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

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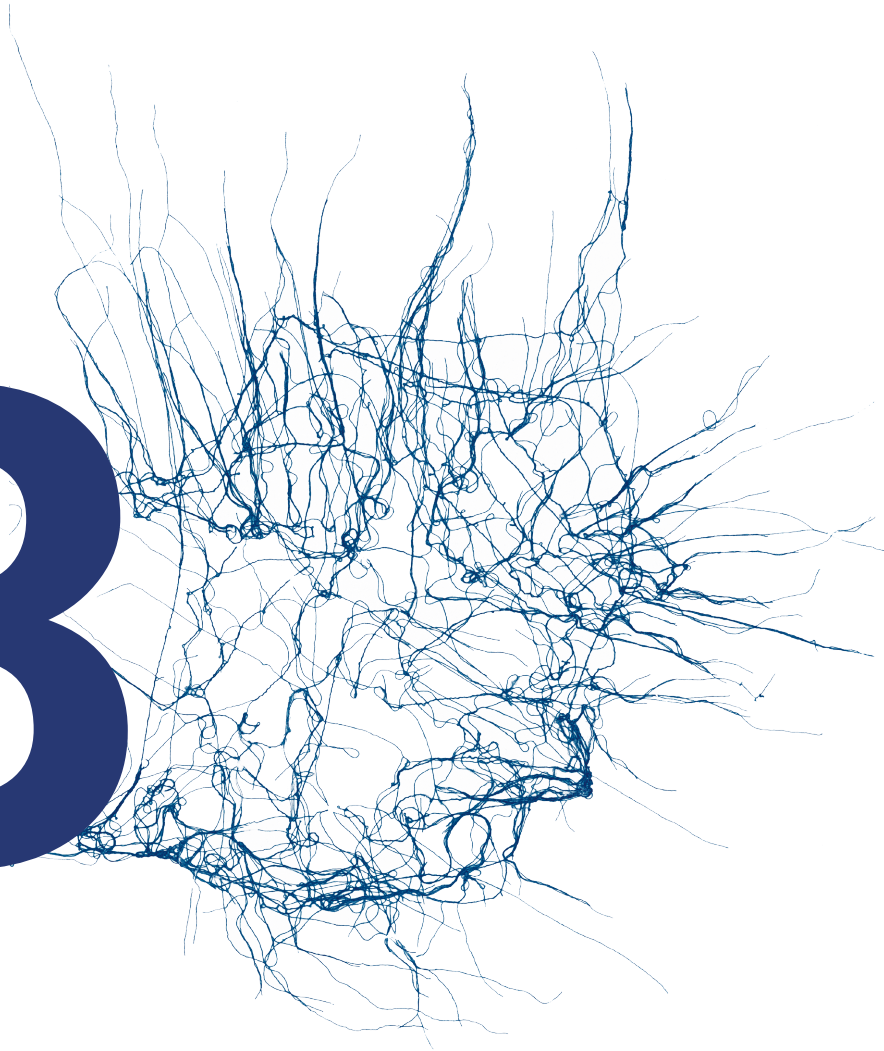

IMMUNOREGULATORY ROLE OF FIBROBLASTS DERIVED FROM CROHN'S DISEASE ASSOCIATED PERIANAL FISTULAS

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ABSTRACT

Around 20% of patients with Crohn's disease (CD) develop perianal fistulas, which significantly impairs the patients' quality of life. Recent studies demonstrated an immunoregulatory function of fibroblasts in the pathogenesis of various diseases. However, in this context little is known about fistula fibroblasts. Investigating the immune regulatory capacity of fistula fibroblasts might provide novel insights into the pathogenesis of perianal fistulas and provide potential therapeutic targets. We have therefore characterized the expression of immune regulatory genes in fistula-derived fibroblasts in relation to surrounding infiltrating immune cells. Fibroblasts isolated from CD fistulas were compared with skin, colon and non-CD fistula derived fibroblasts with regard to the expression of immunoregulatory markers on both mRNA and protein level. Fistula fibroblast from CD patients showed an overall comparable immunoregulatory profile with that of the non-CD fistula fibroblasts. However, a lower programmed death-ligand 1 (PD-L1) membrane expression and higher cyclo-oxygenase 2 (COX-2) expression by CD associated fistula fibroblasts compared with non-CD fistula fibroblasts was found, suggesting inferior immunosuppressive capabilities of the CD fistula fibroblasts. Colon fibroblasts showed significantly higher levels of immunoregulatory markers, including chemokine ligand (CXCL)-1 and interleukin (IL)-6, compared with skin and fistula fibroblasts. Next, the spatial distribution of fistula fibroblasts in relation to both T cell and myeloid cell subtypes was studied in CD perianal tissues in order to identify relevant fibroblast-immune interactions that occur *in vivo*. Moreover, CD fistula fibroblasts expression patterns were subsequently related to immune cell subsets in the CD perianal fistula tissue. Interestingly, we found that a PD-L1^{low}PD-L2^{low}IL-6^{high} fibroblast subset correlated with a high percentage of FOXP3^{pos} cells in the tissue of perianal fistula patients. Finally, immunoregulatory expression patterns were also compared with that of mesenchymal stromal cells (MSCs), in order to study potential working mechanisms of the recently introduced MSC-therapy in CD perianal fistulas. In comparison with MSCs, CD fistula fibroblasts showed high levels of the pathognomonic fibroblast marker podoplanin (PDPN), but low levels of immunoregulatory CXCL-12 and colony stimulating factor (CSF)-1. Altogether, these data reveal a potential specific immunoregulatory role for fibroblasts in CD-associated fistulas and show the anatomical context in which these fibroblast-immune interactions occur in perianal fistulas of CD patients.

INTRODUCTION

Perianal fistulas are a common complication of Crohn's disease (CD), with a cumulative risk of 21% at 10 years after diagnosis of CD[1]. Patients with perianal fistulas present with anal pain, discharge and faecal incontinence, which directly influences their quality of life. Both systemic anti-tumour necrosis factor α (TNF-α) therapy[2-4] and local mesenchymal stromal cell (MSC)-therapy[5,6] showed significant improvement in fistula closure in randomized, placebo-controlled multi center trials. Furthermore, surgical therapies are used to drain the perianal fistulas, followed by closure of the internal opening or complete removal of the fistula tract[7]. However, even with combined surgical and pharmacological approaches, before MSC-therapy was available, only 37% of the patients with complex perianal fistulas in CD achieved complete healing after a median follow-up of 10 years[8].

Perianal fistulas are thought to develop from ulcers in the inflamed rectum. The formation of a fistula tract is supported by epithelial-to-mesenchymal transition, the process in which epithelial cells develop a mesenchymal phenotype and are able to migrate and penetrate in adjacent tissues[9]. Through epithelial-to-mesenchymal transition, transitional cells, which show characteristics of both epithelial and mesenchymal cells, are being formed and can be found along the lining of the fistula tract[10,11]. Besides transitional cells, other mesenchymal cells, like fibroblasts, are present in these fistulas. Fibroblasts are a heterogenous cell population that are capable of producing various cytokines and chemokines, leading to recruitment and activation of different subsets of immune cells[12]. Kinchen et al[13] recently investigated changes in this fibroblast heterogeneity in the inflamed colon using a single cell RNA sequencing approach. A fibroblast subset, characterized by the expression of podoplanin (PDPN) and interleukin (IL)-33, was shown to be scarcely present in the healthy colon but expanded in the intestines of ulcerative colitis (UC) patients. This population of fibroblasts showed upregulation of several markers involved in T cell chemotaxis and activation. Also in CD patients colonic fibroblasts showed a differential expression for some immunoregulatory markers compared with healthy intestinal fibroblasts[14]. If and how fibroblasts play a role in regulating immune cells in CD-associated fistulas, however, has not yet been investigated.

Although perianal fistulas are clearly associated with CD, they can also develop in humans without inflammatory bowel disease (IBD). These cryptoglandular fistulas origin from an inflamed anal gland eventually leading to the formation of an inflamed fistula track[15]. Interestingly, while in patients with CD the perianal fistulas are treated with immunomodulating and MSC-therapies, in cryptoglandular fistulas surgical interventions combined with antibiotics are the corner stone of therapeutic intervention.

The present study aimed at characterizing CD fistula-derived fibroblasts, with emphasis on their role in immune cell recruitment and activation. Fibroblasts from perianal CD fistula tissue were characterized by immunoregulatory marker expression and compared with skin, colon and non-CD fistula fibroblasts. Fistula fibroblasts immunoregulatory gene expression patterns were related to immune cell subtype compositions and their spatial context in the tissue of CD perianal fistula patients. Contrastingly, allogeneic MSCs isolated from bone marrow or adipose tissue, are currently used as a treatment modality for patients with perianal fistulas. To study differences in immunoregulatory function and thereby a potential working mechanisms of MSC-therapy, gene expression of MSCs was compared with the expression patterns found in CD fistula fibroblasts.

MATERIALS AND METHODS

Human samples

Paraffin-embedded perianal fistula slides from patients with CD that underwent a fistulectomy were obtained from the department of Pathology of the Leiden University Medical Center (LUMC) and conducted in accordance to the Declaration of Helsinki and the Code of Conduct for responsible use of Human Tissue and Medical Research as drawn up by the Federation of Dutch Medical Societies in 2011. This Code permits the further use of coded residual (historical) tissue and data from the diagnostic process for scientific purposes. For all other human samples, protocols were approved by the Biobank committee or MEC of the LUMC and informed consent was obtained before tissue collection (B20.005). Perianal fistula scrapings from patients with and without CD were available from the established biobank of the department of Gastroenterology and Hepatology of the LUMC. Patient characteristics and the usage of immunomodulating medication at the time of fistula surgery were recorded of these patients. One patient had no CD related symptoms at the time of fistula surgery and previous endoscopy showed no signs of CD, however 1.5 years later he was successfully treated with anti-TNF-α therapy, suggesting underlying CD. This patient is included in red in the non-CD group, since at the time of material collection there were no signs for intestinal inflammation. Fistula scraping was collected during surgery. One part was washed and stored in paraformaldehyde 4% for paraffin embedding, while fistula fibroblasts were isolated from the other part. Normal colonic tissue was obtained from patients undergoing colectomy for colorectal cancer, with 10 cm distance from the tumor, and abdominal human skin fibroblasts were obtained from the department of Dermatology. Lastly, human MSCs were isolated from the bone marrow of healthy volunteers. These MSCs were obtained after written informed consent[6].

Fibroblast and MSC isolation and culture

In order to isolate fistula fibroblasts, fistula scrapings were cut in small pieces and depleted of epithelial cells during incubation in 5mM EDTA (Merck Millipore, Burlington, MA, USA) for 30 minutes at 37°C. The small tissue pieces were then placed in 6-wells plates and cultured in Dulbecco's Modified Eagle Medium (DMEM)/F-12 (Gibco) supplemented with 10% fetal calf serum (FCS, Gibco), 1% penicillin/streptomycin (Gibco), 1% gentamycin (Gibco) and 1% fungizone (Gibco). Adherent cells grew from the tissue pieces after 4-7 days. Colonic fibroblasts were isolated in a similar way to fistula fibroblasts as described before[16]. Abdominal skin fibroblasts were isolated by incubation of the dermis of the skin in collagenase (Gibco)/dispase II (Roche, Basel, Switzerland) for 2 hours at 37°C. MSCs were isolated from the bone marrow as described before[6]. All cells were cultured in DMEM/F-12 medium supplemented with 10% FCS and 1% penicillin/ streptomycin. Primary human fibroblasts and MSCs were used for up to 7 passages.

Quantitative Polymerase Chain Reaction (qPCR)

RNA was isolated from fibroblasts or MSCs (passage 4-7) with the NucleoSpin® RNA kit (Macherey-Nagel, Düren, Germany) according to manufacturer instructions. RNA concentration and purity were determined with the NanoDrop 1000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). Complementary (c)DNA was synthesized with the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific). Between 225-1000ng mRNA were used for cDNA synthesis. qPCR analysis was performed according to established qPCR protocols using SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). Primers are indicated in Supplementary table 1. Stability of expression of the various reference genes; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-actin, Beta-2-Microglobulin (B2M), Hydroxymethylbilane Synthase (HMBS) and hypoxanthine phosphoribosyltransferase 1 (HPRT1) was measured. Using the NormFinder software[17], GAPDH was determined to be the best reference gene, with a stability value of 0,084. All values were therefore normalized for cDNA content by GAPDH.

Flow cytometry

Flow cytometry was performed on fibroblasts and MSCs to determine target gene expression on protein level. The fibroblasts/MSCs were detached from the culture plate using TrypLE Select (ThermoFisher Scientific) and stained with 7AAD (Biolegend, San Diego, CA, USA), anti-programmed death-ligand (PD)-L1 (clone M1H1, APC or BV421, eBioscience, Waltham, MA, USA), anti-PD-L2 (clone MIH1, APC, BD Bioscience, Franklin Lakes, NJ, USA), anti-human leukocyte antigen (HLA)-DP/DQ/DR (clone Bu26, FITC, Biorad) and cyclooxygenase (COX)-2 antibodies (clone AS67, PE, BD bioscience). For intracellular staining, cells were fixed (fixation buffer and diluent, eBioscience) and permeabilized

(permeabilization buffer, eBioscience). Unstained samples and isotype controls were used as controls. Compensation was performed with OneComp eBeads Compensation Beads (Invitrogen, Carlsbad, CA, USA), which were prepared according to the manufacturers' protocol. The fibroblasts/MSCs were analysed through flow cytometry with the BD FACS™ LSR-III cytometer (BD Biosciences). Analysis of the data was performed with the FlowJo software version 8.7.1 (Tree Star Inc. Ashland). The mean fluorescent intensity of every sample was corrected for sample specific auto-fluorescence.

Seven colour immunofluorescence and immunophenotyping

In order to define several subtypes of immune cells in fistulectomy (n=8) and fistula scraping material (n=15), sections of 4 µm were cut and mounted onto glass microscope slides. The tissues were then deparaffinized and heat induced epitope retrieval was applied. Sequential tissue slides were stained with two panels, for the T cell panel (n=14), a combination of direct and indirect labelled antibodies for rabbit anti-CD45, mouse anti-CD8, rabbit anti-CD3, mouse anti-FOXP3, mouse anti-CD45RO and rabbit anti-vimentin were used (Supplementary Table 2). For the myeloid panel (n=15), rabbit anti-CD45, rabbit anti-CD11c, mouse anti-HLADR, rabbit anti-CD68, mouse anti-CD163 and rabbit anti-vimentin were used (Supplementary Table 3). The staining procedure was performed as described before[18]. In short, the tissues were stained with primary and secondary antibodies for indirect detection. Next, the tissues were stained with the directly conjugated antibodies. Lastly, the tissues were stained with 4', 6-diamidino-2-phenylindole (1 µm DAPI, ThermoFisher scientific) as a nuclear counterstain. Samples were covered with a cover slip using ProLong Gold Antifade Reagent (Cell Signalling Technology, Danvers, MA, USA). Whole slide fluorescent images of the tissues were obtained with the Vectra 3.0 Automated Quantitative Pathology Imaging System (PerkinElmer, MA, USA). Using the Phenochart software (PerkinElmer), regions of interest were selected randomly, though avoiding areas with high amounts of erythrocytes, for multispectral imaging (MSI) analysis. For each sample 5 1338 µm x 1000 µm fields or 10 669 µm x 500 µm fields were selected. The Inform Advanced Image Analysis Software (PerkinElmer) was used to count and phenotype cells in the acquired MSI fields. The first step in the Inform algorithm was preparing the MSI images for analysis through a process called 'spectral unmixing' to decompose the image into its various components. The selected spectra were Alexa 488 (CD45), Alexa 546 (CD8/CD11c), Alexa 594 (CD3/HLADR), Alexa 633 (FOXP3/CD163), Alexa 647 (CD45RO/vimentin), Alexa 680 (Vimentin/CD68) and DAPI. Once the images were prepared, the algorithm then created a cell segmentation map. Nuclei segmentation was based on DAPI. Detection of cell membrane was based on membrane markers CD3, CD8 and CD45RO for the T cell panel and based on CD163 and CD68 for the myeloid panel. Once the cell segmentation map was created, each identified cell was associated with the according

set of pixels, so that marker signals could be extracted. The segmented cells could then automatically be analysed for phenotyping. $CD3^{pos}/CD8^{neg}$, $CD3^{pos}/CD8^{pos}$ and $CD3^{pos}/FOXP3^{pos}$ phenotypes could be discriminated within the same image. In order to analyse the myeloid panel, separate analyses were run for each marker and cells were defined as either positive or negative for CD68, CD11c, HLADR, and CD163. Using R (R Foundation, Boston, MA, USA), a script was created to combine the phenotypes for each cell, based on the cell coordinates. $CD11c^{pos}$, $CD11c^{pos}HLADR^{pos}$, $CD68^{pos}$, $CD68^{pos}HLADR^{pos}CD163^{neg}$ and $CD68^{pos}HLADR^{pos}CD163^{pos}$ phenotypes were identified. Images displaying vimentin+ and CD45- signals were also obtained to identify fibroblasts. Total cell count and cell counts of each phenotype were obtained for each image. The T cell versus myeloid ratio was defined for each fistula sample by the % $CD3^{pos}$ cells versus the % $CD11c^{pos}$ or $CD68^{pos}$ cells of all counted cells.

Statistical Analysis

Statistical analysis of all data was performed with the GraphPad Prism 7 version 7.02 software. Analysis of two groups was done with an unpaired, non-parametric Mann-Whitney test. Statistical analysis of more than two groups was done with an unpaired, non-parametric Kruskal-Wallis test, with a Dunn's post-analysis. The correlation between fibroblast mRNA expression and the number of immune cells was analysed with the Spearman correlation. All data are presented as mean with standard deviation.

RESULTS

Colon fibroblasts show the highest expression of genes involved in immunoregulation

Perianal fistulas connect the lumen of the colon with the perianal skin and, therefore, fistula-derived fibroblasts (from both CD and non-CD patients) were compared with colon and skin-derived fibroblasts. First, we characterized all cells for known fibroblast markers (Supplementary Figure 1A) and absence of markers for immune (CD45), endothelial (CD31) and epithelial cells (keratin 20) on qPCR (data not shown). All fibroblast markers were expressed by the cultured cells, thereby confirming their origin. Colon fibroblasts showed higher FAP and α-SMA expression compared with skin and colon fibroblasts (Supplementary Figure 1A).

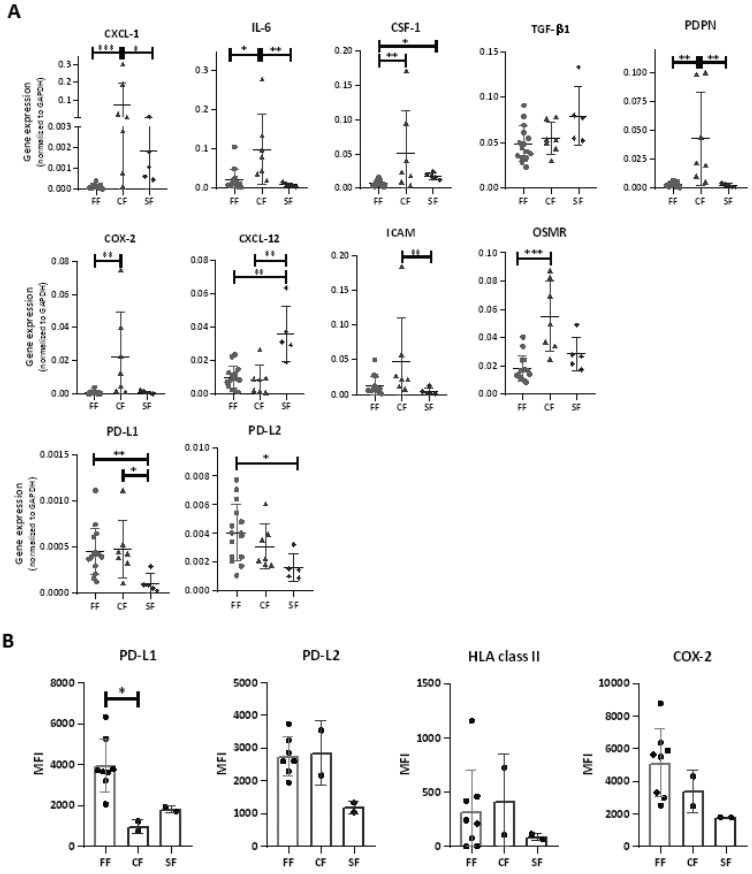

FIGURE 1. Fibroblasts derived from fistula (both CD- and non-CD), colon and skin show different expression of immunoregulatory markers. A. Comparison of mRNA levels in fibroblasts derived from fistulas (FF, n=15), colon (CF, n=6) and skin (SF, n=5). B. Comparison of PD-L1, -L2, HLA class II and COX-2 mean fluorescent intensity (MFI), corrected for auto-fluorescence, of FF (n=8), CF (n=2) and SF (n=2). *p<0.05, **p<0.01, ***p<0.001

Analysis of expression profiles (Figure 1A) showed higher levels of immunoregulatory markers in colon fibroblasts, like chemokine ligand (CXCL)-1, interleukin (IL)-6, cyclo-oxygenase 2 (COX-2), intracellular adhesion molecule (ICAM) and oncostatin M receptor (OSMR). Also for podoplanin (PDPN), a marker associated with pathogenic fibroblasts in both rheumatoid arthritis[19,20] and IBD[13,21] higher expression was found in fibroblasts derived from the colon (Figure 1A). Colony stimulating factor (CSF)-1 showed in both colon and skin fibroblasts higher expression compared with fistula fibroblasts. In contrast, CXCL-12 was found to be highest in skin fibroblasts. CD274/PD-L1 and PD-L2/CD273 mRNA expression, two checkpoint molecules regulating T cell activity, were found highest in

fistula and colon fibroblasts (Figure 1A). To investigate if altered mRNA expression levels also result in altered protein expression, flow cytometry analysis was performed. These data revealed that in contrast to the mRNA expression, the highest level of membrane bound PD-L1 and COX-2 was found in the fistula fibroblast group (Figure 1B). Protein levels for PD-L2 and HLA class II molecules were comparable between the 3 groups, although the sample size was too small to draw firm conclusions (Figure 1B). Together, these data show differences in the expression of immunoregulatory markers in fibroblasts isolated from different locations.

TABLE 1. Patient characteristics. 1 patient was 1.5 yr after fistula surgery diagnosed with CD, but is still included in non-CD group. Of CD patients, n=10 fistula fibroblasts were analysed by qPCR, n=15 fistula scrapings were analysed on IHC for immune cell analysis, of which one only for the myeloid panel, n=10 were analysed for both fistula fibroblast qPCR and IHC for immune cells.

	CD patients (n=15)	Non-CD patients (n=5)
Female (n, %)	10	1
Medication		
Anti-TNF	6	-
Vedolizumab	4	-
Corticosteroids	4	-
5-ASA	2	-
Other immunomodulators	6[#]	-
No immunomodulators	1	5

[#] thiopurines, allopurinol and dasatinib (cancer therapy)

Different COX-2 and PD-L1 expression by CD and non-CD fistula fibroblasts

Since perianal fistulas develop both in patients with and without CD, we aimed to compare the fibroblasts derived from these two groups of patients. Patient characteristics and immunomodulating medication at the time of fistula surgery are shown in Table 1. None of the patients experienced fistula closure after an average follow-up of 17 months. Comparison of fistula fibroblast derived from CD and non-CD patients revealed similar expression patterns for fibroblast markers and most genes involved in immunoregulation (Figure 2A, Supplementary Figure 1B). However, CD fistula fibroblasts, in comparison to non-CD fistula fibroblasts, seemed to be characterized by higher COX-2 expression both on mRNA (Figure 2A) and protein (Figure 2B) level. COX-2 is important for the conversion of arachidonic acid towards prostaglandin E_2 (PGE_2), which suggest the presence of higher PGE_2 levels in the CD fistula microenvironment. Furthermore, also Twist-1 showed significantly higher expression in CD fistula fibroblasts, and a trend towards higher expression was found for its downstream target CXCL-12 (Figure 2A)[22], which is known to be involved in immune cell recruitment after epithelial damage[23]. Although no differences in

PD-L1 mRNA levels were observed, PD-L1 protein expression showed a trend towards lower levels of membrane bound PD-L1 in CD versus non-CD fistula fibroblast samples (Figure 2B). This trend was also observed with another PD-L1 antibody (Supplementary Figure 2).

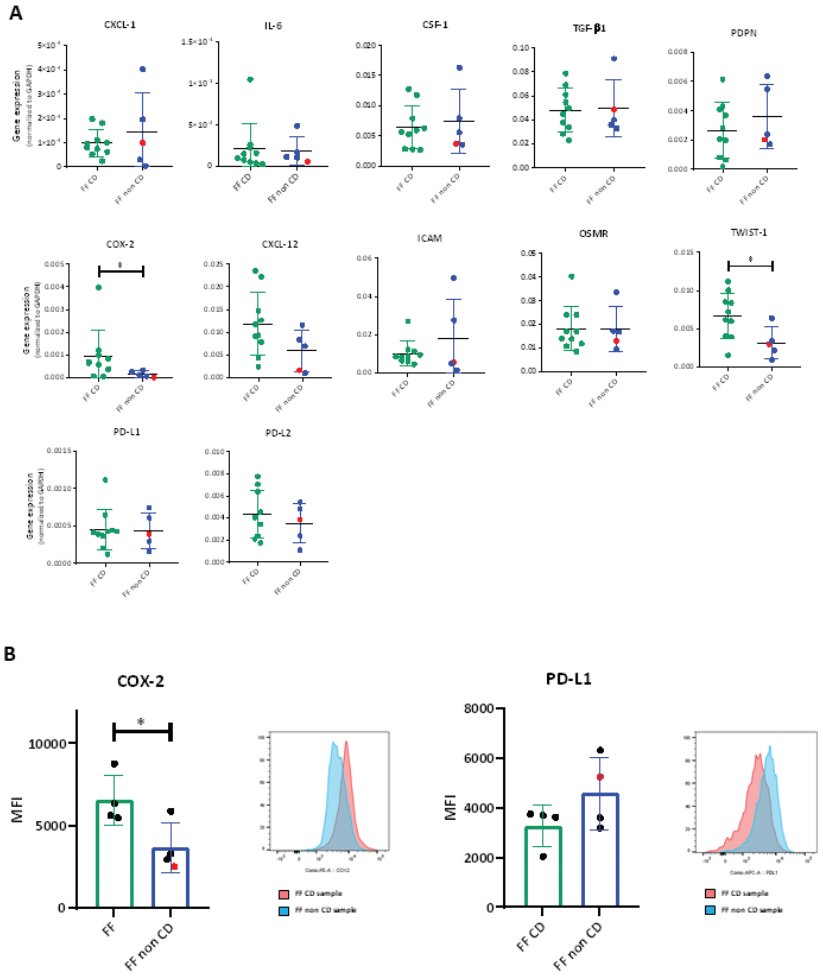

FIGURE 2. Higher expression of Twist-1 and COX-2 in fistula fibroblast isolated from Crohn's disease patients. A. Comparison of mRNA levels for immunoregulatory markers in fistula fibroblasts (FF) derived from Crohn's disease (CD, n=10) and non-CD (n=5) patients. B. Comparison of COX-2 and PD-L1 mean fluorescent intensity (MFI), corrected for auto-fluorescence (FF CD n=4, FF non CD n=4). Red data point represents fibroblasts from a patient that was diagnosed with CD later on.*p<0.05

Both T cell and myeloid cell abundant fistulas in CD patients

If fibroblasts play an instrumental role in the recruitment or activation status of immune cells in perianal fistulas, colocalization of fibroblasts and immune cells would be expected. Therefore the location of fibroblasts in relation to immune cells was investigated in the fistula resection specimens using 7 colour immunofluorescence. Fistula tracts from CD patients obtained after fistulectomy showed the presence of fibroblasts, defined by vimentin^{pos}CD45^{neg} cells with an elongated phenotype, around the fistula tract (Figure 3). Presence of α-SMA^{pos} fibroblasts along the fistula tract was also confirmed using immunohistochemistry (Supplementary Figure 3). Close to the fibroblasts, different subtypes of T cells, defined by CD3^{pos}CD8^{neg} (mainly CD4 T cells), CD3^{pos}CD8^{pos} (CD8 T cells) and CD3^{pos}FOXP3^{pos} (regulatory T cells) cells, were observed (Figure 3B). Also different types of myeloid cells, like CD11c^{pos} cells and CD68^{pos} macrophages were found around the fibroblasts (Figure 3C). In fistula scrapings, obtained during curettage and from which fistula fibroblasts were isolated, fibroblasts and different immune cells populations were also observed in close proximity (Supplementary Figure 4). CD3^{pos}CD8^{neg} T cells were the most common T cells in the fistula scraping (Supplementary Figure 5A), while 3.3% of all cells were found to be FOXP3^{pos} regulatory T cells. Of the myeloid cells, macrophages (CD68^{pos}) were the most dominant subset in CD fistulas (Supplementary Figure 5B). Although the importance of T cells in CD has been reported before[24], in the here investigated CD patients both T cell (CD3^{pos}) and myeloid (CD11c^{pos} or CD68^{pos}) populations could be abundant in fistulas (Figure 4).

Higher percentage of FOXP3^{pos} cells in fistulas with PD-L1^{low}PD-L2^{low}IL-6^{high} fibroblasts

Next, to test whether fistula fibroblasts could be involved in immune cell regulation in perianal fistulas, we correlated the percentage of different subsets of immune cells in CD fistula tissue with the mRNA expression of immunoregulatory markers on CD fistula fibroblasts isolated from the same tissue. Interestingly, we found that the number of FOXP3^{pos} regulatory T cells in CD perianal fistulas correlated with PD-L1, PD-L2 and IL-6 expression on fistula fibroblasts (Figure 5A), suggesting that PD-L1^{low}PD-L2^{low}IL-6^{high} fibroblasts in perianal fistulas are associated with a high number of regulatory T cells. In contrast, patients with PD-L1^{high}PD-L2^{high}IL-6^{low}fibroblasts isolated from the perianal tissue showed low amounts of regulatory T cells in the their perianal tissue. Also OSMR and CSF-1 were higher expressed in fibroblasts that were associated with high levels of regulatory T cells (Figure 5B). No other T cell subtypes correlated with fibroblast phenotypes while for the myeloid subtypes only weak correlations were identified (data not shown).

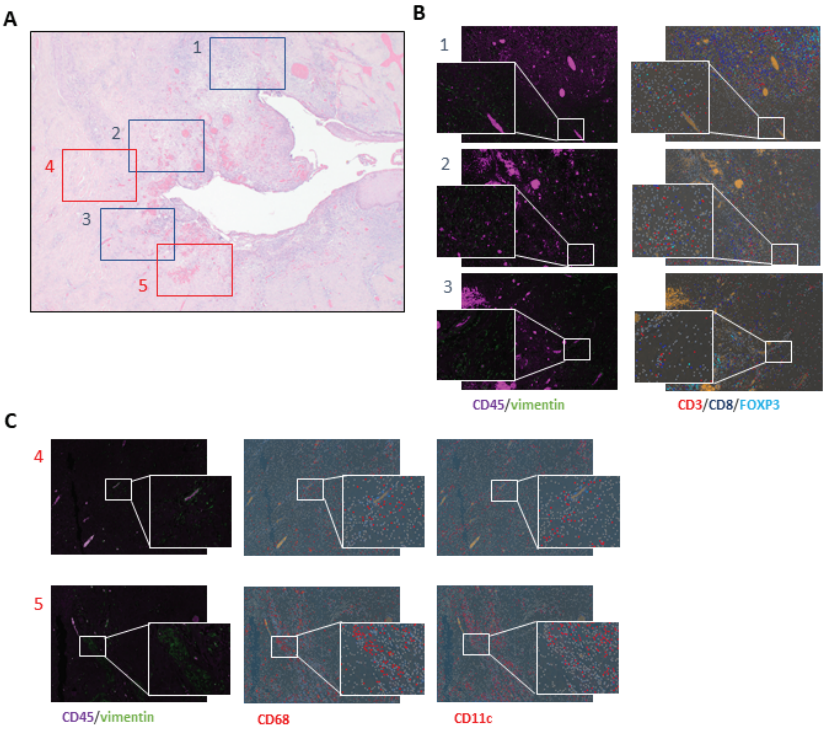

FIGURE 3. Different subsets of immune cells are located in close proximity to fistula fibroblasts. A. H&E staining of perianal fistula tissue obtained after fistulectomy from a patient with Crohn's disease. Magnification 20x. B. Identification of fibroblasts ($CD45^{neg}vimentin^{pos}$ cells) and different subsets of T cells in the perianal tissue. Magnification 50x. C. Identification of fibroblasts ($CD45^{neg}vimentin^{pos}$ cells) and different myeloid markers (CD68 and CD11c). Magnification 50x.

Fistula fibroblasts differently express immunoregulatory markers compared with MSCs

Finally, since allogeneic MSCs are an effective therapy for CD-associated perianal fistulas and known for their immunoregulatory capacities, we compared the immunomodulating profile of fistula fibroblasts from patients with CD with bone marrow-derived MSCs. Fistula fibroblasts expressed significantly lower mRNA levels of transforming growth factor-beta 1 (TGF-β1), CSF-1, CXCL-12, COX-2 and OSMR compared with MSCs (Figure 6A). While PDPN, associated with an inflammatory fibroblast phenotype, was significantly higher in fistula fibroblasts. Interestingly, we found a lower PD-L1 mRNA and surface protein expression in MSCs compared with fistula fibroblasts (Figure 6A and B), together with a lower mRNA expression of PD-L2 (Figure 6A). The differential gene expression of these checkpoint molecules could arise from the different inflammatory stimuli these cells encountered *in vivo*, but could also reveal their intrinsic immunomodulatory capacities.

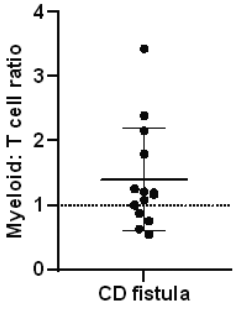

FIGURE 4. Both T cell and myeloid cell dominant perianal fistulas exist. Myeloid vs T cell ratio in perianal fistula scrapings from Crohn's disease patients based on 7 colour immunofluorescence

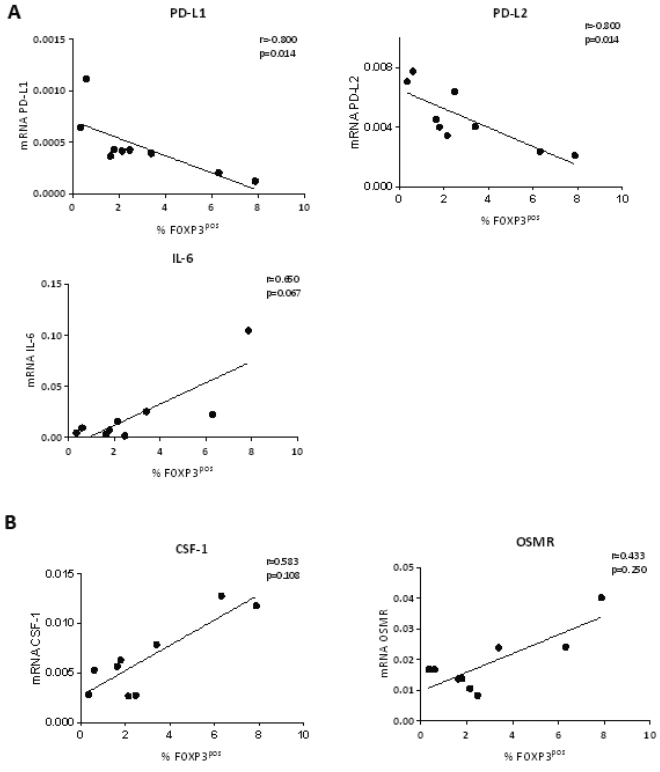

FIGURE 5. High expression of IL-6 and low expression of PD-L1 and PD-L2 by fistula fibroblasts is associated with a higher percentage of FOXP3^{pos} cells in Crohn's disease (CD) perianal fistulas. Correlation between the percentage of FOXP3^{pos} cells in the perianal fistula scraping of CD patients and the mRNA level of fistula fibroblasts derived from the same patients for (A) PD-L1, PD-L2, IL-6 (B) OSMR and CSF-1. Pearson correlation.

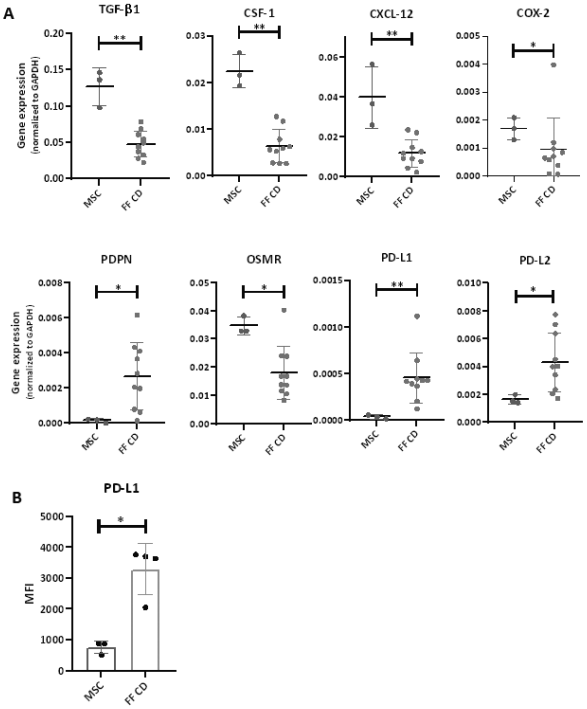

FIGURE 6. Fistula fibroblasts show lower levels of most immunoregulatory molecules compared with allogeneic MSC-therapy. A. Comparison of mRNA levels for immunoregulatory markers in bone marrow-derived mesenchymal stromal cells (MSC, n=3) and fistula fibroblasts from Crohn's disease patients (CD FF, n=10). B. Comparison of PD-L1 surface expression on MSCs (n=3) and CD FF (n=4). Mean fluorescent intensity (MFI) is corrected for auto fluorescence. *p<0.05, **p<0.01

DISCUSSION

In this study we characterized the immunoregulatory phenotype of perianal fistula fibroblasts derived from CD patients and compared them with skin, colon and fistula fibroblasts from non-CD patients. Furthermore, we related the phenotype of CD fistula fibroblasts to immune cell subtype compositions of CD perianal fistula patients. Unravelling the immunoregulatory function of fistula fibroblasts can provide insights in the pathogenesis of CD associated fistulas. Finally, since MSC-therapy is a new stromal therapy for perianal fistulas, we studied the differences in immunoregulatory function of CD fistula fibroblasts compared with MSCs.

Although fistula fibroblasts from CD and non-CD patients showed comparable expression patterns, CD fistula fibroblasts showed higher levels of COX-2 and lower levels of PD-L1

expression. In the CD fistula tissue we identified the location of fibroblasts and the close proximity of different subsets of immune cells around the fibroblasts. Interestingly, we found both T cell and myeloid abundant CD perianal fistulas and a $CD3^{pos}CD8^{neg}$ T cell dominance in the T cell phenotype distribution. This is in line with a previously published study, in which CD fistula tissues were analysed using CyTOF[25]. In addition we confirmed that a substantial part of the myeloid cells consisted of macrophages. Furthermore, the number of regulatory T cells in perianal fistulas was correlated with the expression of PD-L1, PD-L2, IL-6, OSMR and CSF-1 by CD fistula fibroblasts.

Previous research already revealed that the location of fibroblasts determines to a considerable extent their phenotype[26]. In accordance, we found the largest differences in expression patterns for immunoregulatory markers between the fibroblast isolated from the three different anatomical locations, skin, colon and fistula. Since previous work showed changes in the stromal intestinal component of patients suffering from ulcerative colitis[13], the direct comparison of CD fistula fibroblasts with matched rectum fibroblasts is important in future research. It would also be interesting to take along skin fibroblasts from these patients, since extra-intestinal manifestations of IBD in the skin are known. Despite overall comparable expression levels in fistula fibroblasts isolated from CD and non-CD patients, differences in COX-2, Twist-1/CXCL-12 and PD-L1 were observed.

COX-2 is able to metabolize arachidonic acid into PGE_2 and is typically induced by inflammation[27]. The prostaglandin PGE_2 has a dual role in inflammation, it is able to suppress effector functions of macrophages and neutrophils[28-30], but also has pro-inflammatory functions by promotion of T helper (Th) 1 cell differentiation and Th 17 cell proliferation[31]. Furthermore, high levels of PGE_2 can exacerbate the inflammatory process in murine models through dendritic cell-derived release of IL-23[32]. In accordance with our observation in CD fistula fibroblasts, COX-2 expression was also upregulated in a subset of fibroblast-like cells in UC patients[13] and in ileum-derived CD fibroblasts[33]. Interestingly, COX-2 ablation in intestinal myofibroblasts was associated with increased susceptibility to dextran sodium sulphate (DSS)-induced colitis, especially in the initial phase of the colitis[34]. The higher expression of COX-2 found in CD fistula fibroblasts could be the direct result of the underlying inflammatory disease. Whether this is a protective mechanism of fistula fibroblasts, like suggested in DSS-induced colitis, needs to be elucidated. *In vitro* cocultures between CD and non-CD fistula fibroblasts and different immune cells will be helpful in this regard. Since CD patients included in our study were almost exclusively treated with biological therapies, this could have affected the results. In RA it was reported that COX-2 expression in the synovial tissue of RA patients did not change after therapy with either etanercept or infliximab, two anti-TNF therapies[35]. However, there are reports

that showed that TNF-α, at least *in vitro*, is able to upregulate COX-2 in fibroblasts[36], which would suggest an ever larger difference in CD and non-CD fibroblasts COX-2 levels when taking a COX-2-reducing, anti-TNF-α effect into account.

Another interesting finding in our study is the altered expression of genes involved in the Twist-1/CXCL-12 pathway. Westendorp and colleagues showed upregulation of CXCL-12 by intestinal fibroblasts surrounding the epithelial crypts in response to loss of Indian Hedgehog in epithelial cells in DSS-induced colitis[23]. The upregulation of CXCL-12 led to recruitment of lymphocytes towards the damaged tissue. The higher expression of the Twist-1/CXCL-12 pathway in CD fistula fibroblasts will probably also lead to the attraction of more immune cells in CD fistulas compared with non-CD fistulas. In future research it would be interesting to compare the immune infiltrate in CD and non-CD fistulas, since the differences in fibroblast COX-2 and CXCL-12 expression could lead to different immune responses. Besides higher levels of COX-2 and Twist-1/CXCL-12, at trend towards lower levels of surface PD-L1 were observed on CD fistula fibroblasts. Similar results were found in CD intestinal fibroblasts compared with healthy and UC intestinal fibroblast[14]. These low PD-L1 levels in CD have been linked to a reduced capacity to suppress Th1 cell activity leading to the inflammatory milieu in CD[14]. Our data imply that due to lower levels of PD-L1 also CD fistula fibroblasts are less capable of inhibiting T cell responses. The observed difference between surface and mRNA PD-L1 levels is intriguing and might provide additional mechanistic implications. PD-L1 is a cell surface bound molecule, but it can also be released by cells. Extracellular PD-L1 includes exosomal PD-L1[37] and soluble PD-L1 through cleavage of membrane bound PD-L1 by MMP13[38]. Also the immunosuppressive function of exosomal[37] and soluble[39] PD-L1 has been implied before. Interestingly, activated MMP-13 was found specifically in the supernatant of CD fistula colonic lamina propria fibroblasts, where it was almost absent in the supernatant of non-fistula-derived intestinal fibroblasts[40]. Measurement of the amount and function of exosomal and soluble PD-L1 produced by CD and non-CD fistula fibroblasts would be helpful to understand the role of the different forms of PD-L1 in immunosuppression by fibroblasts.

Intriguingly, the CD fistula fibroblast PD-L1 and PD-L2 mRNA expression levels were found to correlate with the number of $FOXP3^{pos}$ cells in the perianal fistula tissue from which the CD fistula fibroblasts were isolated. Low PD-L1 and PD-L2, but high IL-6, OSMR and CSF-1 expression correlated with a high percentage of $FOXP3^{pos}$ regulatory T cells in perianal fistulas. A relation between fibroblasts and $FOXP3^{pos}$ immune cells has been suggested before. In different types of cancer it was found that a subset of cancer-associated fibroblasts (CAFs) is associated with a higher level of $FOXP3^{pos}$ cells and thereby a worse prognosis[41,42]. In one of these studies the role of CXCL-12 was elucidated in the

process of FOXP3 attraction by CAFs[41]. However our data do not suggest a correlation between FOXP3 cell numbers and the expression levels of CXCL-12, although an trend to upregulation of CXCL-12 was found in CD compared with non-CD fistula fibroblasts. The mechanism by which fistula fibroblasts could regulate the number of $FOXP3^{pos}$ in CD fistulas is not clear yet. Potential mechanisms would involve the increase in the number of FOXP3 cells in the perianal tissue by increasing their differentiation from $CD4^{pos}$ T cells, through for example production of TGF-β1, inducing their migration from the blood stream or inducing their proliferation. Our preliminary data show indeed that fistula fibroblasts are capable of inducing $FOXP3^{pos}$ cell differentiation out of $CD4^{pos}CD25^{neg}CD127^{pos}$ cells. New studies need to be performed to reveal if different CD fistula fibroblasts can differently affect regulatory T cell differentiation.

Allogeneic MSCs are a recently approved therapy for CD-associated perianal fistulas[5,6]. One of the MSC mechanisms to induce fistula closure could be normalization of the effects of inflammation-associated CD fistula fibroblasts. Therefore, resident CD fistula fibroblasts were compared with MSCs. PDPN, a marker that is in both rheumatoid arthritis[19,20] and IBD[13,21] associated with fibroblasts derived from inflamed tissue, was almost undetectable in MSCs. It has also been suggested that the use of PD-L1 competent MSCs could potentially be a mechanism for suppressing local inflammatory responses in CD fistulas, by counteracting the reduction of stromal PD-L1 in CD[14]. Our qPCR and flow cytometry data showed however lower PD-L1 and -2 expression by MSCs, which could be explained by the non-inflammatory milieu of the bone marrow where the MSCs are derived from. It would be interesting to see whether MSCs stimulated with pro-inflammatory cytokines are able to upregulate PD-L1 and -2 to a higher extent compared with CD fistula fibroblasts. Interestingly, MSCs mimicked the mRNA profile of the CD fistula fibroblasts associated with a high percentage of regulatory T cells in the fistula tissue, by a lower expression of PD-L1, PD-L2 and a higher expression of OSMR and CSF-1 compared with the average CD fistula fibroblast mRNA expression. The link between MSCs and regulatory T cells in the healing of perianal fistulas was previously shown by Ciccocioppo and collegeaus[43]. In that study it was found that fistula healing after autologous MSC injections was accompanied by increased levels of rectal $FOXP3^{pos}$ cells[43]. Furthermore, a recent paper about a new murine humanized model for perianal fistulas showed the absence of IL-10, a cytokine mainly produced by $FOXP3^{pos}$ cells, in the human transplant in which the fistulas develop[44]. We can speculate that $FOXP3^{pos}$ cells have an important role in the pathogenesis of perianal fistulas, while fibroblasts and MSCs have the ability to increase $FOXP3^{pos}$ cells and thereby diminish inflammation in order to facilitate fistula closure.

In conclusion, our data indicate a role for fistula-derived fibroblasts in the regulation of local immune responses in CD-associated perianal fistulas. Further research is needed to reveal the underlying mechanism and to optimize MSC-therapy in perianal fistulas.

Acknowledgement
We thank Dr. A. el Ghalbzouri and J.J. Out-Luiting, Dept. of Dermatology, LUMC for providing the dermal fibroblasts and Dr. A. Farina Sarasqueta, Dept. of Pathology, LUMC for identification of perianal fistula tracts in histological slides.

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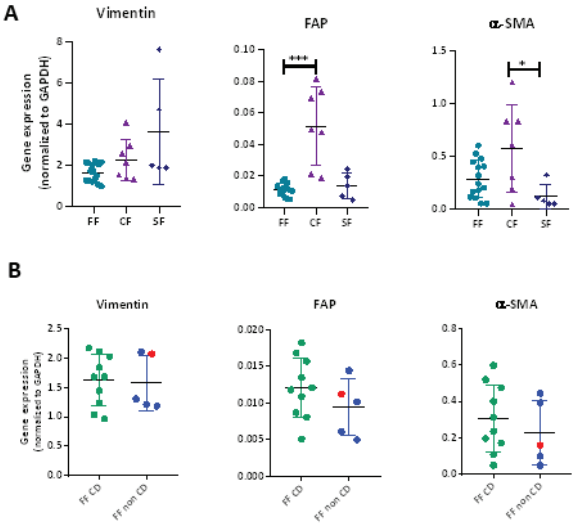
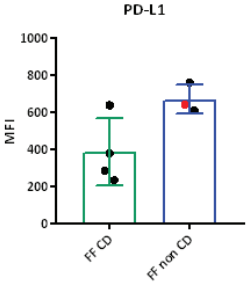
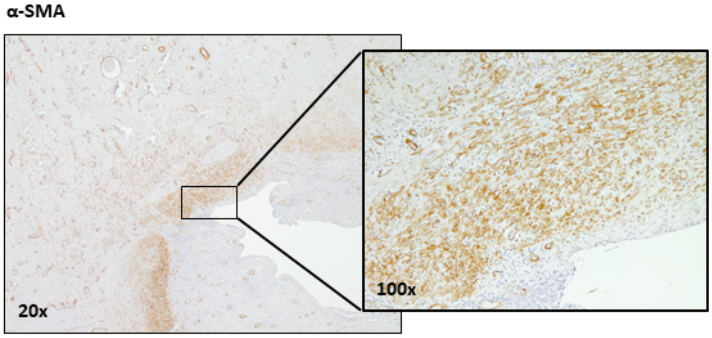
SUPPLEMENTARY FILES



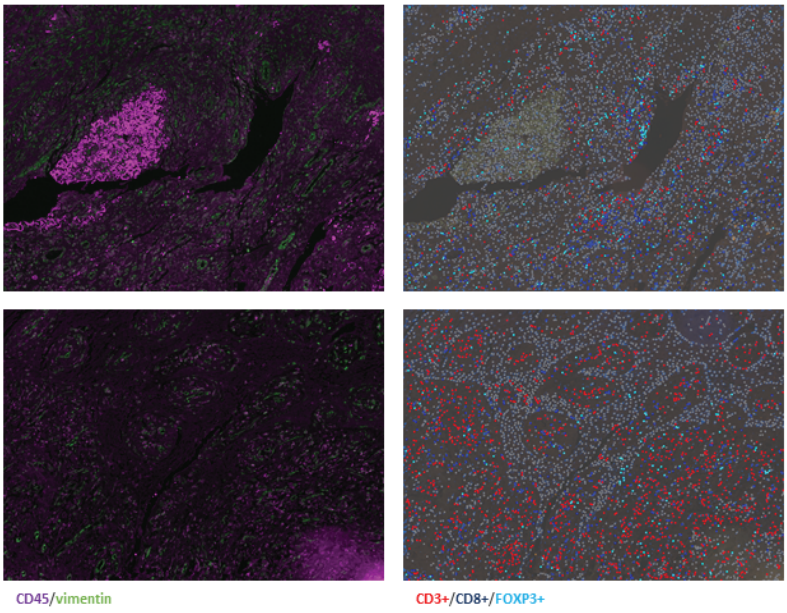
Supplementary FIGURE 1. A. Comparison of mRNA levels of fibroblasts markers vimentin, fibroblast activation protein (FAP) and alpha-smooth muscle actin (α-SMA) on fibroblasts derived from fistulas (FF, n=15), colon (CFs, n=6) and skin (SF, n=5). B. Comparison of fibroblast mRNA levels between FFs derived from Crohn's disease (FF CD, n=10) and non-CD (n=5). Red data point represents fibroblasts from a patient that was diagnosed with CD later on.*p<0.05, ***p<0.001



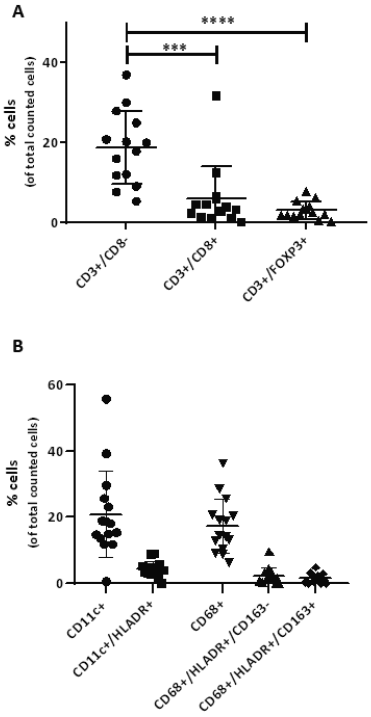
Supplementary FIGURE 2. Comparison of PD-L1 surface expression on Crohn's disease fistula fibroblasts (CD FF, n=4) and non-CD FF samples (n=3, other samples then shown in **FIGURE 2B**) using a BV421-conjugated antibody. Mean fluorescent intensity (MFI) is corrected for auto-fluorescence. Red data point represents fibroblasts from a patient that was diagnosed with CD later on.



Supplementary FIGURE 3. Alpha-smooth muscle actin (α-SMA) staining on perianal fistula tissue from Crohn's disease patients. Magnification 20x, 100x.



Supplementary FIGURE 4. Identification of fibroblasts and different T cell subsets in scraping material from perianal fistulas from Crohn's disease patients. Magnification 50x.



Supplementary FIGURE 5. A. Percentage of T cell subsets in Crohn's disease (CD) associated perianal fistula scrapings. B. Percentage of myeloid subsets in CD associated perianal fistula scrapings. Cells could be included in more than one myeloid subsets, when expressing different markers.**p<0.01, ****p<0.0001

Supplementary Table 1

Human primer sequences used for qPCR analysis.

Human Genes	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
GAPDH	GCACCGTCAAGGCTGAGAAC	ATGGTGGTGAAGACGCCAGT
α-SMA	CCGGGAGAAAATGACTCAAA	GAAGGAATAGCCACGCTCAG
Vimentin	TGTCCAAATCGATGTGGATGTTTC	TTGTACCATTCTTCTGCCTCCTG
FAP	ATCTATGACCTTAGCAATGGAGA ATTTGT	GTTTTGATAGACATATGCTAATT TACTCCCAAC
Keratin 20	CAGACACACGGTGAACTATGG	GATCAGCTTCCACTGTTAGACG
CD45	AACAGTGGAGAAAGGACACA	TGTGTCCAGAAAGGCAAAGC
CD31	GCTGACCCTTC TGCTCTGTT	TGAGAGGTGGTGCTGACATC
PDPN	CCAGGAACCAGCGAAGACC	GCGTGGACTGTGCTTTCTGA
TGF-β1	CCCTGGACACCAACTATTGC	CTTCCAGCCGAGGTCCTT
PD-L1	ATTCCGGCAGTGTACCTTGA	GGCATTCAAGGGTTCAAGCA
PD-L2	CTCACCTCTGGAGCCTATGG	AGTCTGGCAGCAAGAAGGAT
CXCL-1	CAGAAGGGAGGAGGAAGCTC	CTCTGCAGCTGTGTCTCTCT
ICAM	GGCCTCAGTCAGTGTGA	AACCCCATTCAGCGTCA
COX-2	TAAGTGCGATTGTACCCGGAC	TTTGTAGCCATAGTCAGCATTGT
TWIST-1	GTCCGCGTCCCACTAGC	TCCATTTTCTCCTTCTCTGGAA
IL-6	AGTGAGGAACAAGCCAGAGC	GTCAGGGGTGGTTATTGCAT
CXCL-12	CTCCACATCCTCCACGTTCT	GCTTTGGTCCTGAGAGTCCT
CSF-1	GTGAGATTCCCGTACCCCAA	GAGAGGAAGTTGCTGGGTCT
OSMR	GCAAGTCAAGGAAATGTCAGTG	CCCCAAGGCAGTGTCCGTCC

Supplementary Table 2

Antibodies included in the T cell panel for 7 colour immunofluorescence.

Target	Clone	Species	Isotype	Detection	Fluorochrome	Supplier
CD45	D9M8I	Rabbit	IgG	Direct	Alexa488	Cell signalling Technology
CD8	4B11	Mouse	IgG2b	Indirect	CF555	DAKO
CD3	D7A6E	Rabbit	IgG	Direct	Alexa594	Cell signalling Technology
FOXP3	236A/ E7	Mouse	IgG1	Indirect	CF633	Thermo fisher scientific
CD45RO	UCHL1	Mouse	IgG2a	Indirect	CF647	Cell signalling Technology
Vimentin	D21H3	Rabbit	IgG	Indirect	Alexa 680	Cell signalling Technology
DAPI						

Supplementary Table 3

Antibodies included in the myeloid panel for 7 colour immunofluorescence.

Target	Clone	Species	Isotype	Detection	Fluorochrome	Supplier
CD45	D9M8I	Rabbit	IgG	Direct	Alexa488	Cell signalling Technology
CD11c	EP1347Y	Rabbit	IgG	Direct	Alexa546	Abcam
HLA-DR	TAL 1B5	Mouse	IgG1	Direct	Alexa594	Thermo fisher scientific
CD163	10D6	Mouse	IgG1	Indirect	CF633	Thermo fisher scientific
CD68	D4B9C	Rabbit	IgG	Indirect	Alexa680	Cell signalling Technology
Vimentin	D21H3	Rabbit	IgG	Indirect	Alexa 647	Cell signalling Technology
DAPI						