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*J Clin Invest.* 2024. [https://doi.org/10.1172/JCI173835](https://doi.org/10.1172/JCI173835).

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Title: Intercellular interaction between FAP$^+$ fibroblasts and CD150$^+$ inflammatory monocytes mediates fibro-stenosis in Crohn’s disease.

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Abstract

Crohn's disease (CD) is marked by recurring intestinal inflammation and tissue injury, often resulting in fibro-stenosis and bowel obstruction, necessitating surgical intervention with high recurrence rates. To elucidate the mechanisms underlying fibro-stenosis in CD, we analysed the transcriptome of cells isolated from the transmural ileum of CD patients, including a trio of lesions from each patient: non-affected, inflamed, and stenotic ileum samples, and compared them with samples from non-CD patients. Our computational analysis revealed that pro-fibrotic signals from a subset of monocyte-derived cells expressing CD150 induced a disease-specific fibroblast population, resulting in chronic inflammation and tissue fibrosis. The transcription factor TWIST1 was identified as a key modulator of fibroblast activation and extracellular matrix (ECM) deposition. Genetic and pharmacological inhibition of TWIST1 prevents fibroblast activation, reducing ECM production and collagen deposition. Our findings suggest that the myeloid-stromal axis may offer a promising therapeutic target to prevent fibro-stenosis in CD.

Brief summary: Crosstalk between pro-fibrotic fibroblasts and inflammatory monocytes regulate fibro-stenosis in Crohn’s disease.
Introduction

Crohn’s disease (CD) is a chronic inflammatory condition affecting the gastrointestinal tract, characterised by transmural inflammation that often leads to complications such as strictures, fistulas, and abscesses. The disease presents with considerable variability in both its presentation, severity and progression. Although patients suffering from CD share common clinical characteristics, the natural disease course is rather heterogeneous, in which the disease behaviour can remain indolent or progress rapidly toward severe comorbidities (1–3). Strictures develop in approximately 30% to 40% of CD patients over time, leading to surgery in 70% of cases and recurrent postoperative complications in nearly 40% within a decade (4–6). Ileal stricturing results from excessive extracellular matrix (ECM) deposition and muscular hyperplasia due to repeated cycles of inflammation and tissue repair, leading to fibrosis and bowel obstruction, commonly referred to as fibro-stenosis (7, 8).

Current CD therapies aim to suppress inflammation through the administration of corticosteroids, immunosuppressive agents, and/or biologicals such as anti-tumour necrosis factor (TNF) antibodies, anti-integrins, anti-IL-12/23 agents or JAK inhibitors (9). While these therapies lead to symptomatic disease remission in approximately 40% of patients, recurrent flares still cause cumulative tissue damage and remodelling of the gut wall, often leading to fibro-stenosis (10). As no anti-fibrotic drugs are currently available for CD patients, the incidence of fibro-stenosis, and consequently that of surgical resection remain very high (11). Most of the existing research on mucosal inflammation and cellular heterogeneity in IBD has relied on biopsies procured through ileo-colonoscopy which do not include the deeper layers of the gut (12–17). Consequently, these studies have been limited by their sampling methodology, failing to address transmural inflammation and fully elucidate the underlying causes of bowel remodelling and obstruction in the ileum and colon of CD patients.

Our study used single-cell RNA sequencing (scRNA-seq) to analyse full-thickness transmural terminal ileum samples from fibro-stenotic CD patients undergoing ileocecal resection. We profiled three stages of lesions: proximal non-affected ileum, inflamed ileum with ulceration, and stenotic ileum, aiming to characterise disease progression. Our study extends the knowledge on cell heterogeneity in the transmural ileum of fibro-stenotic CD patients and highlights complex intercellular interactions between immune cells and fibroblasts, driving the process of tissue remodelling and the establishment of fibrosis in CD. We found that during inflammation and stenosis, pathogenic CD150 Inflammatory monocytes promote tissue remodelling and fibrosis by inducing the differentiation of FAP fibroblasts leading to excessive ECM deposition via the transcriptional regulator TWIST1. Overall, our
findings suggest that targeting the myeloid-mesenchymal axis during inflammation could be an effective strategy to prevent fibro-stenosis in patients with CD.
Results

Uncover the heterogeneity of fibroblasts in fibrostenotic CD

Despite CD being a transmural disease, the use of endoscopic sampling has been a persistent limitation in previous studies (15–18). Therefore, we aimed to investigate the cellular landscape and intercellular interactions in the transmural ileum of CD patients to identify potential therapeutic targets. First, we classified transmural biopsies from resected ileal tissue within each patient based on macroscopic features. Then, we determined inflammatory and fibrotic activity in the proximal healthy margin, inflamed, and stenotic ileum of CD patients, and control non-CD healthy ileum from colorectal cancer (CRC) patients using hematoxylin and eosin (H&E) and Masson’s trichrome staining (Figure S1A). To evaluate our sample classification microscopically, we modified a histopathologic scoring system based on previous studies to assess inflammation and fibrostenosis in CD ileum (Supplemental data) (19, 20). We observed moderate to severe degrees of inflammation and fibrosis, including fissuring ulceration and abscess in inflamed ileum and stenotic ileum (Figure S1B, left and middle). Although similar fibrosis features were found in both inflamed and stenotic ileum, stenotic ileum had a significantly higher level of overall collagen deposition (Figure S1A, shown in blue) compared to inflamed ileum ($p<0.05$) (Figure S1B, right). Altogether, these findings confirmed that our method of classification was reliable to study the progression of tissue through inflammation to fibrostenosis.

To uncover the interplay between immune and stromal cells during fibrosis in CD, we profiled the transcriptome of 169,205 cells from transmural terminal ileum of CD patients ($n = 10$, a trio of lesions from each; proximal as unaffected, inflamed, and stenotic ileum) and CRC patients ($n = 5$; control ileum) using scRNA-seq (Figure 1A). A similar number of cells were profiled from each of the three CD lesions; Proximal = 33.78%, Inflamed = 32.48%, Stenotic = 33.77% (Figure S1C to S1E). Unsupervised clustering followed by annotation of the integrated gene expression data identified several clusters which were classified into 8 compartments, including T cells and ILCs (CD3D, CD3E, CD4, CD8A, NKG7, GNLY, KLRB1), B cells (CD19, CD79A, MS4A1), plasma cells (IGKC, MZB1, JCHAIN), myeloid cells (CD68, LYZ), epithelial cells (EPCAM), endothelial cells (VWF, PECAM1), enteric glial cells (PLP1, S100B) and mesenchymal cells (LUM, PDGFRA, DCN) (Figure 1B to 1D). To identify the major ECM-producing cell compartments, we compared expression of ECM core genes between each cell compartment. This comparison confirmed the mesenchymal compartment as the major source of ECM proteins during inflammation and stenosis (Figure S1F). Thus, we
proceeded with a deeper characterisation of the mesenchymal compartment to address their heterogeneity in different disease states.

Re-clustering of the mesenchymal compartment identified two clusters of mural cells: pericytes (RGS5) and contractile pericytes (ADIRF), one cluster of smooth muscle cells (MYOC, MYH11, ACTG2) and seven clusters of fibroblasts: myofibroblasts (SOX6, ACTA2), ADAMDEC1 fibroblasts (ADAMDEC1, CCL11), ABL2 fibroblasts (ABL2, PLIN2, CLDN1), GREM1−CD34+ fibroblasts (MFAP5, CD55), GREM1+CD34+ fibroblasts (GREM1, C3, C7), FAP fibroblasts (FAP, CD82, TWIST1, POSTN) and a cluster of proliferating FAP fibroblasts (MKI67, TOP2A, CENPF, FAP, CD82, TWIST1, POSTN) (Figure 2A and 2B). FAP fibroblasts and ABL2 fibroblasts were found to be unique to inflamed and stenotic ileum compared to control and proximal ileum (Figure 2C, and S2A). To identify the stromal cluster responsible for pathological ECM deposition, we developed a collagen module score utilising the core matrisome collagen gene signature from MatrisomeDB. This approach identified FAP fibroblasts as the primary ECM-producing cells among all mesenchymal subsets (Figure 2D). This was confirmed by Gene ontology (GO) enrichment analysis, which showed that FAP fibroblasts were significantly enriched in GO processes associated with extracellular matrix organisation (Figure 2E).

Next, to confirm the presence of FAP fibroblasts in inflamed and stenotic samples, we performed flow cytometry on transmural ileum of CD patients. After gating out leukocytes (CD45), endothelial cells (CD31) and epithelial cells (CD326), we used CD90 and podoplanin (PDPN) to identify fibroblasts (Figure 2F and S2B). Using this approach, we confirmed that FAP fibroblasts (FAP+CD34−) were unique to the inflamed and stenotic ileum and absent in the control and proximal ileum (Figure 2G, S2 and S2D). In line, FAP enzymatic activity was significantly elevated in the inflamed and stenotic CD ileum compared to unaffected margins (Figure S2E) (21). In addition, we investigated the spatial distribution of the fibroblast subsets using multiplexed immunofluorescence staining (PDPN, CD34, FAP and ADAMDEC1). Importantly, we observed high expression of FAP in the submucosa and deeper layers of CD ileum where excess ECM deposition is observed. On the other hand, ADAMDEC1 fibroblasts were predominantly present in the healthy mucosa (Figure 2H and S2F).

The FAP fibroblasts not only express higher ECM genes but are also characterised by an activated phenotype with overexpression of CD90, PDPN, and FAP proteins, pro-fibrotic autocrine loop molecules (IL6, IL11, TGFBI), neutrophils chemoattracting chemokines (CXCL1, CXCL5, CXCL6) and monocyte chemoattracting chemokines (CCL2, CCL5, CCL7) (Figure S2G). Moreover, single sample gene set enrichment analysis (ssGSEA) confirmed that
FAP fibroblasts are enriched for processes such as inflammatory response and leukocyte chemotaxis (Figure S2H) (22). Overall, these results suggested a key role for FAP fibroblasts in perpetual recruitment and potential activation of myeloid cells into the tissue during chronic inflammation.

Eventually, we compared our data to previously published IBD scRNA-seq data sets of mucosal biopsies from CD and ulcerative colitis (UC) patients using Seurat integration and label transferring (Figure S8A) (15–17). Cross-dataset cell type prediction score showed low to moderate similarity of FAP fibroblasts across these three datasets (Figure S2I). To further validate the presence of FAP fibroblasts across both CD and UC in colon, we analysed transmural samples from healthy CRC colon (unaffected), healthy CD colon (non-inflamed), inflamed/stenotic CD colon (granulating ulcer and thickened bowel wall) and UC colon (inflamed) using flow cytometry (Figure S2J). Of note, high proportion of FAP fibroblasts was predominantly observed in the inflamed/stenotic colons of patients with CD rather than in those with inflamed colons due to UC (Figure S2K). These findings imply that FAP fibroblasts represent a distinct pathogenic subset that emerges during chronic inflammation and fibrosis, characterised by excessive ECM deposition in CD.

**Transcriptional regulation and pro-inflammatory properties of FAP fibroblasts**

Given the unique involvement of FAP fibroblasts in ECM deposition, we set out to investigate the upstream transcriptional regulators driving their transcriptional pathological state. Using single-cell regulatory network inference and clustering (SCENIC) analysis, TWIST1, SIX2, PRRX2, MSX2, and HIF1α were identified as the top regulons (a network of a transcription factor and corresponding targets) active in FAP fibroblasts (Figure 2I) (23). When performing gene enrichment analysis of top regulons, TWIST1 target genes stood out with significant enrichment of several GO terms associated with excessive ECM deposition such as collagen containing extracellular matrix as well as the gene set containing the core constituents of ECM (Figure 2J). Using multiplex immunofluorescent staining (CD34, FAP and TWIST1), we confirmed that in the inflamed ileum of CD patients, TWIST1 is expressed in the nuclei of FAP expressing fibroblasts (Figure 2K).

Consistent with the established role of TWIST1 in epithelial-mesenchymal transition (EMT), ssGSEA demonstrated a significant enrichment of EMT gene signatures in FAP fibroblasts (Figure S2L) (24, 25). In addition, our ssGSEA analysis revealed a significant enrichment of terms associated with cellular senescence, including KEGG’s cellular senescence and Reactome’s senescence-associated secretory phenotype in FAP fibroblasts (Figure S2M) and enrichment of genes associated with the senescence-associated secretory
phenotype (SASP) (Figure S2N) (26–28). Overall, these results suggest that TWIST1 drives a pro-fibrotic phenotype in FAP fibroblasts and activates cellular senescence pathways.

**GREM1-CD34+ fibroblasts as potential precursors of FAP fibroblasts**

To gain insight into how FAP fibroblasts appear during chronic inflammation, we exploited the gene expression data to construct a differentiation trajectory for FAP fibroblasts. After identifying three fibroblast clusters topologically connected to FAP fibroblasts using partition-based graph abstraction (PAGA), we applied monocle3 on these four clusters (Figure S3A) (29, 30). Trajectory analysis revealed that GREM1-CD34+ fibroblasts differentiated into FAP fibroblasts during inflammation (Figure 3A). Along the differentiation trajectory towards FAP fibroblasts, fibroblasts exhibited a loss of CD34 expression and an acquisition of TWIST1, FAP, and COL1A1 expression (Figure 3B and S3B). Multiplex immunofluorescent staining also showed the expression of CD34 in FAP fibroblasts together with TWIST1, indicating the differentiation status (Figure 2K). We hypothesised that the markedly distinct inflammatory microenvironment in CD may induce FAP fibroblasts differentiation from GREM1-CD34+ fibroblasts. To identify the specific factors driving this differentiation, we used CellPhoneDB, a computational tool that estimates intercellular interactions between cell types and fibroblasts based on a curated database of ligand-receptor interactions (31). Using this approach, we observed an intense crosstalk within the mesenchymal compartment and between mesenchymal, endothelial, and myeloid cells. Notably, myeloid cells were responsible for most of the predicted interactions with the disease associated mesenchymal cells in general and particularly with FAP fibroblasts as lesions progressed from inflammation to stenosis (Figure 3C and S3C).

**Stromal-immune cell interaction induces FAP fibroblast differentiation and activation**

To better understand the signalling cues involved in the transcriptional shift to FAP fibroblasts in CD ileum, we employed a recently described computational tool called NicheNet, which enables the prioritisation of ligands responsible for inducing alterations in a specific gene set (32). Based on the differentially regulated genes between GREM1-CD34+ fibroblasts and FAP fibroblasts, NicheNet predicted IL1A, IL1B, IL6, CXCL12, IFNG, LIF, OSM, TNF, and TGFB1 as the top ligands associated with the transcriptional reprogramming of GREM1-CD34+ fibroblasts towards FAP fibroblasts (Figure 3D and 3E). Next, we compared the gene expression levels of these ligands between cell compartments, and we found that the myeloid compartment exhibited the highest gene expression levels of the highest ranked ligands including IL1A, IL1B, TNF, TGFB1, and OSM. In contrast, IFNG expression was predominantly found in T cells/ILCs, while IL6 and CXCL12 were mainly expressed by
mesenchymal and endothelial cells (Figure 3F, and S3C). The activation of these signalling pathways was also confirmed by ssGSEA analysis (Figure S3D). Taken together, ligand–target analysis suggested myeloid cells as the major source of pro-fibrotic ligands, leading to FAP fibroblast cell state differentiation and activation.

**scRNA-seq analysis reveals presence of a pro-fibrotic monocyte subset specific to inflammation and stenosis**

To identify the pro-fibrotic myeloid cell subsets involved in driving the activation of FAP fibroblasts, we performed unsupervised re-clustering of the myeloid compartment (LYZ, CTSG, CD68, CSF1R) and we identified a total of fourteen clusters in the CD ileum and in the healthy CRC control ileum (Figure 4A, 4B and S4A). Two clusters of mast cells (KIT, CTSG) were identified in all segments, with minimal differences in gene expression observed between the two clusters (data not shown). Four distinct dendritic cell (DC) clusters were also identified including cDC1 (CCND1, CLEC9A), cDC2 (LTB), lymphoid DCs (CCR7, LAMP3) which were enriched in inflammation and plasmacytoid DCs (pDCs) which initially clustered along with B cells (GZMB, TCF4, IRF7) (Figure 4C). The macrophage and monocyte clusters exhibited remarkable variations across the different tissue conditions. Two macrophage clusters, distinguished by LYVE1 or IGF1 expression, were mainly present in non-inflamed ileum as opposed to inflamed and stenotic ileum. These clusters also displayed other typical mature resident macrophage markers such as C1QA and C1QB but lacked CCR2 expression. Three clusters, namely CCR2 monocytes, MMP9 macrophages, and neutrophils were predominant in inflamed and stenotic ileum (Figure S4A). We also observed an inflammatory monocyte cluster (Inflammatory monocytes), which could be identified by the expression of SLAMF1 (CD150) and common monocyte markers, including CD300E, FCN1 and CCR2. Most strikingly, this cell subset was exclusively present during inflamed and stenotic ileum (Figure 4B and S4A). To identify the subset(s) of myeloid cells inducing FAP fibroblast differentiation during chronic inflammation, we evaluated the inflammation specific appearance and secretion of myeloid-derived pro-fibrotic ligands. Most importantly, Inflammatory monocytes had the highest expression of the pro-fibrotic ligands (IL1A, IL1B, TNF, and TGFB1) predicted by the NicheNet analysis (Figure 4D).

**Inflammatory monocytes as a hyperinflammatory activation state of monocytes**

Given the potential role of inflammatory monocytes in fibrosis, we compared the transcriptional profile of the four myeloid clusters (Neutrophils, CCR2 monocytes, Inflammatory monocytes and MMP9 macrophages) predominantly present in inflamed and stenotic ileum. The functional gene enrichment analysis employing GO terms revealed a
significant enrichment in inflammation and fibrosis associated GO terms such as (regulation of) interleukin-1 production, tissue remodelling, and wound healing, indicating their high inflammatory and pro-fibrotic profile compared to the other three myeloid subsets (Figure 4E). Similarly, Reactome pathway analysis revealed enrichment of pathways associated with extracellular matrix organisation, chemokine receptors bind chemokines, and interleukin-1 family signalling in Inflammatory monocytes. This underscores the inflammatory and pro-fibrotic characteristics of these cells (Figure S4B). Furthermore, we quantified the intercellular interactions between Inflammatory monocytes and other cell types using CellPhoneDB. Our analysis revealed that FAP fibroblasts exhibited a higher number of ligand-receptor interactions with Inflammatory monocytes in comparison to other immune cell subsets (Figure 4F and S4C). Of note, this analysis indicated a potential role for FAP fibroblasts in the recruitment of monocytes via CCL2, CCL5 and CCL7 (Figure S2G and 4F).

Further, to distinguish the main transcription factors driving the pro-fibrotic phenotype of Inflammatory monocytes, we performed SCENIC analysis. ELK3, MSC, STAT4, HIF1A, and IRF7 were found to be among the top significantly enriched regulons in Inflammatory monocytes (Figure S4D). To understand the monocyte dynamics across disease states, we performed trajectory analysis of clusters connected with monocytes as identified by PAGA (data not shown). Nevertheless, we removed pDCs from the trajectory analysis as they have been reported to be not of monocyte origin (33). Trajectory analysis showed two branches of monocyte differentiation through pseudotime (Figure S4E and S4F). One branch of CCR2 monocytes gave rise to IGF1 macrophages, with a subsidiary branch giving rise to cDC2. This branch was predominant in control and proximal ileum. The other branch depicted CCR2 monocyte differentiating into Inflammatory monocytes and further to MMP9 macrophages was predominant in inflamed and stenotic ileum (Figure 4B, S4E and S4F).

**Inflammatory monocytes are predominantly present in inflamed and stenotic ileum**

To definitely confirm the association of the Inflammatory monocytes with inflammation and stenosis in transmural CD ileum, we performed flow cytometry and immunofluorescence staining, using CD150 as a reliable marker to identify Inflammatory monocytes within the myeloid compartment. In the diseased ileum, CD14<sup>dim</sup> cells (as Neutrophils), CD150- monocytes (as CCR2 monocytes, CCR2<sup>+</sup> and CD150<sup>+</sup>) and CD150<sup>+</sup> monocytes (as Inflammatory monocytes, CCR2<sup>+</sup> and CD150<sup>-</sup>) were significantly increased, compared to the control and proximal ileum ($p < 0.005$) (Figure 4G, 4H, S4G and S4H). Similarly, with multiplex immunofluorescent staining, we observed an increase of CD68<sup>-</sup>CD150<sup>-</sup> cells (as Inflammatory monocytes) in the deeper muscularis layer of diseased
CD ileum, which co-localised with FAP expression (Figure 4I and S4I). We confirmed CD150 as a surface marker for Inflammatory monocytes within the myeloid compartment, facilitating their isolation for in vitro experiments. Taken together, Inflammatory monocytes represent a pathogenic subset of myeloid cells secreting excess of inflammatory and pro-fibrotic mediators and promoting differentiation of FAP fibroblasts and tissue remodelling.

To explore the potential of CD150 as a biomarker for CD in blood, we conducted flow cytometry analysis to assess the abundance of CD150+ monocytes in peripheral blood mononuclear cells (PBMC). Notably, in patient with CD undergoing surgery for fibro-stenosis we found a significant increase in CD150+ circulating monocytes (Figure S4M) but we did not observe an elevation in CD150+ monocyte levels in PBMC.

**CD150 Inflammatory monocytes represent a specific feature of CD**

To contextualise our findings within the broader IBD research landscape, we reanalysed and compared our data to three IBD scRNA-seq datasets derived from mucosal biopsies of both CD and UC patients using Seurat integration and label transferring (Figure S8B) (15–17). Similar myeloid clusters were identified in all three IBD datasets with low similarity score in UC patients and moderate similarity score in CD samples (Figure S4J). To further confirm the presence of Inflammatory monocytes across UC and CD, we performed flow cytometry analysis by using transmural colon from CD and UC patients (Figure S4K). Notably, the presence of CD150+ monocyte population was unique to CD in the colon but absent during UC (Figure S4K). Together with our findings in CD ileum, these results suggest that CD150 Inflammatory monocytes are a unique subset with highly inflammatory and pro-fibrotic properties, specific to CD patients in both inflamed ileum and colon.

**Highly multiplex spatial transcriptomics revealed co-localisation of FAP fibroblasts and Inflammatory monocytes in the inflamed and stenotic ileum**

In order to define the spatial distribution of cell clusters and delineate the fibroblast-myeloid cell niche in the CD affected ileum, we conducted a multiplex spatial transcriptomic analysis on 99 genes (Figure S5A) selected based on our scRNA-seq data. We examined transmural terminal ileum samples from 3 CD patients, including proximal non-affected ileum, inflamed ileum with ulceration, and stenotic lesions. Upon cell segmentation analysis and unsupervised clustering, followed by annotation, we could observe the specific spatial locations of various cell types we identified in our scRNA-seq. These included mesenchymal cells (PDGFRα, PDGFRβ), myeloid cells (CCR2, CD68, C1QA), T cells (CD3E, CD4, CD8α), B cells (CD19, MZB1), epithelial cells (ELF3), endothelial cells (ACKR1, CLDN5, PECAM1, VWF), neurons (UCHL1), enteric glial cells (PLP1), and smooth muscle cells (MYOCD).
(Figure 5A and S5A). The spatial map revealed the specific tissue locations of the disease-associated cell clusters (Figure 5B), confirming an increased proportion of FAP fibroblasts and inflammatory monocytes in the inflamed and stenotic ileum of CD patients (Figure 5C). Notably, these cells were absent in the healthy ileum of the same patients. Spatial localisation of FAP and PDGFRA (FAP fibroblasts), and CCR2, CD68, SLAMF1 (Inflammatory monocytes) were primarily found in the submucosa of inflamed and stenotic ileum in CD patients (Figure 5D). The spatial map further underscored the close co-localisation of FAP fibroblasts and inflammatory monocytes (Figure 5E). Collectively, these findings point towards intensive fibroblast-myeloid cell interactions during inflammation and fibro-stenosis in the CD ileum.

**Inflammatory monocyte derived pro-fibrotic cues activate FAP fibroblasts via TWIST1**

To validate the expression and functional relevance of pro-fibrotic ligands in Inflammatory monocytes as observed in our scRNA-seq data, we quantified the gene expression of pro-fibrotic ligands across different FACS-sorted myeloid subsets: HLA-DR$^+$ cells, CD14$^{dim}$ neutrophils (CD14$^{low}$, HLA-DR$^{neg}$), CD150- monocytes and CD150$^+$ monocytes (Figure S6A). In line with our scRNA-seq data, Inflammatory monocytes (CD150$^+$ monocytes) showed the highest gene expression of IL1A, IL1B, TNF, TGFB1, and SLAMF1 but not OSM, which was expressed mainly by the CD14$^{dim}$ neutrophils as previously reported (Figure 6A) (18). Secretion of high levels of IL-1α, IL-1β, TNFα and TGF-β1 from the CD150$^+$ monocytes was also confirmed at the protein level (Figure S6B).

To confirm that Inflammatory monocyte-derived ligands modulate fibroblast activation as seen in our scRNA-seq data, we sorted CD14$^{dim}$ neutrophils, CD150$^-$ monocytes and CD150$^+$ monocytes from 5 CD patients undergoing surgery for ileal stenosis and collected cell supernatant after 16 hours of culture. Subsequently, the supernatants from FACS-sorted myeloid cells were used to stimulate CCD-18Co fibroblasts (CRL-1459, ATCC). In line with our computational prediction, the supernatants of Inflammatory monocytes (CD150$^+$ monocytes) isolated from inflamed ileum induced an activated fibroblast state with increase expression of TWIST1, FAP and type III collagen compared to the supernatants of other FACS-sorted myeloid subsets (Figure 6B, 6C and S6C). To further confirm that ECM deposition induced by Inflammatory monocyte-derived ligands modulate fibroblasts, we co-cultured CCD18-Co fibroblasts and FACS-sorted myeloid cell supernatants with ascorbic acid for three weeks and performed immunofluorescent staining without permeabilisation (Figure S6D). Cell supernatants of Inflammatory monocytes (CD150$^+$ monocytes) promoted significantly higher
FAP expression and higher extracellular deposition of type I collagen, and type III collagen by fibroblasts, compared to the control and CD14dim neutrophils (Figure 6D, 6E and S6E).

Next, to assess the potency of different cytokine combinations in inducing fibroblast activation, we monitored response of primary ileal fibroblasts isolated from transmural ileum, sourced from healthy regions of CRC patients to different cytokine combinations using high-content imaging. The combination of IL-1α, IL-1β, TNFα, TGFβ1, OSM and IFNγ (henceforth referred to as pro-fibrotic cues) resulted in the highest expression of TWIST1, FAP and type III collagen among different cytokine combinations after 48 hours of stimulation (Figure S6F). Computational analysis had indicated a specific transcriptional regulatory role for TWIST1 in the excess ECM deposition exhibited by FAP fibroblasts. Consequently, we explored whether TWIST1 knockdown, achieved through lentivirus transduction, could influence pro-fibrotic cues-induced fibroblast activation. Of note we found that knockdown of TWIST1 expression significantly mitigated fibroblast activation and reduce collagen production in CCD-18Co fibroblasts (Figure 6F, 6G and 6H).

To further validate if pharmacological inhibition of TWIST1 could affect the profibrotic nature of activated fibroblasts, we used Harmine, a recently identified TWIST1 inhibitor (34, 35). Following treatment with Harmine, primary ileal fibroblasts stimulated by pro-fibrotic cues exhibited a notable decrease in the expression levels of FAP, TWIST1, and type III collagen compared to those treated with the vehicle (Figure 6I and 6J). Notably, Harmine treatment also significantly reduced ECM deposition in ileal fibroblasts stimulated by pro-fibrotic cues, with a marked decrease in the extracellular deposition of fibronectin, and types I, III, and IV collagen, compared to vehicle-treated cells (Figure 6K and 6L, S6H and S6I). Furthermore, utilising a 3D in vitro model based on human iPSC-derived intestinal organoids (IOs) that comprise both epithelial and stromal cells, we demonstrated that pro-fibrotic cues led to a significant increase in the expression of FAP and PDPN, which was substantially reduced in the presence of Harmine (Figure 6M and 6N). Altogether, our results provide solid evidence that Inflammatory monocyte-derived pro-fibrotic cues modulate FAP fibroblast activation and ECM secretion in a TWIST1-dependent manner.

Genetic deletion or pharmacological inhibition of TWIST1 attenuates chronic colitis-induced intestinal fibrosis

To verify the role of TWIST1 as a major driver of fibroblast activation and intestinal fibrosis in vivo, we generated fibroblast-specific Twist1-deficient mice (Twist1Δ/ΔCol1a2) by breeding Twist1-floxed mice (Twist1fl/fl) with Col1a2-CreER mice. Then we subjected Twist1Δ/ΔCol1a2 mice and their littermates to chronic DSS colitis upon tamoxifen administration.
Of note, Twist1Δ/ΔCol1a2 mice showed a significant improvement in the overall disease activity index (DAI) when compared with their littermates (Figure S7A to S7D). Additionally, Twist1Δ/ΔCol1a2 mice exhibited diminished collagen accumulation in the colon and reduced colon tissue size compared to their littermates (Figure S7E to S7G). In line, Twist1Δ/ΔCol1a2 mice post DSS-induced colitis also revealed significant lower tissue damage as showed by the Mouse Colitis Histology Index (CHI) (Figure S7H to S7L). TWIST1 deletion in vivo was also associated with a reduction in FAP expression in fibroblasts isolated from the colon of Twist1Δ/ΔCol1a2 mice compared to their littermates after DSS-induced colitis (Figure S7M and S7N). In line, Cre recombinase deletion of TWIST1 in primary mouse colonic fibroblasts isolated from Twist1β/β mice, resulted in with lower induction of FAP and type III collagen expression in response to pro-fibrotic cues (Figure S7O and S7P).

Finally, pharmacological blockade of TWIST1 with Harmine led to diminished collagen deposition and reduced TWIST1 and FAP expression in the colon during chronic DSS colitis (p < 0.05), with no significant effect on the CHI (Figure S7Q to S7T) or colitis severity (Figure S7U to S7X). Our results confirmed that both genetic and pharmacological inhibition of TWIST1 mitigates gut fibrosis in chronic intestinal inflammation.
Discussion

Lack of transmural sampling has been a long-standing limitation in CD studies despite the disease occurring in all layers of the gut wall (36). So far, scRNA-seq studies on CD have mostly focused on characterising mucosal inflammation using endoscopic biopsies and hence do not appreciate the transmural heterogeneity in CD ileum (15–17). In our study, we utilised scRNA-seq to analyse transmural ileal biopsies from each fibrostenotic CD patient, covering a trio of lesions including proximal non-affected ileum, inflamed ileum with ulceration, and stenotic ileum. This approach allowed to fully characterise the different stages of disease progression up to fibro-stenosis within the deeper layers of the gut, where significant tissue remodelling is commonly observed in affected patients. Our data revealed a previously unknown heterogeneity in the transmural CD ileum, with the fibroblast and myeloid compartments showing remarkable differences across lesions. Deeper analysis of mesenchymal compartment revealed FAP fibroblasts as the key pathogenic cell subset uniquely present in inflamed and stenotic CD ileum, responsible for excessive deposition of ECM. Of note, FAP fibroblasts were spatially enriched in the deeper submucosa and muscularis layers of fibro-stenotic CD ileum in close proximity with Inflammatory monocytes expressing SLAMF1 (CD150). Additionally, FAP fibroblasts exhibited elevated expression of collagen and ECM genes, along with an activated phenotype characterised by overexpression of profibrotic autocrine loop molecules, such as IL11 and IL6, and chemokines for neutrophils (CXCL1, CXCL5, CXCL6) and monocytes (CCL2, CCL5, CCL7), suggesting a critical role in perpetuating a feed-forward loop that sustains chronic inflammation within the tissue. Trajectory analysis indicates that FAP fibroblasts differentiate from homeostatic CD34+ GREM1− fibroblasts during inflammation, which aligns with prior studies demonstrating that CD34 mesenchymal cells are essential for maintaining intestinal homeostasis and differentiate into myofibroblasts via the TGFb-Smad2 signalling pathway in response to inflammation (37). Similar observation has been made in the heart, where depletion of CD34+ cells lead to reduced myocardial fibrosis and improved cardiac function (38).

Transcriptional regulatory network analysis revealed TWIST1 as the main transcriptional regulator driving the excess ECM gene expression by FAP fibroblasts. TWIST1 has been primarily investigated in cancer for its role in EMT (24, 25). Concordantly, our gene enrichment analysis indicated an activation of ECM related pathways in FAP fibroblasts. In line with our findings, TWIST1 has also been implicated in other fibrotic diseases such as pulmonary fibrosis and renal fibrosis (39). Moreover, TWIST1 has demonstrated pro-fibrotic properties in human fibroblasts by enhancing matrix stiffness (40). In our study, both
pharmacological inhibition using Harmine and genetic deletion of TWIST1 reduced fibroblast activation and ECM protein production in activated human and mouse intestinal fibroblasts (34, 35). Similar results were observed in a murine model of chemically induced chronic colitis, where TWIST1 deletion in fibroblasts (Twist1^−/−Col1a2^−/− mice) as well as treatment with Harmine attenuated ECM deposition in the gut. The anti-fibrotic effects of TWIST1 deletion are in line with recent literature reporting reduced ECM accumulation in bleomycin-induced dermal fibrosis and skin wound healing (41, 42). Currently, several Phase I studies are assessing the toxicity of Harmine in patients (NCT05526430, NCT05780216, NCT05829603, and NCT04716335), and if successful, inhibition of TWIST1 via Harmine may represent a future anti-fibrotic treatment, particularly for preventing intestinal fibrosis.

Overall, our findings are consistent with the evolving paradigm highlighting the crucial role of stromal cells, including FAP fibroblasts, in chronic inflammation and cancers across various organs such as the liver, lung, heart and joint (43–48). Consistently, depletion of FAP fibroblasts using engineered FAP CAR T cells resulted in reduced tissue damage and ECM deposition in experimental models of AngII/PE-induced cardiac damage and bleomycin-induced lung fibrosis as well as reduced leukocyte infiltration and disease severity in a murine model of arthritis (49–51). Further, we looked into the environmental cues driving the differentiation of FAP fibroblasts and identified an extensive crosstalk between FAP fibroblasts and myeloid cells, in which myeloid-derived cytokines, including IL1β, IL1α, OSM, and TNFα, promoted the differentiation of FAP fibroblasts (52). Although the plasticity of monocytes in response to the altered tissue microenvironment, and their capacity to direct stromal cells towards either a regulated wound healing process or dysregulated tissue remodelling, and fibrosis have been widely described (53). In our study, Inflammatory myeloid cells, differentiated from monocytes during inflammation, presented with a hyperinflammatory signature with high expression of IL1A, IL1B and TNF. Re-clustering of the myeloid cells revealed that pro-inflammatory ligands were secreted by a specific subset of monocytes identified by the unique expression of CD150, which co-localised with FAP fibroblast in the deeper submucosa and muscularis layer in inflamed and stenotic CD lesions (54–56). Our observations are in line with a recent investigation demonstrating that, when stimulated with lipopolysaccharide (LPS), blood-derived monocytes from CD patients resistant to anti-TNF therapy display a hyperinflammatory phenotype, characterised by increased release of TNFα, IL-23, and IL-1β (57). Taken together, these findings suggest a potential association between a dysregulated immune response in monocytes, activation of stromal cells, and resistance to anti-TNF therapy in certain CD patients with a high risk of developing fibro-stenosis.
To contextualise our findings within the broader understanding of cellular heterogeneity in IBD, we compared our inflammatory monocyte and FAP fibroblast transcriptional signatures with those from recently published scRNA-seq datasets (15–17). Despite including patients with B2 type stricturing CD, the signature scores for FAP fibroblasts and inflammatory monocytes in ileum samples from Kong et al.’s data were consistently low. In contrast, in Martin et al., a study using surgical samples from CD patients, we have identified high gene signature scores consistent with our FAP fibroblast and CD150 monocyte subsets in the ileal lamina propria of a distinct subset of patients resistant to anti-TNF therapy (15). This discrepancy may be attributed to Kong et al.’s sampling strategy lacking transmural sampling (16). Thus, we postulate that the key difference between fibro-stenosis and chronic inflammation in patients may lie in the localisation of FAP fibroblasts in the deeper layers. In line, our pathological FAP+ stromal cells state resembled the ECM high fibroblasts identified by Mukherjee et al. in full-thickness ileal samples as a major driver of stricture formation in CD patients (58). Eventually, and in line with data published by Smillie et al., we could not consistently identify activated FAP fibroblasts and CD150 monocytes in transmural colonic samples from UC patients, suggesting that FAP fibroblasts and CD150 inflammatory monocytes may predominantly represent a feature of CD (17).

Overall, our study extends our knowledge on cellular heterogeneity in the transmural ileum of fibro-stenotic CD patients, highlighting key interactions between immune cells and fibroblasts. We discovered that inflammatory monocytes drive tissue remodelling and fibrosis by promoting via TWIST1 a pro-fibrotic fibroblast state during inflammation and stenosis. Ultimately, our research has revealed multiple potential therapeutic targets, offering promise for developing more effective treatments for fibro-stenotic CD.
Methods

Sex as a biological variable

Sex was not considered as a biological variable; therefore, human and mouse studies included both genders.

Human specimens

The resected terminal ileum was collected immediately after surgery from patients with Crohn’s disease (CD) or colorectal cancer (CRC) under the supervision of the specialised IBD-pathologist (GDH). The healthy ileum was macroscopically classified as proximal tissue, while the ulcerative ileum exhibiting a non-thickened bowel wall was categorised as inflamed tissue. Conversely, the non-ulcerative ileum displaying a thickened bowel wall at the location of a narrowed lumen was designated as stenotic tissue.

Histological slides of the terminal ileum

Transmural biopsies from the terminal ileum were fixed in 4% formaldehyde, embedded in paraffin and 5µm thick sections were cut for histological analysis. Hematoxylin and eosin (H&E) and Masson's trichrome staining were performed in the Department of Imaging & Pathology at the UZ Leuven. The pathological score system was modified from Gordon et al. and examination was performed by a specialised IBD-pathologist (GDH) (19, 20). To quantify the relative histologic area of collagen on Masson’s trichrome stained slides, the average of ten images in each sample was taken and quantified with Image J. The slides were imaged on the Marzhauser Slide Express 2 (Nikon) at the VIB Leuven.

Single-cell isolation from the terminal ileum

Single-cell suspensions were prepared from the transmural terminal ileum. Briefly, healthy (as the proximal tissue), granulating ulcerative (as the inflamed tissue) and thicken (as the stenotic tissue) biopsies of CD ileum and healthy (as the control group) ileum of CRC were treated with 1 mM DTT and 1 mM EDTA in 1x Hank’s balanced salt solution (HBSS), and 1 mM EDTA in HBSS at 37 °C for 30 minutes, respectively. Then the tissue was minced and digested with 5.4 U/mL collagenase D (Roche Applied Science), 100 U/mL DNase I (Sigma), and 39.6 U/mL dispase II (Gibco) in a sterile gentleMACS C tube for 20 minutes at 37 °C at 250 to 300 rpm after dissociating with the gentleMACS™ Dissociator (program human_tumor_02.01). After being treated with Red Blood Cell Lysis Buffer (11814389001, Roche), single-cell suspensions were used for scRNA-seq, cell culture and flow cytometry. Single-cell-RNA-sequencing and data analysis

Cell suspensions were processed with a 10x3’ v3 GEM kit and loaded on a 10x chromium controller to create Single Cell Gel beads in Emulsion (GEM). A cDNA library was
created using a 10x 3’ v3 library kit and was then sequenced on a NovaSeq 6000 system (Illumina). Pre-processing of the samples including alignment and counting was performed using Cell Ranger Software from 10x.

Doublet score was calculated using three methods (scDbllfinder, scrublet and DoubletFinder) on each sample separately and corresponding doublet scores were added to the metadata (59–61). Also using the DropletQC package, a QC metric indicating the fraction of reads exclusive to nuclear reads was calculated (62). Thus, our QC metrics included fraction of nuclear reads, doublet scores and nUMI.

Data were analysed using Seurat v3 SCTransform-Integration workflow with each patient as a batch. Only cells with more than 199 unique genes and less than 30 % mitochondrial genes were included in the analysis. Next, SCTransform function from Seurat was used to scale, normalise and transform each sample (10x channel) with method set as ‘glmgGamPoi’ and percentage of mitochondrial genes as the variable to regress (63–65). Next, 3000 features were selected for integration and a reference-based integration with 10 out of the 35 samples as reference samples was performed. After PCA, 80 principal components were used to find shared nearest neighbours and compute UMAP. Next, the shared nearest neighbour graph was used for clustering the cells at a resolution of 2.

After clustering, 2 clusters (which clustered in the middle of the UMAP) with low UMI and without expressing distinguishable markers for any cell type were removed. Some clusters such as neutrophils had lower number of genes expressed but showed distinct neutrophil markers. A cluster with a high number of doublets as indicated by the doublet scores was also removed. Differential gene expression between clusters was performed using the Wilcoxon test implemented in Seurat using FindAllMarkers or FindMarkers functions.

**Annotation and subsetting of the data**

Small intestine data model pre-trained on the human ileal single cell data of the Human Gut Atlas was downloaded from the CellTypist website and was used to annotate the clusters with CellTypist (66, 67). Each of the 73 clusters were then classified into 8 different compartments – Mesenchymal, Myeloid, T/NK cells, B cells, plasma cells, epithelial cells, endothelial cells and EGCs based on the cell typist annotation. The annotation was additionally manually curated using canonical markers as shown in Figure 1D. Each compartment except EGCs were then separately re-clustered to reveal detailed heterogeneity. First re-clustering within each compartment at high resolution revealed low quality cells and doublets which were filtered out and only high-quality cells were retained for the second round of re-clustering.
Overall, an effort was made to stay consistent in annotation with earlier single cell atlas publication – the human gut atlas (67). For the mesenchymal cells and myeloid cells, Celltypist annotation based on human gut atlas was used wherever possible. For T cells, B cells, epithelial cells and plasma cells, manual annotation was performed after curating Celltypist annotations based on 2 publicly available annotated single cell RNA sequencing data sets of similar tissue. After re-clustering followed by filtering and annotation of subclusters in each compartment, all retained cells were used to compute the UMAP of all cells as in Figure 1B. The fine annotations of subclusters obtained from re-clustering each compartment was carried over to the metadata of all cells for downstream analyses such as CellPhoneDB.

**Gene regulatory network analysis**

Gene regulatory network analysis was performed using the python implementation of single-cell regulatory network inference and clustering (SCENIC) (23). Specifically, GRNBoost was used to construct a gene regulatory network from log normalised counts. Identified networks were then pruned using DNA motif analysis to remove indirect targets or associations and enrichment of each regulon in single cells were quantified using AUCell algorithm included in SCENIC. Further wilcoxon rank sum test as implemented in the Seurat R package was used to identify top significant transcription factors in each cluster. The analysis was performed using pySCENIC (0.11.1) - the python implementation of SCENIC (68).

**Trajectory analysis**

PAGA was used to estimate the connectivity between the Seurat clusters (29). 4 clusters including FAP fibroblasts identified by PAGA as connected were then used with Monocle3 to learn the differentiation trajectory (30). Healthy tissue specific GREM1\textsuperscript{+}CD34\textsuperscript{+} fibroblasts were annotated as the root of the trajectory prior to ordering cells along the pseudotime. Similarly, for myeloid cell trajectory, PAGA was employed first to identify connected clusters. Connected clusters except pDCS were then used with monocle3 to construct the trajectory. Cells were ordered with CCR2 monocyte at the beginning of pseudotime.

**Intercellular interaction and signalling analysis**

Overall ligand receptor interactions among cell compartments were analysed using CellPhoneDB (31). CellphoneDB analysis was separately performed for each of the 4 tissue segments on all cells using subcluster annotations. Specific ligands involved in altering gene expression leading to differentiation of FAP fibroblasts were done using the NicheNet R package with Kyoto Encyclopedia of Genes and Genomes (KEGG) database used for ligand-receptor pairs (26, 32). Genes differentially expressed between ABL2 fibroblasts, GREM1\textsuperscript{+} CD34\textsuperscript{+} fibroblasts, GREM1\textsuperscript{+}CD34\textsuperscript{+} fibroblasts, and FAP fibroblasts were considered as the
geneset of interest. Visualisations were prepared using the ggplot2 R package except for Heatmap visualisations which were prepared using the heatmap R package.

**In-silico gene functional analysis**

Functional analysis of upregulated genes in specific clusters or a regulon was done using an enricher function from the ClusterProfiler package (69, 70). Also, for single cell data of myeloid and mesenchymal compartments, single sample gene set enrichment score (ssGSEA) as implemented in the scGSVA package was used with a combined database of KEGG, Reactome, Gene Ontology (GO), HALLMARK, BIOCARTA and Human Phenotype (HP) (26, 28, 71–73). The Wilcoxon test was used to estimate the statistical significance of the term enrichment in the clusters. For specific custom geneset of Core matrisome collagens or core ECM genes, gene sets were downloaded from the matrisomeDB database, and the AddModuleScore function from Seurat package was used to create a module score at the single cell level (74).

**Flow cytometry, sorting and analysis**

To validate cell proportion of scRNA-seq in fibroblast subsets, cell suspensions were labelled with CD45-PE (1:300, clone 2D1, BioLegend), CD326-PE (1:300, clone 9C4, Biolegend), CD31-PE (1:300, clone WM59, Biolegend), CD90-BV421 (1:400, clone 5E10, Biolegend), podoplanin (PDPN)-APC (1:300, clone NC-08, Biolegend), CD34-FITC (1:200, clone 4H11, eBioscience), and FAP-Alexa fluor 750 (1:300, clone 427819, R&D Systems). 7-AAD (1:100, 559925, BD Biosciences) was used to determine cell viability. The combination of CD45, CD31 and CD326 was used as lineage to eliminate immune cells, endothelial cells and epithelial cells.

CD45-FITC (1:400, clone HI30, Biolegend), CD3-APC/Cy7 (1:300, clone UCHT1, Biolegend), CD56-APC/Cy7 (1:300, clone HCD56, Biolegend), CD19-APC/Cy7 (1:300, clone HIB19, Biolegend), HLA-DR-APC (1:300, clone L243, Biolegend), CD150 (SLAMF-1)-BV421 (1:100, clone A12, BD Horizon), CD14-PE/Cy7 (1:400, clone 63D3, Biolegend), CCR2-PE (1:400, clone LS132.1D9, BD Pharmingen) and CD206-PE/CF594 (1:300, clone 19.2, BD Horizon) were used to identify different subsets of myeloid cells. The combination of CD3, CD19 and CD56 was used as lineage to eliminate T cells, B cells and NK cells. Flow cytometry and sorting were performed on the MA900 multi-application cell sorter (Sony) with 100 µm sorting chips.

Sorted-HLA-DR+ cells, CD14dim cells, CD150CCR2+ cells and CD150+CCR2+ cells (10,000 cells per well) were seeded in a 96-well clear round bottom plate (3788, Corning) with 100 µL of RPMI-1640 medium, supplied with 5% FBS (BWSTS181H, VWR), 1% HEPES
(15630056, Gibco), 1% L-glutamine (A2916801, Gibco), 1% sodium pyruvate (11360070, Gibco), and 1% antibiotic/antimycotic solution (A5955, Sigma-Aldrich) for 16 hours. The cell supernatants were collected and used to stimulate fibroblasts.

**Spatial transcriptomics**

Human ileum was embedded in Tissue-Tek® O.C.T.TM Compound (Sakura) and snap frozen in isopentane (Sigma) chilled by liquid nitrogen. Embedded tissues were stored at -80°C. Human ileum was sectioned, placed within capture areas on Resolve BioScience slides (8x8mm). The samples were analysed as previous described (75). In brief, thawed tissue sections were fixed, and subjected to Molecular Cartography™ (100-plex combinatorial single molecule fluorescence in-situ hybridization) using the manufacturer’s instructions (protocol 3.0). The probes were designed using Resolve’s proprietary design algorithm. Cell segmentation was performed following of Resolve BioSciences pipeline (76, 77). After segmentation, we filtered the cell-wise gene expression data, retaining only cells with a minimum of three detected transcripts. This data was analyzed using the Seurat R package (v3), where SCTransform normalization was applied, followed by Principal Component Analysis (PCA) using the 'RunPCA' function, computation of shared nearest neighbour graph using with 'FindNeighbours’. Next the 'RunUMAP' and 'FindClusters' functions were used for data visualisation and clustering, respectively. The 2D spatial component of the data were visualised by incorporating centroid coordinates from the segmentation analysis.

**Primary human ileal fibroblasts and CCD18-Co colonic human fibroblast**

Single-cell suspensions were directly cultured in a T-25 flask (90026, TPP) with RPMI-1640 medium (31870074, Gibco), supplied with 10% FBS (BWSTS181H, VWR), 1% HEPES (15630056, Gibco), 1% L-glutamine (A2916801, Gibco), 1% sodium pyruvate (11360070, Gibco), and 1 % antibiotic/antimycotic solution (A5955, Sigma-Aldrich). 0.25% Trypsin-EDTA (25200056, Gibco) was used to detach the cells. Primary human ileal fibroblasts were obtained, purified confirmed by using vimentin (1:500, clone 280618, R&D systems) and alpha-smooth muscle actin staining (1:500, NB300-978, Novus Biologicals) (data not shown) and used after two passages (81). CCD18-Co colonic fibroblasts (CRL-1459) were obtained from ATCC and cultured according to the ATCC culture guides.

For generating CCD18-Co cells with stable TWIST1 shRNA mediated knockdown, lentiviral particles were generated using third-generation packaging vectors in human embryonic kidney (HEK)293T cells using the MISSION pLKO.1-puro vector. Short hairpin (sh)RNA knockdown constructs for TWIST1 were obtained from the Mission TRC shRNA library (Sigma-Aldrich) with the following sequences (TRCN0000020541, NM_000474.2-
As a non-targeting control, a non-human shRNA targeting sequence (SHC002, Sigma-Aldrich) was used. Cells were infected with viral particles overnight and subsequently selected/cultured with 2 µg/ml of Puromycin (Sigma). To confirm TWIST1 knockdown, total RNA was isolated using the NucleoSpin RNA isolation kit (Macherey-Nagel) according to manufacturer’s instructions. cDNA was synthesized with the RevertAid First strand cDNA synthesis kit (Thermo Fisher Scientific). RT-qPCR was performed with SYBR Green Master mix (Bio-Rad laboratories, Nazareth, Belgium) using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Target genes were amplified using specific primers (Supplemental data).

Primary ileal fibroblasts and CCD-18Co fibroblasts were cultured in a 96-well plate for cell proliferation assay, scratch wound assay, in vitro-extracellular matrix deposition and immunofluorescent staining to determine fibroblast phenotypes and therapeutic function of Harmine (5 µM, 286044, Sigma-Aldrich). Active concentrations of Harmine were determined by using IncuCyte proliferation assay (data not shown). Different combinations of 5 ng/mL human IL-1α (R&D Systems), 5 ng/mL human IL-1β (R&D Systems), 5 ng/mL human TGFβ1 (R&D Systems), 5 ng/mL human TNFα (R&D Systems), 5 ng/mL human IFNγ (R&D Systems) or 5 ng/mL human OSM (R&D Systems) were used to stimulate fibroblasts along with 5 % FBS RMIP-1640 medium.

Data availability

Read count matrix of the scRNA-seq is deposited under restricted access in the European Genome-phenome Archive (EGA; study submission number: EGAS50000000382). Requests for the data will be reviewed by the UZ Leuven-KU Leuven data access committee. Any data shared will be released via a Data Transfer Agreement that will include the necessary conditions to guarantee protection of personal data (according to European GDPR law).

Any additional information required to re-analyse the data reported in this paper is available from the lead contacts upon request.

Statistical analysis

Data are shown as mean ± standard error of the mean (SEM) with individual data values produced by using the GraphPad Prism V.9.1.0 software (GraphPad Inc). Multiple groups were compared by one-way analysis of variance (ANOVA) with Tukey's multiple comparison test.

Study approval

The protocol for human specimens was approved by the Institutional Review Board (IRB) of the University Hospitals Leuven, Belgium (B322201213950/S53684, CCARE, S-53684 and S64914). All recruitment was performed after ethical approval and oversight from
the IRB and informed consent was obtained from all participants before surgery. Clinical information and metadata for the samples in this study were provided in Supplemental data. The protocol for animal studies was approved by the Animal Ethics Committee at the KU Leuven (project 188/2019).
Author contributions
Conceptualisation: BJK, SA, BV, GDH, SV, GM; Methodology: BJK, GD, SA, GB, VDS, PVDV, IDM, JF, GDH, GM; Software: SA, SS; Formal analysis: BJK, SA; Investigation: BJK, SA, GZ, GD, FB, SS, AZ, LVB, VB, YVR, EV, SaV, CB, SL, LH, BV, GDH; Resources GB, AW, ADH, GDH, SA, BV, SV, GM; Data Curation: BJK, SA, BV; Visualisation: BJK, SA; Funding acquisition: MS, EK, SV, GM; Project administration: SV, GM; Supervision: SV, GM; Writing – original draft: BJK, SA; Writing – review & editing: all.
Acknowledgments

We thank all patients for their participation and tissue donations. Brecht Creyns (KU Leuven) collected samples, with technical assistance from Iris Appeltans, Karlien Vranken, and Renata Siqueira de Mello (TARGID, KU Leuven). Sales Ibiza Martinez (University of Antwerp) offered experimental setup suggestions. The IBD Leuven group aided in biobanking and administration. Reena Chinnaraj and Vera Dermesrobian (FACS Core, KU Leuven) provided flow cytometry and sorting support. The Genomics Core (UZ Leuven) assisted with scRNA-seq, and Nikky Corthout and Axelle Kerstens (BioImaging Core Leuven) helped with the PerkinElmer Operetta CLS system and Nikon-Marzhauser Slide Express 2. Stef Janson (Leiden University Medical Center) and Gaia Sarcinelli (Humanitas University) conducted lentivirus transduction and siRNA TWIST1 silencing. Tobie Martens (TARGID, KU Leuven) supported with the Zeiss LSM 780 – SP Mai Tai HP DS. Imaging was done at the Zeiss LSM 780 – SP Mai Tai HP DS (CIC, supported by Hercules AKUL/11/37 and FWO G.0929.15 to Pieter Vanden Berghe, KU Leuven). Computational resources were from the VSC (Flemish Supercomputer Center), funded by FWO and the Flemish Government. BioRender was used to generate graphical images under publication license (Agreement number: MY26OV5OK9). This study was supported by funding from Taiwan (Ministry of Education)-KU Leuven Scholarship Programme (BJK), Fundamental Research by the Stichting tegen Kanker (VDS), FWO grants (G086721N, GM and S008419N, SV and GM), FWO SB fellowship (1S64222N, YVR), FWO postdoctoral fellowship (SaV), KU Leuven Global PhD Partnerships with the University of Edinburgh (GPUE/20, FB), KU Leuven Global PhD Partnerships with the University of Melbourne (GPUM/22/020, SS), KU Leuven Internal Funds (C12/15/016, GM and C14/17/097, GM, CB and SV), Clinical Research Fund (KOOR) at the University Hospitals Leuven (CB, BV), Research Council at the KU Leuven (BV), European Union’s Horizon 2020 research (SL), innovation programme under the Marie Sklodowska-Curie grant (101029427, SL), ECCO Pioneer award 2023 (LH and GM), European Union’s Horizon Europe Research & Innovation programme (FIBROTARGET, 101080523, BJ, SV and GM), European Union–Next Generation EU (MEYS, LX22NPO5107, VB and JF), Ministry of Health of the Czech Republic-DRO (Institute of Hematology and Blood Transfusion- UHKT (00023736, JF) and Boehringer Ingelheim opnMe initiative (GM), FWO-WOG (W001620N, GM).
Declaration of interests:

SV has received grants from AbbVie, J&J, Pfizer, Galapagos and Takeda; consulting and/or speaking fees from AbbVie, Abivax, AbolerIS Pharma, AgomAb, Alimentiv, Arena Pharmaceuticals, AstraZeneca, Avaxia, BMS, Boehringer Ingelheim, Celgene, CVasThera, Dr Falk Pharma, Ferring, Galapagos, Genentech-Roche, Gilead, GSK, Hospira, Imidomics, Janssen, J&J, Lilly, Materia Prima, MiroBio, Morphic, MrMHealth, Mundipharma, MSD, Pfizer, Prodigest, Progenity, Prometheus, Robarts Clinical Trials, Second Genome, Shire, Surrozen, Takeda, Theravance, Tillots Pharma AG and Zealand Pharma.

GM has received research support from Boehringer Ingelheim and speaking fees from Janssen.

GDH’s institution KULeuven has received payments for his involvement as central pathology reader in clinical trials of J&J, Galapagos, Takeda and Genentech-Roche.

BV has received research support from AbbVie, Biora Therapeutics, Landos, Pfizer, Sossei Heptares and Takeda; Speaker’s fees from Abbvie, Biogen, Bristol Myers Squibb, Celltrion, Chiesi, Falk, Ferring, Galapagos, Janssen, MSD, Pfizer, R-Biopharm, Takeda, Truvion and Viatris; Consultancy fees from Abbvie, Alimentiv, Applied Strategic, Atheneum, Biora Therapeutics, Bristol Myers Squibb, Galapagos, Guidepont, Mylan, Inotrem, Ipsos, Janssen, Progenity, Sandoz, Sossei Heptares, Takeda, Tillots Pharma and Viatris.

CB has received consultancy fees from Ablynx.

EK is an employee of Boehringer Ingelheim Pharmaceuticals, Inc., with no equity (e.g., stocks/shares).

All other authors declare no conflict of interest.
References


Figure 1. Single-cell profiling of fibro-stenotic ileal from CD and control ileum from CRC. (A) Experimental workflow for scRNA-seq of ileum using the 10x Chromium platform and further analyses and validations in this study. (B) Uniform Manifold Approximation and Projection (UMAP) embedding of showing ileal single-cell transcriptomes from 169,547 cells from 10 CD patients with a trio of lesions (proximal, inflamed and stenotic) and 5 CRC control
ileum depicting cell compartments. (C) UMAP in Figure 1B split by disease segments. (D) Heatmap depicting relative expression of distinguishing marker genes in each cell compartment.
Figure 2. Heterogeneity of stromal cells in fibro-stenotic CD. (A) UMAP representation of re-clustered mesenchymal cells across different lesions of the terminal ileum. (B) Heatmap showing relative expression of top marker genes in each subset. (C) Cell subset composition
across different lesions of the terminal ileum. (D) Bar plot showing gene set module score for core matrisome collagen genes in each stromal cell subset in different lesions. Horizontal lines indicate medians of respective lesions. Enrichment analysis for Reactome biological pathways in FAP fibroblasts (logFC, >0.5; FDR, <0.1). (F) Flow cytometry gating strategy for fibroblast subsets and (G) the plot of FAP expression in pan fibroblasts (7-AAD CD45 CD31 CD326 PDPN THY1) in different lesions of terminal ileum from 19 CD patients and 8 CRC control ileum. Data are shown as box and whisker plots. Statistically significant differences were determined using a one-way ANOVA test corrected with Tukey's multiple comparisons test (**p < 0.01, ***p < 0.005, ****p < 0.001). (H) Immunofluorescence staining for PDPN, ADAMDEC1 (indicated by white arrows), CD34 and FAP expression in healthy ileum and CD diseased ileum. CD34 and FAP co-localisation is indicated by orange arrows (scale bar = 200 µm). (I) Heatmap showing relative transcription factor activity in each stromal cell subset based on Single-cell regulatory network inference and clustering (SCENIC) analysis. (J) Heatmap showing selected terms after functional enrichment analysis of top 5 regulons using GO terms and core ECM gene set from MatrisomeDB (*indicate statistically significant terms after one sided Fisher’s exact test and multiple correction by Benjamini & Hochberg method). (K) Immunofluorescence staining for TWIST1 and CD34 expression in FAP fibroblasts in CD diseased ileum (indicated by arrows, scale bar = 50 µm).
Figure 3. Trajectory analysis of fibroblast subset and stromal-immune interactions. (A) Pseudo-time trajectory projected onto a UMAP of selected fibroblast subsets. (B) Normalised expression levels of selected markers visualised along the pseudo-time. (C) Heatmap showing number of interactions (Ligand-Receptor pairs) between cell compartments and mesenchymal subsets. (D) Niche signalling driving FAP fibroblast differentiation, predicted by NicheNet; Regulatory potential of each target gene in columns by ligands in rows. (E) Circos plot
depicting links between predicted ligands by NicheNet and their receptors. (F) Dot plot showing expression of NicheNet-predicted ligands in all cell compartments.
Figure 4. Heterogeneity of myeloid cells in fibro-stenotic CD. (A) UMAP representation of re-clustered myeloid cells and (B) cell subset composition across different lesions of the terminal ileum. (C) Heatmap showing the expression of the top marker genes of each myeloid subset. (D) Dotplot showing NicheNet predicted ligands expressed by myeloid cell subsets. (E) Selected Gene Ontology terms significantly enriched myeloid cell subsets. (F) Cellphone DB dot plot showing ligand-receptor interactions between FAP fibroblasts and Inflammatory
monocytes or Neutrophils. First and second interacting molecules correspond to first and second cell types on the y axis respectively. Black circles indicate significant interactions (G) Flow cytometry gating strategy for myeloid cell sub-populations and (H) the plot of CD150 (SLAMF7) expression in CD14⁺ myeloid cells (7-AAD CD45⁺CD3⁺CD19⁻CD56⁻HLA-DR⁺⁻) in different lesions of terminal ileum from 19 CD patients and 8 CRC control ileum. Data are shown as box and whisker plots. Statistically significant differences were determined using a one-way ANOVA test corrected with Tukey's multiple comparisons test (**p <0.01, ***p <0.005, ****p <0.001). (I) Immunofluorescence staining for CD68, CD150 and FAP expression in healthy ileum and CD diseased ileum. Original image composed of stitched 25× images. The scale bar represents a distance of 200 µm in the upper panel and 100 µm in the lower panel. The white arrows indicate the spot of co-localisation.
Figure 5. Spatial co-localisation of FAP fibroblasts and inflammatory monocytes in inflamed and stenotic ileum of fibro-stenotic CD patients. (A) UMAP representation of cell type across different lesions of the terminal ileum from 3 fibro-stenotic CD patients. (B) Spatial map showing the location of cell types across different lesions of the terminal ileum. (C) Bar plot showing the proportion of cell types across different lesions of the terminal ileum. (D) Molecular Cartography of indicated genes in the full thickness of proximal and inflamed ileum and in the mucosa/submucosa layer of stenotic ileum. (E) Spatial map showing the co-
localisation of FAP fibroblast and Inflammatory monocytes in different lesions of terminal ileum.
Figure 6. CD150+ monocytes-derived cytokines promote FAP fibroblast activation and extra-cellular matrix protein deposition under TWIST1 regulation. (A) Heatmap showing
relative expression of NicheNet-predicted ligands expressed by FACS-sorted myeloid cell subsets (n=4). (B) Immunofluorescence staining and (C) Heatmap showing relative expression of FAP, TWIST1 and type III collagen in monocyte-stimulated CCD-18Co fibroblasts (Scale bar = 100 µm). (D) Immunofluorescence staining (10× image) and (E) Bar plot showing relative expression of FAP, type I and type III collagen in monocyte-stimulated CCD-18Co fibroblasts. Data are shown as bar plots with SEM. Statistically significant differences were determined using a one-way ANOVA test corrected with Tukey's multiple comparisons test (**p <0.005, ****p <0.001) (Scale bar = 1 mm). (F) Bar plot showing TWIST1 expression level after lentivirus transduction. Data are shown as bar plot with SEM. Statistically significant differences were determined using T test (*p <0.05). (G) Immunofluorescence staining (25× image) and (H) Heatmap showing relative expression of FAP, TWIST1 and type III collagen in pro-fibrotic cues-stimulated TWIST1 knockdown CCD-18Co fibroblasts (Scale bar = 100 µm). (I) Immunofluorescence staining and (J) Heatmap showing relative expression of FAP, TWIST1 and type III collagen in pro-fibrotic cues-stimulated CCD-18Co fibroblasts (Scale bar = 100 µm). (K) Immunofluorescence staining and (L) Bar plot showing relative expression of FAP, type I and type III collagen in pro-fibrotic cues-stimulated CCD-18Co fibroblasts after TWIST1 inhibition. Data are shown as bar plot with SEM (Scale bar = 1 mm). (M) Immunofluorescence staining and (N) quantitative analysis (mean fluorescence intensity) of FAP and PDPN in pro-fibrotic cues-stimulated iPSC-derived intestinal organoids with or without Harmine. Data are shown as bar plot with SEM. Statistically significant differences were determined using a one-way ANOVA test corrected with Tukey's multiple comparisons test (*p <0.05, **p <0.01, ***p <0.005, ****p <0.001) (Scale bar = 300 µm).