair were measured in (stimulated) MSC culture supernatants 24 and 48 h poststimulation. Upregulation of G-CSF, IL-2, IL-6, IL-8, CCL2, CCL5, VEGF, and CXCL1 was found upon stimulation with all mixtures mimicking the different IBD environments (Mix-1, -2 and -3, Fig. 2C, 2D). Mix-1, reflecting the inflamed UC colon, induced the highest expression of IL-6 and VEGF. IL-6 is a known inducer of angiogenesis by stimulation of VEGF production,[39] which could suggest autocrine signaling in MSCs. On the other hand, CCL5 levels showed the strongest increase after stimulating MSCs with the cytokines mimicking the fistula environment (Mix-2 and -3). GM-CSF was secreted in low amounts by both unstimulated and stimulated MSCs, except for MSCs stimulated with Mix-3, which showed degradation of available GM-CSF in the non-conditioned medium. In line with the qRT-PCR data for TGF-β1, less TGF-β1 protein was found in the supernatant of MSCs after stimulation with the cytokines reflecting the IBD environment. Lastly, HGF production significantly differed between the different MSCs already before stimulation, suggesting that besides the cytokine environment also the MSC donor influences the regenerative phenotype of MSCs. This is corroborated by the observation that MSC-30-1 and MSC-30-2, 2 MSC products derived from the same donor, showed a comparable cytokine production profile (Fig. 2C).

Immunoregulatory Functionality in MSCs Depends upon the Inflammatory Environment

To determine whether stimulation with the different cytokine mixes also induces functional differences between MSCs, the influence of stimulated MSCs on T cell proliferation and DC differentiation was assessed (Fig. 3A). These are both established read-outs for assessing MSC function.[40,41] To correct for potential donor variation for both immune cells and MSCs, an MSC product derived from one donor was first tested using PBMC obtained from 3 different donors (Fig. 3B). In addition, 3 (T-cell proliferation) or 6 (DC differentiation) different MSC products were tested, in combination with PBMCs from the same donor (Fig. 3C). Addition of unstimulated MSCs to PBMCs already resulted in slightly decreased T-cell proliferation, while the inhibitory effect of MSCs on T-cell proliferation was significantly increased following cytokine stimulation. MSCs stimulated with IFN-γ alone or with Mix-4 showed the strongest inhibitory effects on T-cell proliferation, ie, T-cell proliferation levels of less than 30% as compared to T cells that were incubated with unstimulated MSCs. MSCs stimulated by cytokine mixtures mimicking the inflamed IBD environment (Mix-1, -2, and -3) showed the same, albeit with lower levels of inhibition of T-cell proliferation.

To study the effect of stimulated MSCs on DC differentiation, $CD14^{pos}$ monocytes were cultured in the presence or the absence of MSCs (Fig. 3B, 3C). Unstimulated MSCs inhibited DC differentiation by around 50%, while stimulation with Mix-1 mimicking the first UC profile, further decreased the percentage of DCs to 24%. The most pronounced inhibition of DC differentiation (96% and 94%) was observed with the mixtures mimicking the second UC profile (Mix-2) and the 2 fistula profiles (Mix-2 and -3). Together this indicates that although Mix-1, -2, and -3 reduced T-cell proliferation to the same extent, MSCs stimulated with Mix-2 or -3 are superior in inhibiting DC differentiation.

Inhibition of DC Differentiation by MSCs Correlates with their COX-2 and G-CSF Expression

Finally, we evaluated whether the expression of various immunoregulatory cytokines upon stimulation was related to the functional inhibition of T-cell proliferation or DC differentiation. This poses the opportunity to identify factors that potentially are involved in the immunomodulatory functions of MSCs (Fig. 4). Interestingly, high COX-2, and G-CSF production by MSCs correlated significantly with a lower percentage of DCs (Fig. 4A). Furthermore, decreased T-cell proliferation was correlated with HLA-DR, PD-L1, CD54, IDO, and CCL5 production by MSCs (Fig. 4B). Irrespective of whether these molecules are directly responsible for the immunomodulatory function of the MSCs, these factors might be used as a surrogate markers to measure the immunomodulatory function of MSCs in vitro.

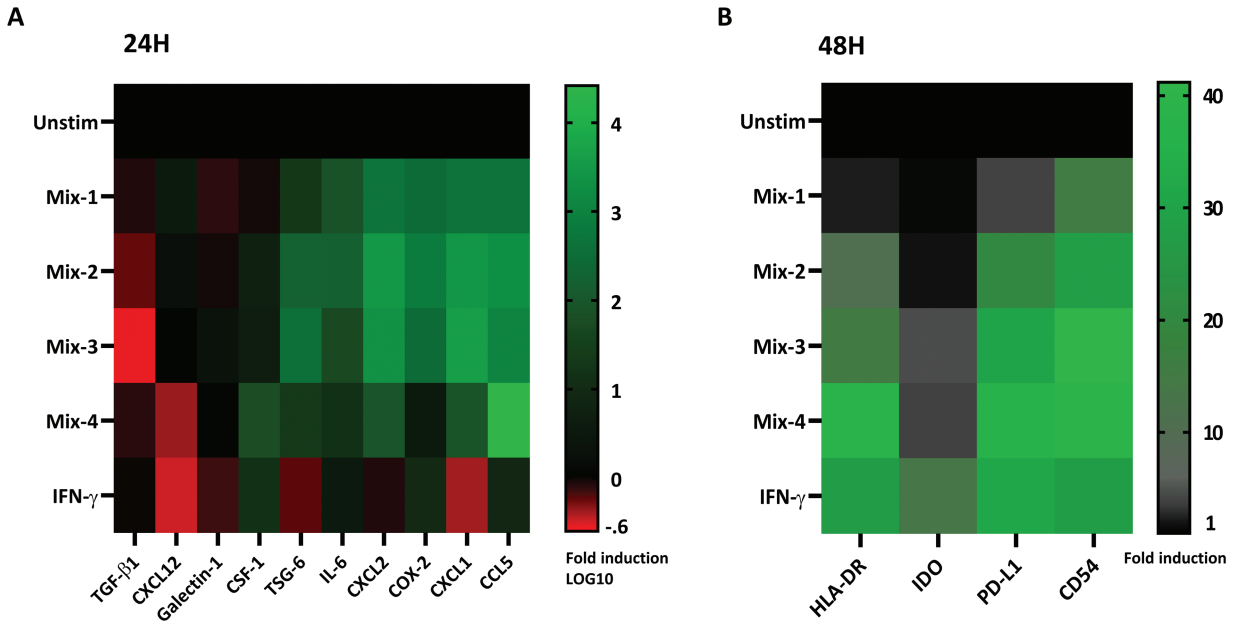

Figure 2. Continued

Discussion

In the present study, we investigated whether cytokine mixtures, mimicking the different inflammatory milieus in IBD patients, can affect the immunomodulatory characteristics of bmMSCs. Our data show that at least 4 disease-specific cytokine profiles exist in IBD patients. Interestingly, MSCs differentially responded to cytokine mixtures mimicking these cytokine profiles both by differential expression of immunomodulatory and tissue regenerative molecules and in their capacity to inhibit DC differentiation. Strikingly, we observed that cytokine mixture enhancing the capacity of MSCs to inhibit DC differentiation did not always result in the strongest inhibition of T-cell proliferation, indicating distinct mechanisms. Finally, the degree of inhibition of DC differentiation was 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 CCL5. These observations indicate the impact the recipient's cytokine milieu can have on the immunomodulatory characteristics of MSCs and potentially thereby the clinical response to MSC treatment.

The analysis of the local cytokine milieu in the colon of patients with UC and in perianal fistulas of patients with CD showed high levels of inflammatory cytokines in both conditions. However, the levels of all cytokines were found to be the highest in perianal fistulas. These high proinflammatory cytokine levels in perianal fistulas could be one of the reasons why MSC therapy is so efficient in fistula healing[9] and suggests that "priming" of the MSCs might be essential to utilize or further enhance their immunomodulatory potential. IFN-γ has been previously shown to enhance the immunosuppressive characteristics of MSCs.[19,20] Surprisingly, in one of the UC cytokine profiles (UC01), IFN-γ was only detected at low levels. Therefore, IFN-γ was not included in the cytokine mix mimicking these patients' profiles (Mix-1). The other UC cytokine profile (UC02) did include IFN-γ and Mix-2, which mimics this profile, and was indeed better capable to inhibit DC differentiation compared to Mix-1, but no difference was found in the ability to inhibit T-cell proliferation. This suggests that priming with IFN-γ does not impact MSCs' ability to suppress T-cell proliferation. Next to the impact of the cytokines on immunomodulation, our data show that Mix-1 induced higher VEGF, TGF-β, and HGF levels, suggesting that MSCs in this milieu have more angiogenic and tissue regenerative properties compared to MSCs stimulated with Mix-2. This indicates that the main mechanism of action of MSCs, either immunomodulatory or tissue regenerative, in UC patients could differ depending on the patients' local cytokine profile. The 2 fistula cytokine profiles only differed in the presence of IL-4. Previously, it was shown that IL-4 hinders the development of T helper 17 cells and IBD patients have lower expression of IL-4 in the lamina propria.[42,43] In our study, comparable levels of IL-4 were found in the colonic tissue of healthy controls and UC patients, although in some UC patients very low IL-4 levels were observed, which was not observed in healthy controls. Stimulation with the 2 cytokine mixtures reflecting the 2 profiles found in perianal fistulas (Mix-2 and -3) showed comparable results on MSC' immunomodulatory properties. Only GM-CSF production by MSCs was tremendously reduced after stimulation with Mix-3, the mix containing IL-4, which could result in less stimulation of granulocytes and monocytes in vivo in a subset of the perianal fistulas after MSC therapy.

By performing multi-cytokine stimulations, our study revealed interesting additive and synergistic effects of cytokines. Previously it was shown that IL-4 and OSM synergistically induced CCL2 and IL-6 expression in intestinal fibroblasts.[44] In our study, the synergistic effect of IFN-γ, TNF-α, IL-13 combined with IL-17, IL-1β, and OSM on G-CSF production by MSCs was found (Fig. 2), albeit we did not pinpoint which single or multiple cytokines were responsible for this effect. In contrast, CXCL1 production was additively and not synergistically increased. These data indicate that the effect of different cytokines on MSCs is not a simple addition of the different single cytokine effects and should be taken into account, for instance in the design of potency assays. The

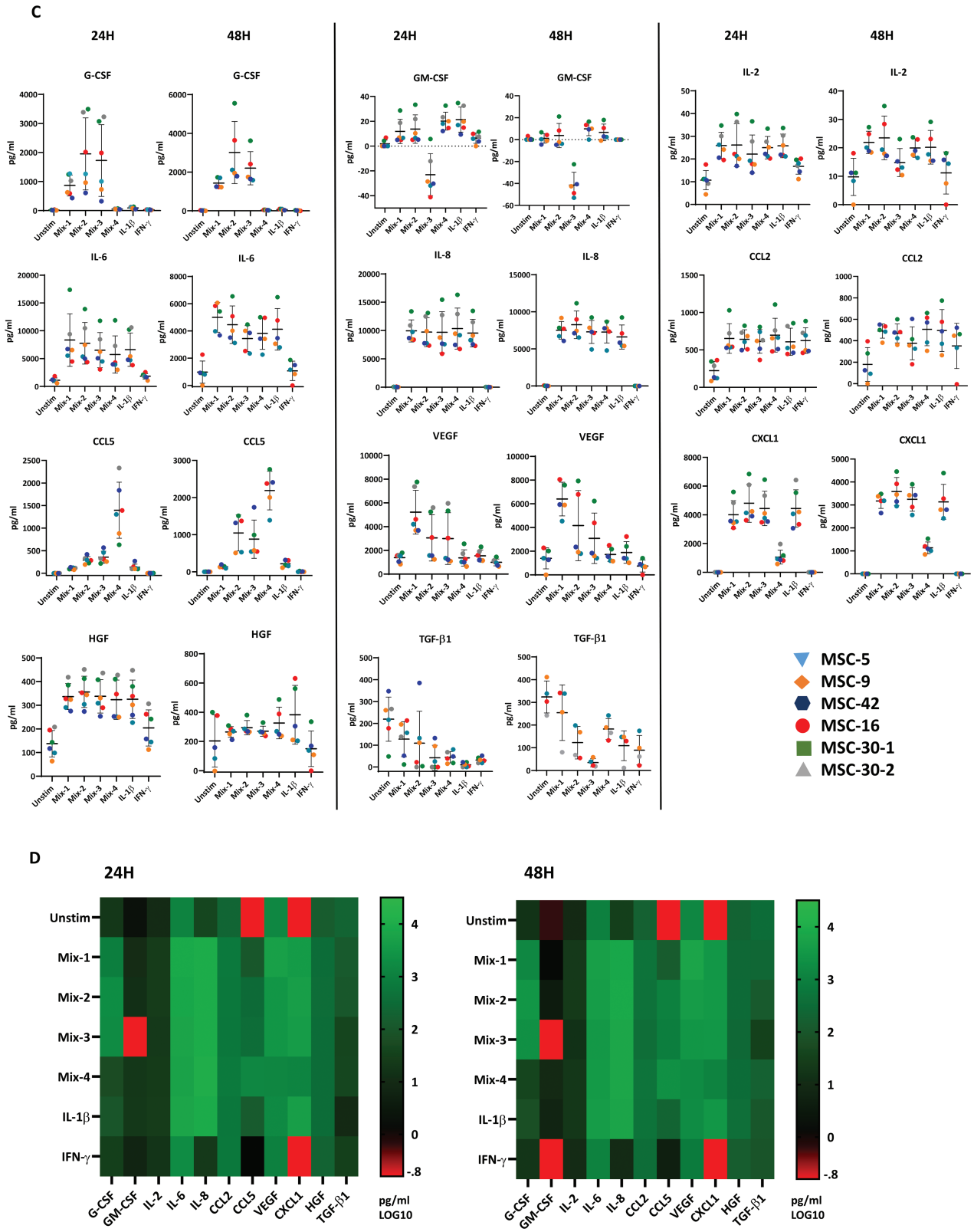

Figure 2. Cytokine mix-specific effects on the expression of mRNA of immunomodulatory molecules by MSCs. (**A**) Heatmap of fold induction (log10 transformation) of mean mRNA expression of different genes in 2 different MSCs after stimulating for 24 h (24H) with Mix-1, -2, -3, -4 or IFN-γ compared to mRNA expression in unstimulated MSCs. Gene expression upon stimulation with Mix-3 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 3 different MSCs upon stimulation for 48 h (48H) with Mix-1, -2, -3, -4 or IFN-γ, compared to unstimulated MSCs. (**C**) Levels of different chemokines and cytokines measured in the supernatant of 6 different MSC products stimulated for 24 h (24H) and 48 h (48H) with different cytokine mixtures (log10 transformation, pg/mL). (**D**) Heatmap of mean cytokine and chemokine levels (pg/mL, log10 transformation) measured in the supernatant of 6 different MSC products stimulated for 24H and 48H with Mix-1, -2, -3, -4 or IFN-γ.

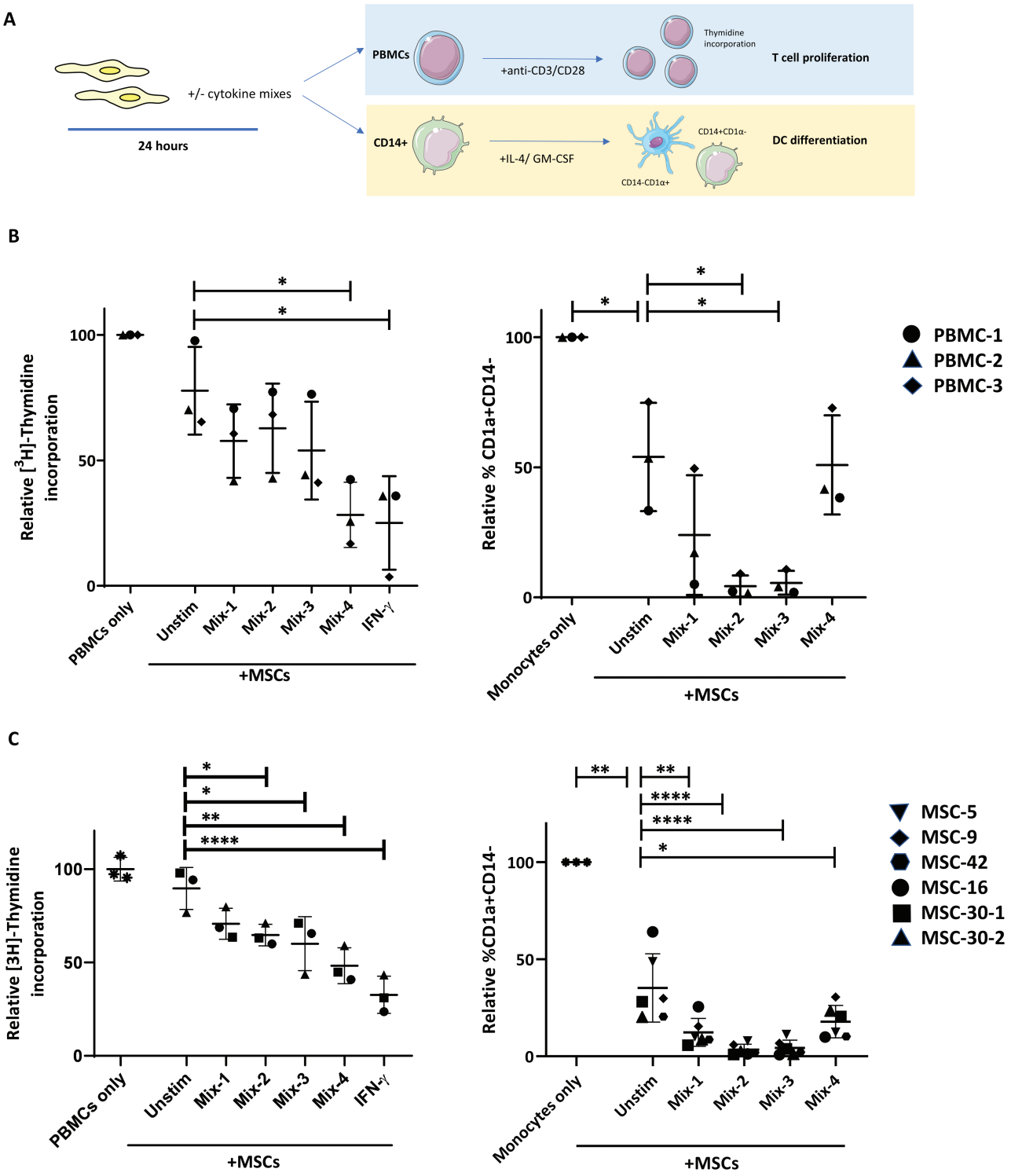

Figure 3. Cytokine-stimulated MSCs show different immunoregulatory properties. (**A**) Schematic overview of the T-cell proliferation and DC differentiation assay. (**B**) T-cell proliferation assay: relative ^{3}H-thymidine incorporation in PBMCs cultured alone compared to PBMCs cocultured with unstimulated 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 to monocytes cultured alone. PBMCs from 3 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**) 3 different MSC products were used in the T-cell proliferation assay as described above. Six MSC products (different symbols) were used in the DC differentiation assay as described above, while using 2 different PBMC products $^{*}P < .05$. Parts of the figure were drawn by using pictures from Servier Medical Art. Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/).

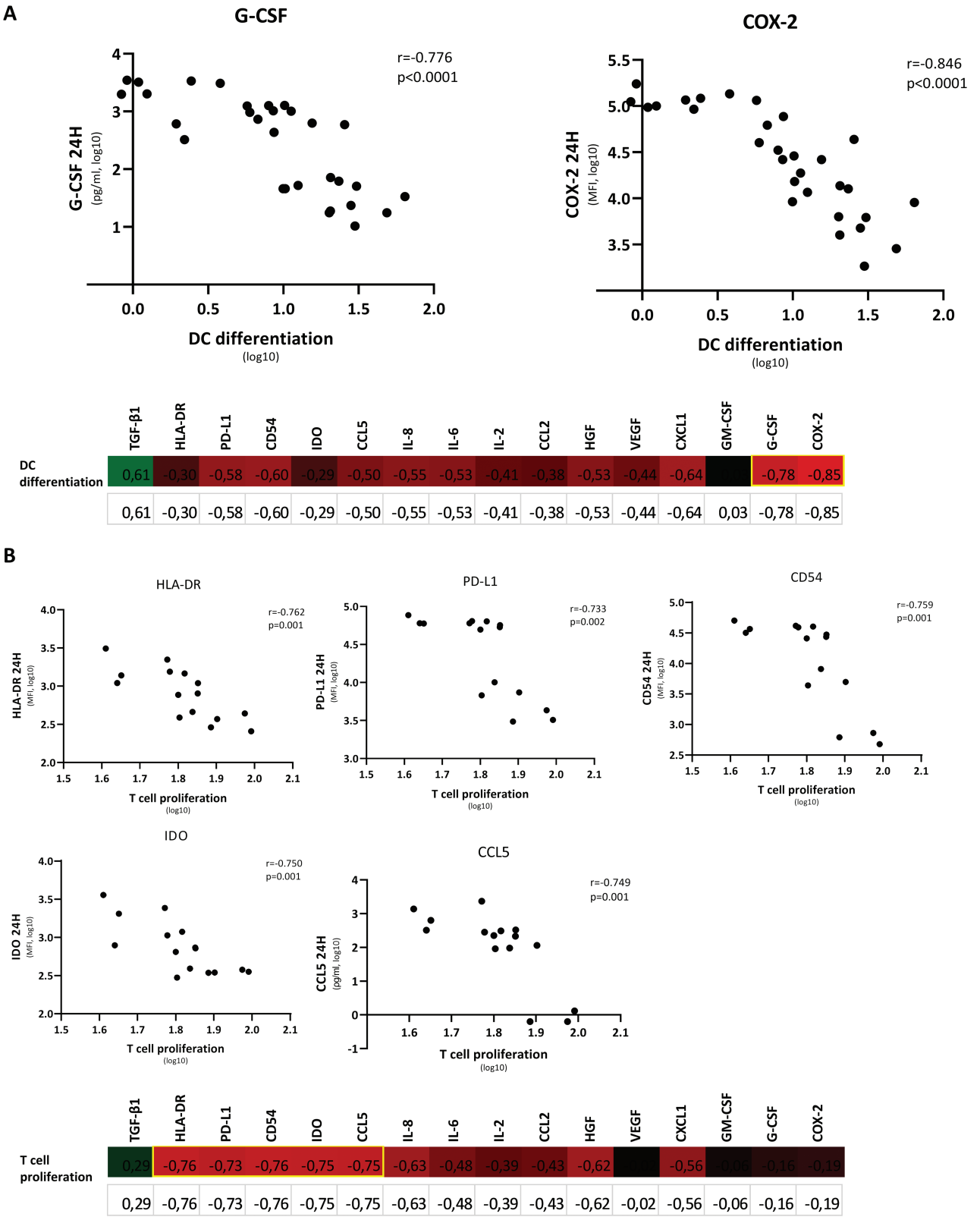

Figure 4. The inhibition of DC differentiation by MSCs correlates with MSCs' COX-2 and G-CSF expression. (**A**) DC differentiation upon coculture with MSCs, measured as the percentage of $CD73^{neg}CD1a^{pos}CD14^{neg}$ cells correlates with G-CSF and COX-2 expression (Pearson correlation, log10 data transformation). Correlation coefficients between DC differentiation and all measured proteins are indicated in a heat-bar. (**B**) T-cell proliferation correlates with HLA-DR, PD-L1, CD54, IDO, and CCL5 production (Pearson correlation, log10 data transformation). Correlation coefficients between T-cell proliferation and all measured proteins are indicated in a heat-bar.

capability of stimulated MSCs to inhibit T-cell proliferation was correlated with PD-L1, CD54, IDO, HLA-DR, and CCL5 expression. This is in line with previous reports that showed the importance of PD-L1, CD54, and IDO in inhibiting T cells using blocking experiments.[36,37,45-47] An inverse correlation was observed between monocyte differentiation into DCs and COX-2 and G-CSF production by MSCs. In addition, the relation between COX-2 and the inhibition of differentiation of monocytes into $CD1\alpha^{pos}$ $CD14^{neg}$ DCs has been described before.[48,49] This knowledge could help to select the best immunomodulatory MSCs for clinical use, for example, based on the level of in vitro COX-2 and/or G-CSF production instead of performing a time-consuming immune cell assay.

MSCs are considered to be immune privileged, based on low expression of HLA class I and II molecules and low levels of co-stimulatory molecules such as CD40 and CD80.[50,51] Currently we know that in some patients after local MSC therapy for perianal fistulizing disease transient donor-specific HLA class I antibodies are found.[8,9,52] This indicates that in therapeutic settings MSCs might not be as immune privileged as suggested before. Interestingly, in our current study, HLA-DR expression by MSCs was found to be upregulated after stimulation with Mix-2, -3 or 4 in contrast to unstimulated or Mix-1-stimulated MSCs. This indicates that the local cytokine milieu could strongly impact the level of HLA II expression and thereby potentiate recognition by the host immune system. Since Mix-1, mimicking one of the two UC cytokine milieus, does not upregulate HLA-DR in contrast to the other mixtures, this could result in MSCs that stay "immune privileged" in some of the UC patients. Currently, it remains unknown whether HLA expression by MSCs correlates to the presence of anti-HLA antibodies in patients. Furthermore, no relation between the presence of anti-HLA antibodies and the safety or therapeutic effects of MSC therapy has been found yet.[10] When clinical implications of anti-HLA antibodies have been assessed in more detail, it could be useful to further analyze if the UC cytokine milieu is responsible for HLA expression by MSCs and ultimately the presence of anti-HLA antibodies.

MSC therapy for the treatment of refractory perianal fistulas has been FDA and EMA-approved since 2018. Given the fact that MSC therapy is expensive, requires extensive logistics and clinical outcomes are variable, there is a clear need to stratify patients for their eligibility for MSC therapy.[53] In this manuscript, we showed the potential of the IBD cytokine milieu to serve as a marker for MSC function. We showed the capacity of different cytokine mixtures, mimicking different IBD cytokine milieus, to regulate the immunomodulatory phenotype and function of bmMSCs. Given the differences in functional responses of MSCs to the cytokine mixtures used (mimicking UC or FC microenvironments), this suggests that, especially for the treatment of luminal UC, the patients' cytokine milieu could be relevant for exerting the MSC regulatory function on DC differentiation. Unfortunately, local cytokine levels in IBD do not completely reflect cytokine levels in serum, which is in accordance with previous literature.[54] This restricts the application of cytokine serum levels as an indicator for the cytokine levels in the local IBD environment and emphasizes the potential importance of analyzing the local cytokine milieu rather than serum cytokine levels prior to MSC therapy. Next to the patients' local cytokine milieu, also differences between the MSCs derived from different donors were observed. Analysis of the cytokine levels in the supernatant after stimulation revealed subtle differences between MSCs from different donors, but a close resemblance between 2 MSC products derived from one donor (MSC-30-1/2). Clinical data from our current clinical trial on the safety and tolerability of local MSC therapy in proctitis in UC will be helpful to further investigate both the impact of different MSC donors and the local cytokine milieu on safety and therapeutic outcome (Dutch trial register: NTR7205).

It is important to mention that this study is not aiming to mimic the full inflammatory IBD milieu in vitro and assess its effect on MSCs. Eleven cytokines known to play a role in IBD were analyzed and used to establish recombinant cytokine mixtures for in vitro stimulation. Therefore, the results obtained cannot be extrapolated directly to the human situation. It is highly likely that locally injected MSCs in IBD patients will be modified by many more cytokines and chemokines as well as via contact-dependent interactions. Furthermore, the possibility of upregulation of cytokine receptors on MSCs upon transplantation in an IBD environment could make them responsive to the additional cytokines for which normally no receptors are present. However, the present data clearly show that components of the inflammatory IBD milieu directly influence the phenotype and in vitro function of MSCs.

In summary, our data suggest that the in vivo immunoregulatory properties of MSCs therapy could be directly 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 the local cytokine milieu.

Acknowledgments

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Conflict of Interest

M.B. declared research funding from an ECCO grant. A.v.d.M-d.J declared advisory role with Ferring; honoraria from Tramedico, Galapagos, Janssen Cilag; research funding from Nestle, Galapagos, Norgine. W.E.F. declared advisory role with Boost Pharma and financial relationship with DSMB Glycostem. The other authors declared no potential conflicts of interest.

Author Contributions

M.C.B.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; A.E.v.d.M.-de.J: conception and design, financial support, provision of study material or patients, data interpretation, final approval of manuscript; E.C.L.M.S., L.G.P: collection and/or assembly of data, data analysis and interpretation, final approval of manuscript; H.W.V., W.E.F.: conception and design, financial support, data interpretation, final approval of manuscript; M.v.P.: conception and design, data analysis and interpretation, final approval of manuscript; L.J.A.C.H: conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript; K.S.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Supplementary Material

Supplementary material is available at *Stem Cells Translational Medicine* online.

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