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

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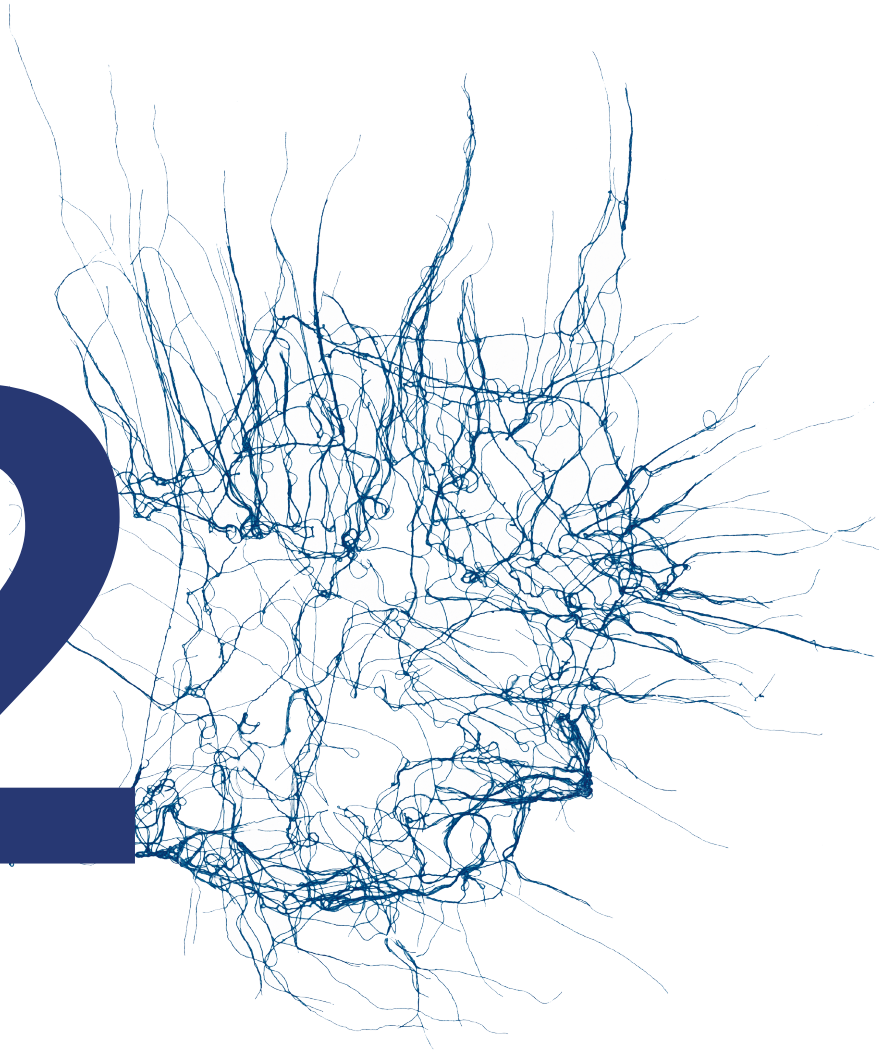

STROMAL CELLS IN THE PATHOGENESIS OF INFLAMMATORY BOWEL DISEASE

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ABSTRACT

Up till now, research on inflammatory bowel disease (IBD) has mainly been focused on the immune cells present in the gastrointestinal tract. However, recent insights indicate an important and significant role for stromal cells in IBD pathogenesis as well. Stromal cells in the intestines regulate both intestinal epithelial and immune cell homeostasis. Different subsets of stromal cells have been identified in other inflammatory diseases (e.g. rheumatoid arthritis) and also in IBD these various stromal subsets are now recognized and seem to carry out specific functions in the inflamed gut. Novel potential therapies for IBD utilize, as well as target these pathogenic stromal cells. Injection of mesenchymal stromal cells into fistula tracts of Crohn's disease patients is already approved and used in clinical settings. In this review we discuss the current knowledge of the role of stromal cells in IBD pathogenesis. We further outline recent attempts to modify the stromal compartment in IBD with agents that target or replace the pathogenic stroma.

INTRODUCTION

Inflammatory bowel disease (IBD) incidence is still increasing worldwide, mostly due to an accelerating incidence in newly industrialised countries[1]. Although clear progress has been made, the exact pathogenesis of IBD is still poorly understood. The current working model of IBD pathogenesis proposes a dysfunctional epithelial barrier that finally leads to an aberrant immune response to the intestinal bacteria. Recent research demonstrates that besides intestinal epithelial and inflammatory cells also stromal cells play an important role in IBD pathogenesis. So far, therapies for IBD have been mainly focused on the targeting of immune cells, and this has given rise to the development of a number of biologic therapies, small molecules and other immunomodulators. Biologic therapies such as anti-TNF-α and anti-IL-23/12 therapies have been successfully introduced into the clinic. However, attempts to block a number of additional cytokine networks, like for example blockage of interferon-γ[2] (IFN-γ) or IL-17A[3], were rather disappointing. With immune modulating therapies, mucosal healing in Crohn's disease (CD) is only achieved in up to 45% of patients[4,5]. Subsequently, the risk of surgery within 10 years after diagnosis is still 46.6% and 15.6% for, respectively, CD and ulcerative colitis (UC)[6]. In addition, a definite curative treatment for IBD patients has not yet been discovered. It might be important to develop alternative therapies that target pathogenic stromal cells in IBD, which could probably intervene earlier in the inflammatory cascade and thereby have a better chance of delaying disease progression.

This review will focus on the role that stromal cells, in particular fibroblasts, play in the pathogenesis of IBD, thereby focussing on their role in the inflamed, non-fibrotic intestinal tissue. First, we will describe the current knowledge regarding the function of stromal cells in the healthy intestine. Thereafter, we will discuss the role of activated stromal cells in diseased tissue and highlight the current literature on stromal cells in IBD, focussing on their interaction with both epithelial cells and immune cells. Finally, the currently discovered opportunities for potential therapies pertaining to targeting stromal cells and replacement of stromal cells, by mesenchymal stromal cell (MSC)-therapy, will be highlighted.

Definitions

There seems to be a lack of consensus pertaining to the nomenclature of stromal cells in general. Terms such as "stromal cell", "mesenchymal cell", "fibroblast" and "fibroblast-like cell" are used seemingly interchangeably within and between studies. In this review we will refer to "stromal cells" as non-hematopoietic, non-epithelial and non-endothelial cells[7]. In general, the most abundant stromal cells are fibroblasts, followed by myofibroblasts, smooth muscle cells, pericytes and mesenchymal stromal cells. In the human intestine, stromal cells can be detected in all layers of the gut wall, from the mucosa to the serosa. Mostly, stromal cells are defined as being negative for cell surface markers, such as cluster

of differentiation (CD)31 (endothelial cells), CD45 (immune cells), keratins or epithelial cell adhesion molecule (EpCAM; epithelial cells)[8-10], while they are positive for the cytoskeletal marker vimentin. Fibroblasts, more specifically, are mostly reported to be positive for collagen (COL) type I and -III, CD90 and fibroblast activation protein (FAP)[11-13]. However, as we will discuss later in detail, subsets of fibroblasts have been identified that are negative for FAP and CD90, indicating that fibroblasts also form a heterogenous group of cells. Furthermore, fibroblasts are recognizable through their distinct morphology *in vitro*, as spindle-shaped cells with a flat nucleus and slender cytoplasmic processes[8]. However their morphological properties are more difficult to detect in tissues. MSCs, known for their therapeutic capacity after culture, are defined as CD105-, CD73- and CD90-positive cells, that are able to differentiate (*in vitro*) into osteoblasts, chondrocytes and adipocytes[14]. For pericryptic myofibroblasts, which show properties of both fibroblasts and smooth muscle cells[15], there is consensus in the nomenclature, since these cells are defined as cells that are vimentin- and alpha smooth muscle actin (α-SMA)-positive, but do not express the smooth muscle cell marker desmin[12].

STROMAL CELLS IN INTESTINAL HOMEOSTASIS

Most stromal cells in the gut wall derive from the serosal mesothelium, which originates from the mesoderm, during embryonic development[16,17]. Furthermore, stromal cells in the inflamed gut may also develop from other cell types through the process of epithelial-to-mesenchymal transition (EMT) or endothelial-to-mesenchymal transition (EndoMT)[18-21]. Finally, stromal cells, and especially MSCs and circulating fibrocytes, are able to migrate from the bone marrow towards the intestines[22].

The gut stroma

The gut stroma provides structure and form, and primarily consists of stromal cells and extracellular matrix (ECM). Within the stroma, fibroblasts are mainly known for their role in the production and remodelling of the ECM, by secreting type I, -III and -V collagens, fibronectin, but also matrix-remodelling, proteolytic enzymes including matrix metalloproteinases (MMPs)[23]. A well-known complication of excessive ECM production by fibroblasts in IBD is fibrosis. In this review we will not focus on fibrosis, since excellent reviews have already been published on the role of fibroblasts in fibrosis[24-26]. It is, however, an over-simplification to see fibroblasts only as passive matrix-depositing cells, thereby providing epithelial support and tissue structure. Recent literature shows that fibroblasts also play an important role in maintaining tissue homeostasis by their interaction with both epithelial and immune cells.

Epithelial cell homeostasis

The intestine is covered by a monolayer of epithelial cells. These cells are generated from stem cells in the base of intestinal crypts and then migrate along the crypt lining, while they differentiate into specialized epithelial cells like absorptive enterocytes, goblet cells, enteroendocrine cells, tuft cells, M-cells and Paneth cells[27]. They have a rapid turnover and eventually the mature epithelial cells are shed at the top of the crypt into the lumen, renewing the crypt every 4-5 days[28]. Epithelial cell homeostasis is important because epithelial cells form the first line of defence against pathogens, and they are also responsible for the absorption of nutrients.

Myofibroblasts are described as the stromal cells that are important for maintaining epithelial homeostasis. In the human intestine, myofibroblasts are found along the crypts, surrounding also the intestinal stem cell niche, comprising of $Lgr5^+$ stem cells and Paneth cells[29]. These myofibroblasts have an important role in the process of intestinal epithelial cell renewal via paracrine interactions[30]. Various pathways, such as the Wnt and bone morphogenetic protein (BMP) pathways, are able to modulate stem cell function and differentiation in these intestinal niches[11]. Wnt signaling is necessary for maintaining non-differentiated proliferating $Lgr5^+$ stem cells, while BMP signaling antagonizes Wnt signaling signature genes and induces differentiation of epithelial cells[31-34]. Multiple studies have shown that myofibroblasts play an important role in both of these pathways by secreting, for example, Wnt ligands and BMP antagonists[11,35,36]. Myofibroblasts, specifically in the basal part of the colon crypt, express the BMP antagonists gremlin and noggin, suggesting that they inhibit BMP signaling in the basal crypt regions, yet allow BMP signaling to take place in the upper crypt regions[36]. This differential expression of BMP signaling in specific places in the intestinal crypt suggests heterogeneity within the myofibroblast population. Degirmenci and colleagues identified $Gli1^{pos}$ fibroblasts in mice, with a close relation to the bases of intestinal crypts, to be important for epithelial integrity by production of Wnt and thereby stem cell renewal[37]. Another study further subdivided the $Gli1^{pos}$ cells into CD90-positive and -negative fibroblasts[38]. Those authors found that $CD90^{pos}$ fibroblasts, in contrast to $CD90^{neg}$ fibroblasts, produce BMP antagonists and Wnt ligands, like gremlin and Wnt2b and support organoid growth[38]. Interestingly, the $CD90^{pos}$ fibroblasts could be further divided in an α-SMA-positive and -negative population. Since myofibroblasts are defined as being α-SMA positive cells, this suggests that fibroblasts also play a role in epithelial homeostasis and barrier function, which is often disturbed in IBD[37]. Moreover, in human samples it was also found that a specific fibroblast population contributes to the maintenance of the epithelial homeostasis[39]. This population, identified by CD142 expression, was found close to the epithelial monolayer and single-cell RNA-sequencing (scRNA-seq) revealed the expression of different BMP and Wnt ligands. Overall, evidence

of the specific physical location of these intestinal (myo)fibroblasts, close to the epithelial layer, and their expression of relevant markers, shows that they are able to regulate the function and fate of epithelial progenitors and thereby intestinal epithelial homeostasis.

Immune cell homeostasis

Besides epithelial cell homeostasis, stromal cells also influence intestinal immune cell homeostasis in the intestine. This is the process in which immune cell responses are in a steady-state condition, because pathogens are recognized and cleared at an early stage without immunogenic responses towards non-pathogenic peptides. The intestinal mucosal immune system consists of a variety of immune cells that reside in the healthy gut, either organized in Peyer's patches, in lymph nodes or scattered in the various layers of the gut. Upon encountering foreign proteins, antigen presenting cells, like dendritic cells, present the peptides to lymphocytes in the organized immune structures in the gut, which activates and attracts other lymphocytes to the gut[40].

Stromal cells influence immune cell homeostasis via direct cell-cell contact with immune cells or through the production of chemokines and cytokines[41]. Intestinal fibroblasts are able to produce for example interleukin (IL)-6, IL-8, chemokine ligand 2 (CCL2/MCP-1)[41-44] and chemokine ligand 5 (CCL5/RANTES)[45]. CCL2 binds to chemokine receptor 2 (CCR2), mainly expressed by monocytes, whereas CCL5 binds to several receptors, mainly expressed by T cells, and thereby fibroblasts facilitate the recruitment of both myeloid cells and lymphocytes to the site of inflammation. Myofibroblasts and fibroblasts are also able to affect mucosal T cells via direct cell-cell contact. In non-diseased human colonic lamina propria these stromal cells express programmed death-ligand 1 (PD-L1) and PD-L2[46], which are immune checkpoints that bind PD1 on T cells during antigen presentation[47]. Fibroblasts are able to suppress the proliferation of $CD4^{pos}$ T cells via PD-L1 and PD-L2 and thereby prevent autoimmunity[46]. Colonic fibroblasts can also indirectly affect T cells by induction of retinoic acid production in dendritic cells[48], which is able to block T helper (Th)1 and Th17 differentiation and to enhance regulatory T cell (Treg) differentiation. Furthermore, fibroblasts have been described as being part of the innate immune system because of their ability to recognize pathogen invasion or cell damage[13,49,50]. They can detect pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) through toll-like receptors (TLRs), which triggers the release of chemokines[51]. Indeed, $CD90^{pos}$ fibroblasts are known to express various TLRs[50]. By the expression of MHC class II molecules colonic myofibroblasts are, upon activation, also able to act as non-professional antigen presenting cells[13,52]. Through both MHC class-II expression and the production of prostaglandin E2, human colonic (myo)fibroblasts from non-diseased mucosa have been reported as contributing to the maintenance of

colonic immunological tolerance by promoting the expansion of regulatory FOXP3^{pos} T cells (Tregs)[53]. Together, these observations show that intestinal stromal cells are able to modify the mucosal immune landscape via different pathways. However, some caution and careful interpretation of the data is needed since most of these studies used allogeneic immune cells and *in vitro* cultured stromal cells, which could have gained their activated immunoregulatory phenotype through culturing.

STROMAL CELLS IN DISEASED TISSUE

Upon organ damage, resident stromal cells become activated. In inflammatory diseases, especially in rheumatoid arthritis (RA), there has been more focus on the role of stromal cells in the last decade. In this review we will use current literature in RA on stromal cells to understand more about the role and function that stromal cells might have in other inflammatory conditions and thereby IBD. RA, characterized by painful swellings of joints that will eventually lead to bone erosion and joint deformation[54], shows immunological similarities with IBD and many immunomodulating therapies currently used in IBD were initially explored and approved in RA. In the inflamed joints, leukocytes and a variety of innate effector cells accumulate in the synovium, similar to what occurs in the bowel of IBD patients, together with expansion of the already present lining of fibroblast-like synoviocytes (FLSs)[55]. Hyperplasia of this specific type of fibroblasts found in the synovium, is one of the hallmarks of RA and therefore several studies have been performed to identify and characterize the potential pathogenicity of FLSs in RA. Both the activation of the immune system and disrupted matrix production by the hyperplastic FLSs contribute to cartilage damage and bone erosion[56]. In addition to RA, we will also shortly touch on stromal subsets identified in cancer.

Stromal cell subsets in RA

In RA, several attempts have been undertaken to identify different subtypes of FLS in the inflamed joint. scRNA-seq of RA synovial knee tissue revealed the presence of at least two main fibroblasts clusters[57]. CD55^{pos} fibroblasts, defining subset 1, were mainly found in the synovial lining and showed expression of hyaluronan synthase 1, which is important for the production of synovial fluid[57]. On the other hand, CD90^{pos} fibroblasts, defining subset 2, were found in the synovial sub-lining of the joint and showed high expression of C-X-C motif chemokine 12 (CXCL12). In accordance, another group showed that within the FAPα^{pos} fibroblasts population in the mouse synovium CD90-positive and -negative fibroblasts were also found to have different functions and location[58]. Interestingly, the severity of the joint inflammation correlated with the number of FAPα^{pos}CD90^{pos} cells and not with the number of FAPα^{pos}CD90^{neg} cells. In the murine intestine, similar to the situation

described above, these CD90^{pos} fibroblasts were also identified and found to be specifically located at the base of the crypt[38], which could indicate that CD90^{pos} fibroblasts have an organ-specific cellular location. Another recent study in RA identified three major stromal subsets defined by CD90 and CD34 expression[59]. One of these subsets, CD34^{neg}CD90^{pos} cells, was a specific expanded FLS subset in RA-affected synovium. This population of FLSs showed involvement in bone destruction in RA by high tumor necrosis factor ligand superfamily member 11 (TNFSF11) expression levels, which is a key factor for osteoclast differentiation and activation. In contrast, CD34^{neg}CD90^{neg} fibroblasts were less abundant in RA affected tissue, and especially in swollen RA joints. Most of the fibroblasts detected in RA affected joints also showed podoplanin (PDPN) expression[59,60]. Although PDPN was first identified as a lymphatic vessel marker, cancer-associated fibroblasts (CAFs) were also found to express PDPN. PDPN expression on CAFs was associated with enhanced tumor progression[61] and inhibition of T cell proliferation[62].

Stromal cell subsets in cancer

Given the immunosuppressive environment in tumors, cancer can be seen as the counterpart of IBD, which is defined by an overactive immune response. The role of CAFs in cancer has already been discussed in various excellent recent reviews[63-65]. In the present review we will only highlight the most important findings, which have relevance for the role of stromal cells in IBD. CAFs have been associated with increased cancer cell proliferation, cell invasion and the formation of distant metastasis[63,66]. Transforming growth factor (TGF)-β1 is one of the most abundant cytokines produced by CAFs. It was shown that high TGF-β1 levels, which are associated with a poor prognosis[67], are an immunosuppressive mechanism of CAFs, promoting T cell exclusion and the blocking of the T helper 1 (Th1)-effector phenotype acquisition[68-71]. Interestingly, dual treatment with anti-TGF-β and anti-PD-L1 in a murine breast cancer model changed peritumoral stromal fibroblasts and increased cytotoxic T cell counts in the tumor, leading to a significant reduction in tumor burden only in mice treated with both antibodies[72]. This would indicate that most CAFs are tumor promoting and targeting them inhibits tumor progression. However, targeting all α-SMA^{pos} CAFs in mice with pancreatic cancer increased the number of Tregs in the tumors and led to more aggressive tumors and decreased survival[73]. This indicates that different subpopulations exist, with distinct roles in tumor progression. In colorectal cancer, scRNA-seq profiling of the tumor and matched non-tumor samples revealed the presence of three clusters of fibroblasts, of which two were defined as CAFs[74]. CAF-A, which was the only CAF population showing FAP expression, showed high expression of MMP2 and COL1A2. In contrast, CAF-B had a more myofibroblast-like phenotype with high expression of α-SMA. Two different CAF types were also found in pancreatic cancer tissue by using FAP and α-SMA staining, and defined as inflammatory (i)CAFs and myofibroblastic (my)

CAFs[75]. iCAFs were described as activated stellate cells, forming the dense tumor stroma and being the main source of IL-6 and IL-11, whereas myCAFs were defined by high α-SMA expression and their periglandular location. Besides α-SMA, many other markers have been proposed as distinguishing certain subtypes of fibroblasts. CD146[76] or CD29[77], among others, have been associated with breast cancer CAF subpopulations. Periostin (POSTN), myosin (MYH)-11 and PDPN[78] have also been associated with pancreatic cancer CAF subpopulations. These non-overlapping markers show that at least up till now, robust markers identifying specific CAF subsets have not been established yet. The CAF subpopulations exert different functions, both on cancer and immune cells. Two studies demonstrated the effect of a CAF subpopulation, defined by expression of CD10/GPR77 or fibroblasts growth factor 5 (FGF5) respectively, on the promotion of cancer stem cells[79,80]. Givel et al[81], on the other hand, observed that in ovarian cancers that are enriched for the α-SMA expressing CAF-S1 subset, there is increased accumulation of Tregs. These CAFs were able to recruit, retain and increase survival of $CD4^{pos}CD25^{pos}$ T cells and then promote differentiation of these T cells into Tregs. CXCL12β was highly expressed in this CAF subset compared with other CAF subsets and knockdown of CXCL12 in CAF-S1 reduces $CD4^{pos}CD25^{pos}$ recruitment *in vitro*. In summary, it seems plausible, that as in the healthy colon, also in cancer there are different types of stromal cells that have distinct effects on tumor cell growth and/or immune cell homeostasis.

STROMAL CELL SUBSETS IN IBD

Although stromal cell research in IBD is in its infancy, various mechanisms have been discovered through which stromal cells affect wound healing and modulate the immune milieu in the inflamed intestine. Three major contributions towards understanding the role of stromal cells in IBD were the recent studies from Kinchen[39], Smillie[82] and Martin[83], in which the stromal cell subsets in the colon of IBD patients were analysed using scRNA-seq[39,82,83] and mass cytometry time-of-flight (CyTOF)[39,83]. In the study from Kinchen and colleagues, 12 different non-epithelial and non-immune cell clusters could be detected in the colon of patients with UC. In addition to the myofibroblasts, four different clusters of fibroblast-like cells could be defined (S1-4). Cluster S1 was characterized by the expression of non-fibrillar collagens and elastic fibres, whereas cluster S2 showed high CD142 expression, cluster S3 showed high CD55 and COX-2 expression and cluster S4, which was barely detectable in the healthy gut, yet expanded in UC, showed PDPN and IL-33 upregulation. Smillie and colleagues found eight fibroblast clusters in UC tissue, which also included one myofibroblast population. The clusters mainly differed by expression of Wnt and BMP signaling genes, suggesting their different positions along the intestinal crypt. They also identified one fibroblast population, termed inflammation-associated fibroblasts,

that was expanded in inflamed tissue of UC patients and showed enrichment for genes like IL-11, FAP, and IL-13RA2. In contrast, Martin and colleagues analysed lamina propria cells from ileal tissue from CD patients and identified four stromal clusters; pericytes, smooth muscle cells, fibroblasts and activated fibroblasts[83]. The two fibroblasts subtypes were characterized by expression of platelet-derived growth factor receptors and genes encoding for ECM proteins. Interestingly, activated fibroblasts strongly expressed CD90 and also PDPN. The different functions assigned to the various stromal clusters are discussed below and the most important changes in stromal cells in IBD are summarized in Figure 1.

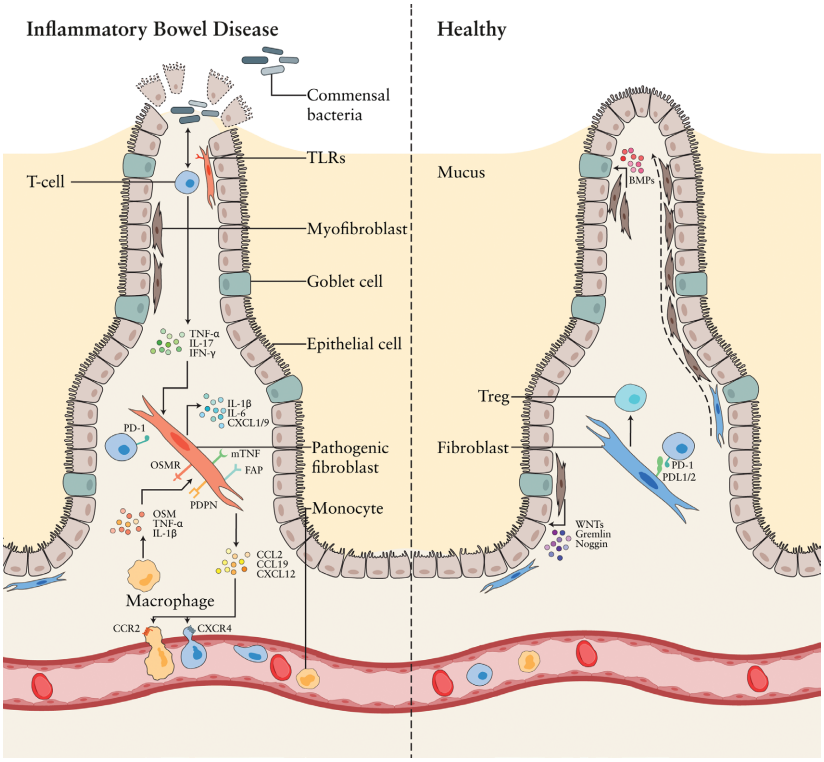

FIGURE 1. Stromal cells in the intestine of IBD patients versus healthy individuals. Different stromal subsets are present in the inflamed bowel. Diminished migration capacity in fibroblasts and less stromal cells (orange) supporting epithelial cells are found in IBD. Stromal cells directly (via TLRs) and indirectly (via microbiota-reactive memory T cells) respond to microbiota by the production of several pro-inflammatory factors. Pathogenic fibroblasts (pink) show expression of PDPN, OSMR, mTNF and FAP, while they produce among others IL-6, IL-13, TNFSF14 and IL-1β Through for example CCL2 and CXCL12 they recruit respectively monocytes and T cells towards the inflamed tissue. Treg – regulatory T cell, PD-L – programmed death-ligand, PDPN – podoplanin, OSMR – oncostatin M receptor, FAP – fibroblast activation protein, IFN-γ - interferon gamma, CXCL – C-X-C motif chemokine, IL-– interleukin, TNFSF-14 – tumor necrosis factor superfamily 14, mTNF – membrane bound tumor necrosis factor, CCL – chemokine ligand, BMP - Bone Morphogenetic Protein.

Wound healing by IBD stromal cells

In order to restore the damaged epithelium in IBD, the migration of fibroblasts, collagen deposition and controlled rebuilding of the epithelial layer is essential[84]. Already some years ago, it was found that the migratory capacity of human colonic lamina propria fibroblasts is altered in IBD. *In vitro* studies showed reduced migratory capacity of fibroblasts from IBD patients compared with control intestinal fibroblasts[85]. This is even further decreased in fibroblasts derived from CD fistula patients[86]. Furthermore, fibroblasts derived from CD or UC inflamed intestines proliferated faster and produced an increased amount of collagen *in vitro* compared with fibroblasts from healthy individuals[87]. This might explain the increased risk of fibrosis in IBD patients, although proliferation and collagen production is also needed for epithelial layer repair. Regarding the role of stromal cells in restoring the epithelial cell layer, it was shown that the CD142^{pos} fibroblast-like subpopulation S2, which is located next to the epithelial monolayer and characterized by the expression of sheet collagens and different Wnt and BMP ligands, was diminished in the colon of UC patients[39]. Previously, it has been shown that in CD-inflamed small intestines the fibroblastic sheath surrounding the crypt contained less SMA^{pos} and tenascin-C^{pos} cells in comparison with controls[88]. These observations suggest dysregulation in the fibroblasts surrounding the crypts in both forms of IBD. In addition, after induction of dextran sodium sulfate (DSS) colitis in mice, increased numbers of Gli1^{pos} mesenchymal cells, the previously mentioned Wnt-secreting subtype of stromal cells surrounding the crypts, were found, suggesting their contribution to restoration of epithelial homeostasis[37]. Together, these studies show the mutual interaction between epithelial and stromal cells in wound healing responses in the inflamed intestine.

IBD stromal cell-responses to microbiota

When the epithelial barrier is not intact, intestinal fibroblasts are able to directly respond to microbial stimuli, like lipopolysaccharides or lipoteichoic acid through expression of TLRs. Activation of TLRs increases, among other cytokines, IL-8, IL-6 and IL-1β production by intestinal fibroblasts[89,90]. Besides TLRs, the expression of nucleotide-binding oligomerization domain-containing protein 2 (NOD2) on fibroblasts is also able to recognize bacterial products, in particular peptidoglycan-derived molecules containing muramyl dipeptide that are produced by both Gram-negative and Gram-positive bacteria[91]. Loss of function mutations in NOD2 were one of the first risk factors identified for ileal CD[92,93]. More recently, Kim and colleagues indicated colonic stromal cells as important producers of CCL2 in response to *C. rodentium* infection by activation of NOD2[44]. CCL2 is in turn responsible for the recruitment of monocytes. Whether NOD2-signaling in IBD stromal cells is altered in response to bacteria is not elucidated as yet. On the other hand, intestinal fibroblasts upregulate IL-17- and IFN-γ-induced cytokines, like IL-6, CXCL1 and

CXCL9, upon stimulation with cell-free supernatants of microbiota-reactive memory T cells ($CD4^{pos}CFSE^{low}ICOS^{high}$) from IBD patients *in vitro*[94]. These studies show both the direct and indirect impact of the intestinal microbiota on stromal cells.

Immunoregulation by IBD stromal cells

Alongside the effects of intestinal stromal cells on wound healing and their response towards microbiota, their role in immunoregulation has also been investigated in IBD. Diminished capacity of IBD human colon-derived (myo)fibroblasts to induce $FOXP3^{pos}CD127^{neg}$ Treg differentiation has been reported. Instead, a $FOXP3^{pos}CD127^{pos}$ T cell phenotype was generated, which showed a decreased expression of TGF-β1 and no expression of IL-10 and thereby reduced immunosuppressive capacities[53]. Another way in which IBD-derived stromal cells are able to affect T cells was highlighted by a recent study showing that expression of the immune checkpoint PD-L1 by (myo)fibroblasts is significantly decreased in inflamed CD colon compared with that in non-inflamed matched colon samples and colons from healthy controls[95]. The decreased PD-L1 expression could lead to a decreased suppression of IFN-γ production by Th cells. Surprisingly, PD-L1 expression by (myo)fibroblasts in UC tissue was increased compared to healthy controls, which has been linked to an increased capacity to suppress Th1 cell activity in the inflamed colon. This observation also suggests a different role for stromal cells in UC and CD. Unfortunately, in contrast to UC, no stromal subset cell analysis has as yet been performed in colonic CD, only in ileal CD so far. In the inflamed colon in UC, the abundance of both the S2, already described above, and S4 fibroblast-like population was changed[39]. While the S4 stroma subset was barely detectable in the healthy colon it was markedly expanded in UC and was found to be involved in leukocyte migration with the expression of markers like CCL19, lysyl oxidases, IL-33 and TNFSF14. This was confirmed in another recent paper, showing a comparable expanded fibroblast population (inflammation-associated fibroblasts) in UC[82], which showed enrichment for inflammation-associated genes like IL-1R1, TNFSF11, IL-13RA2. Interestingly, the expanded S4 population[39], activated fibroblasts[83] and inflammation-associated fibroblasts were associated with high expression of PDPN, a marker which has been identified to be abundantly present in the affected tissue of patients with CD or UC[96], as reported in RA.

Stromal cells both produce and respond to cytokines and chemokines. The recent scRNA-seq data set of IBD tissue revealed that fibroblasts in the inflamed bowel produce, among others, monocyte chemoattractant factors (like CCL2, CCL7)[83], T cell recruitment factors (like CXCL2, CCL19, CCL21 and CXCL12)[39,82], neutrophil attractants (like CXCL2, CXCL8 and CXCL1)[83,82] and factors involved in fibrosis (like IL-11, which is also part of the IL-6 family)[82,83,97]. Fibroblasts in the inflamed murine colon start producing CXCL12 in

response to epithelial damage, which will recruit lymphocytes towards the mucosa[98]. The importance of fibroblast derived CXCL12 on immune cell recruitment has not only been shown in intestinal epithelial damage, but also in cancer and RA. In RA, the CD34^{pos} subset of stromal cells defined by Mizoguchi and colleagues expressed CXCL12 and also other inflammatory genes like CCL2 and IL-6[59]. The CD90^{pos} subset found in RA by Stephenson and colleagues was also characterized by high expression of CXCL12 in comparison with the CD90^{neg} subset[57]. In contrast, a recent paper from Smillie and colleagues showed higher expression of CXCL12 by fibroblasts in the healthy colon compared with in UC inflamed colon[82] highlighting the need to further explore these findings in follow-up studies.

One of the cytokines that stromal cells can respond to is oncostatin M (OSM) by expression of its receptor OSMR or leukemia inhibitory factor receptor (LIFR) and GP130. OSM is produced by hematopoietic cells and was shown to regulate stromal cells in the bone marrow by suppressing their differentiation into adipocytes[5,99,100]. In peripheral tissues, OSM induces a wide range of inflammatory factors in stromal cells, like cytokines, chemokines and leukocyte adhesion factors[97]. The OSM axis is one of the pathogenic stromal signaling pathways in IBD and is implicated in anti-TNF drug resistance[96]. OSM mRNA expression is significantly increased in both CD and UC intestinal mucosal biopsies compared to non-IBD controls and its receptor, OSMR, which is mainly expressed in fibroblasts, is also highly expressed in IBD-tissue[96]. A close correlation between OSM/OSMR expression and histopathological disease severity has been reported for IBD[96]. In particular the inflammation-associated fibroblasts, which expanded during inflammation in the UC colon, showed high OSMR expression[82]. Interestingly, cardiac fibroblasts showed increased CXCL12 production in response to OSM stimulation[101] and could thereby stimulate the recruitment of immune cells by fibroblasts. Unpublished data from our group showed high OSM levels in CD associated perianal fistulas, indicating the importance of this cytokine in severe complications of IBD as well. In addition to OSMR, intestinal fibroblasts also express the IL-17 receptor, which upon stimulation has been shown to induce expression of NF-κβ inhibitor zeta and CXCL1 in CD colonic fibroblasts, leading to their pro-inflammatory phenotype[102]. IL-17 was indeed found to be increased in the intestinal mucosa of patients with IBD[103], thereby potentially modifying the activity and chemotaxis of immune cells by fibroblasts. The importance of the NF-κβ pathway in stromal cells has also been elucidated in a model of colitis-associated cancer, in which a specific knockout of IKKβ, an upstream regulator of NF-κβ pathway, in COL-VI stromal cells, caused reduced colitis and dysplasia development[104]. Interestingly, deletion of the same gene in COL1α2 stromal cells increased the susceptibility to dysplasia and was accompanied by accumulation of Tregs in the tumors[105]. This clearly shows the differential role of certain pathways in disease progression in stromal subsets. Although IL-17 can induce some pro-inflammatory

pathways in stromal cells[106], it was also suggested that IL-17 is able to downregulate the TNF-α-induced CCL5 secretion by subepithelial myofibroblasts and thereby immune cell recruitment[45]. The most well studied cytokine in IBD is TNF-α, since it is the main target of the effective and often prescribed anti-TNF therapy. Although macrophages are the main TNF-α producers, myofibroblasts also signal through transmembrane TNF. CD- and UC-derived myofibroblasts from actively inflamed areas expressed more transmembrane TNF compared with non-inflamed cells or myofibroblasts from healthy controls[107]. Thereby CD and UC myofibroblasts pose a direct target for anti-TNF-α therapy (as discussed in the chapter below). Furthermore, TNF-α-induced genes, like CXCL1, CXCL6 and CCL2 were highly expressed by activated fibroblasts found in inflamed CD tissue[83]. In addition to cytokines, stromal cells also produce the enzyme COX-2, which is important for the conversion of arachidonic acid into prostaglandin E2. COX-2 expression is, compared with in healthy controls, enhanced in the S3 fibroblast subset of UC patients[39]. Upregulation of COX-2 was also shown before in ileum derived CD fibroblasts[102]. Specific COX-2 ablation in intestinal myofibroblasts increased susceptibility to DSS-induced colitis, especially in the initiation phase[108]. These data suggest that COX-2 upregulation by myofibroblasts is a regulatory mechanism for controlling inflammation. However, for many markers expressed by stromal cells in IBD their role in stimulating or inhibiting ongoing inflammatory responses is as yet unknown.

The analysis by Martin and colleagues of inflamed ileal tissue from CD patients revealed that the presence of activated fibroblasts was highly correlated with the presence of inflammatory macrophages, activated dendritic cells, strongly activated T cells, IgG-producing plasma cells and atypical chemokine receptor 1-activated endothelial cells[83]. They also showed that the inflammatory macrophages ($CD68^{pos}CD206^{neg}$) were always in close vicinity of $PDPN^{pos}$ fibroblasts. This cell profile associated with high levels of activated fibroblasts was only found in a subset of patients and did not correlate with, for example, pathologic severity or disease duration. The activated fibroblasts strongly expressed CCL2 and CCL7, ligands for CCR2, which are expressed by circulating classical monocytes and facilitates their recruitment in tissues. On the other hand, the inflammatory macrophages, likely derived from these monocytes, produced inflammatory cytokines like TNF-α, IL-1β, OSM and IL-6, which are all cytokines associated with the activation of fibroblasts[83,96].

These data show the complexity of versatile, sometimes reciprocal, cytokine interactions thereby fine-tuning the function of immune and stromal cells. Taken together, it seems that particular subset of fibroblasts in inflammatory (bowel) diseases can affect the immune system both by the production of soluble factors but also by direct cell-to-cell contact. The first evidence for subpopulations of immunoregulatory fibroblasts, identified by for

example CD90 and CD55 expression, and characteristics of pathogenic fibroblasts, by PDPN or CXCL12 expression, are arising.

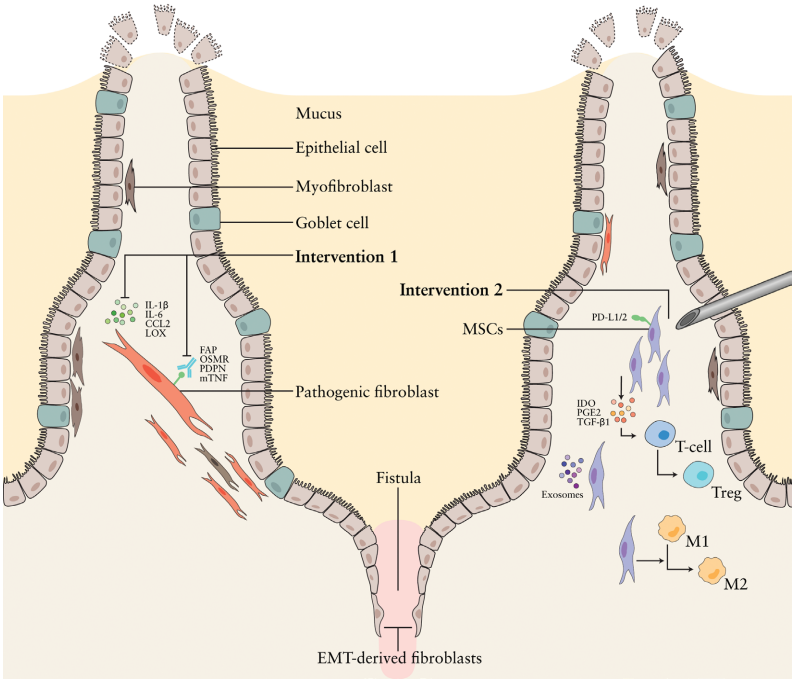

FIGURE 2. Targeting stromal subsets in luminal IBD and CD-associated perianal fistulas. 1: Targeting stromal subsets in IBD. Pathogenic stromal cells could be directly targeted via surface markers like OSMR, mTNF, PDPN and FAP or indirectly by blocking the soluble factors pathogenic stromal cells produce like LOX. 2: Local MSC-therapy. MSCs modulate immune cell responses, thereby reducing the number of proliferating T cells and stimulating the conversion of T cells into regulatory T cells and immunosuppressive 'M2' macrophages. Furthermore they support epithelial regeneration. In these processes soluble factors like IDO, VEGF, HGF, PGE2, surface markers like PD-L1, ICAM and MSC-derived exosomes are involved.
Treg – regulatory T cell, IL- interleukin, LOX – lysyl oxidase, CCL2 – chemokine ligand 2, PDPN – podoplanin, OSMR – oncostatin M receptor, mTNF – membrane-bound tumor necrosis factor, FAP – fibroblast activation protein, PGE2 – prostaglandin E2, IDO – indoleamine, PD-L1 - programmed death-ligand 1, TGF-β – transforming growth factor β.

THERAPEUTIC MODALITIES TO MODIFY THE STROMAL COMPARTMENT IN IBD

The involvement of stromal cells in the pathogenesis of IBD also makes them an interesting therapeutic target. The ultimate goal of stromal IBD therapy would be to normalize the stromal cell compartment in the inflamed gut, which could be performed in two ways (summarized in Figure 2). The first way is to directly target the pathogenic stromal cells that play a role in immune cell recruitment and activation. The identification of these pathogenic stromal cell subsets is still ongoing, but several potential subset targets have been identified, which we will discuss in more detail below. However, because most target molecules will not be organ specific and found on stromal cells throughout the whole body, severe side effects form a potential risk and therefore it might be a safer approach to normalize the stroma in another way. This could be circumvented via the introduction of 'healthy' stromal cells, in order to inhibit the inflammatory immune response and restore the epithelial cell layer. The development of clinical applications using 'healthy' allogeneic MSCs has been an important field of research in several inflammatory diseases, including IBD, in recent years.

Targeting stromal cells

Before defining new therapies to target stromal cells, currently applied IBD medication may also be able to target stromal cells. The presence of transmembrane TNF-α on fibroblasts makes them a target for anti-TNF-α therapy as well. Anti-TNF-*α* treatment with infliximab on CD-myofibroblasts *in vitro* increased tissue inhibitors of metalloproteinases (TIMP)-1 myofibroblast expression and thereby stimulated the migratory potential of the CD-myofibroblasts[107]. In this way anti-TNF therapy could restore the wound healing potential of stromal cells in IBD. Next to directly inhibiting TNF-α function, anti-TNF-*α* therapy is able to induce (indirect) apoptosis in immune cells[109]. Interestingly, CD myofibroblasts revealed to be resistant to infliximab-induced apoptosis *in vitro*, which could be explained by the fact that peripheral blood mononuclear cells (PBMCs) are needed for induction of anti-TNF therapy-induced apoptosis in fibroblasts[110]. In RA, it was found that the TNF-α targeting antibodies infliximab and adalimumab, were less efficient in inducing apoptosis in fibroblasts in the presence of PBMCs than etanercept via upregulating the anti-apoptotic molecule B cell lymphoma (Bcl)-2[110]. In IBD patients, the TNFRII-Fc fusion protein etanercept (binding only soluble and not transmembrane TNF-α) showed, in contrast to the monoclonal antibodies infliximab and adalimumab, no clinical efficacy[111], which could suggest that targeting of stromal cells by anti-TNF therapy is different in IBD compared with in RA. It will be important to unravel to what extent anti-TNF-α therapy is affecting stromal cells in IBD patients and to elucidate a potential subtype of patients that would benefit more from etanercept, perhaps in adjunct to infliximab or adalimumab, since it is thought

to have a higher apoptotic potential for fibroblasts. Interestingly, the intestinal cell profile detected in some of the CD patients in association with high levels of activated fibroblasts, was enriched in non-responders to anti-TNF therapy in a paediatric CD cohort[83,112]. This suggests that a subtype of activated fibroblasts could play a role in resistance to anti-TNF therapy. Also in the inflamed colon of UC patients it was found that the inflammation-associated fibroblasts were especially enriched in pre-treatment samples from patients who did not respond to anti-TNF therapy[82]. So, the presence of activated fibroblasts in CD, (characterized by CD90, PDPN and increased IL-6, IL-11 and CCL2[83]), inflammation-associated fibroblasts in UC, (showing IL-11, IL-25 and IL-13RA2 expression[82]), and OSMR tissue expression[96] was associated with resistance to anti-TNF therapy. Characterizing fibroblasts in inflamed tissue at diagnosis could therefore be helpful in selecting which patient is likely to respond to anti-TNF therapy and in which patients other therapeutic strategies should be used.

Potentially pathogenic (myo)fibroblasts in the intestine of IBD have been shown to express OSMR[82,96,113], PDPN[39,96], and the S4 subset[39] markers in UC: CCL19, LOX, IL-13 and TNFSF14. LOX was also found to be overexpressed by CD stenotic myofibroblasts[114]. LOX inhibition restored both MMP3 activity in stenotic myofibroblasts and prevented aberrant ECM contraction. *In vivo*, the Lox/Loxl1 inhibitor β-aminopropionitrile (BAPN) resulted in reduced disease severity in a mouse model for colitis[39]. Interestingly, the sequencing data of the pathogenic S4 subpopulation[39] and inflammation-associated fibroblasts[82] showed that FAP is also upregulated in UC stroma. FAP is a proline-selective protease, involved in the procession of other proteins and peptides[115]. FAP can directly enhance proliferation, migration, and invasion of cells by which it is expressed. Interestingly, CAFs with high expression of FAP produced more CCL2[116]. Thus targeting FAP, could stop IBD-associated fibroblast proliferation and reduce the production of CCL2 by fibroblasts, and thereby the recruitment of myeloid cells. Anti-FAP therapy to target CAFs has already been tested in clinical trials for several malignancies and could also be a potential therapy to target the S4 fibroblasts/inflammation-associated fibroblasts in UC. The feasibility and safety of targeting FAP in the stroma of patients was demonstrated by Phase I clinical studies, applying monoclonal antibodies to advanced FAP-positive cancer patients[117,118]. No major safety concerns were detected in humans, although ablation of FAP-expressing bone marrow stromal cells was observed in mice treated with anti-FAP[119,120]. In the meantime, many different approaches to potentially blocking FAP via low molecular weight compounds, immunoliposomes, vaccines, and chimeric antigen receptor (CAR) T cells[119] have been developed. Also in a mouse model for RA, FAP deletion, even when only depleted into the joints, showed resolution of the disease[58]. Within the FAP^{pos} cell population, $PDPN^{pos}CD90^{pos}$ cells seemed to contribute the most to the inflammation, since

injection of this specific subpopulation in the joints resulted in more severe and sustained joint swelling, compared with the PDPN^{pos}CD90^{neg} subpopulation. *Ex vivo* inhibition of FAP in CD strictures demonstrated reduced production of type I collagen and TIMP-1[113], which suggest that anti-FAP therapy could be also targeting (IBD-related) fibrosis.

Next to FAP and LOX targeting, the correlation between OSMR on fibroblast-like cells and disease activity in IBD patients gives rise to exploring OSMR targeting. OSMR targeting by a Fc-tagged soluble OSMR-gp130 fusion protein was shown to significantly attenuate colitis in an IBD mouse model resistant to anti-TNF therapy[96]. Furthermore, adenoviral transfer of OSM also reduced the severity of DSS-induced colitis[121]. A phase II clinical trial was performed for an anti-OSM humanized monoclonal antibody (GSK315234) in RA. The data from this study did not show potent clinical efficacy, but it demonstrated the safety of the drug[122]. Further exploring the role of the OSMR on stromal cells in IBD might optimise patient selection for anti-OSM therapy in IBD. In this regard the effectiveness of JAK inhibitors in IBD is interesting, since the JAK pathway is downstream of the OSMR and therefore the effects of JAK inhibitors on stromal cells could teach us more about the OSM-OSMR pathway[123]. As described before, PDPN is also upregulated in pathogenic IBD stromal cells. PDPN regulates cell shape and movement, and is thereby involved in cell migration[124,125]. In the meanwhile it is also the ligand for C-type lectin-like receptor 2, expressed by platelets and some subtypes of myeloid cells, and involved in chemokine and cytokine production[126]. Targeting PDPN could therefore potentially block the interaction with myeloid cells. In preclinical studies, targeting PDPN, using CAR-T cells, antibodies and lectins, successfully inhibited the growth of PDPN^{pos} tumor cells[127]. In RA it was shown that anti-PDPN antibodies protected mice from collagen-induced arthritis by targeting the PDPN expressing synovial fibroblasts[128]. It would be interesting to unravel whether this process is mediated by decreased fibroblast migration or the interaction with platelets or myeloid cells. No studies to target PDPN in mouse models of experimental colitis have been reported yet. Interestingly, Th17 cells also express PDPN[129], suggesting anti-PDPN is able to target both pathogenic stromal and immune cells.

TABLE 1. Clincial trials in IBD applying local injection of mesenchymal stromal cells.

Local MSC administration - fistulising CD												
Indication	***n***	**Placebo-controlled**	**Cell type**	**Dosage**	**Evaluation**	**Efficacy**	**Placebo response rates**	**Follow-up**	**Safety**	**Clinical trial**	**Year**	**Study**
CD fistulas (perianal, rectovaginal, entero-enteric)	4	no	Adipose autologous	3-30 x 10^6	8 w	Healing: 6/8 Partial closure: 2/8	-	12-22 m	No AE	Phase I	2005	Garcia-Olmo *et al.* [146]
Perianal fistulas (cryptoglandular and CD)	49 (24 MSC)	yes	Adipose autologous	20 x 10^6 + F / second dose (40 x 10^6 + F) if incomplete closure after 8 w	8 w	Healing: 17/24 - 11 with single injection - 6 after 2nd injection	Healing: 4/25	12 m (38 m#)	2 SAE (not MSC-related)	Phase IIb	2009	Garcia-Olmo *et al.* [147] #Guadalajara *et al.* [148]
Perianal CD fistulas	10	no	Adipose autologous	10 x 10^6, 20 x 10^6 or 40 x 10^6 MSC/ml (proportional to fistula size - total number: 30-400 x 10^6)	8 w	Healing: 3/10 Partial closure: 7/10	-	8 m	No AE	Phase I	2013	Cho *et al.* [149]
Perianal CD fistulas	43 (completed 33)	no	Adipose autologous	30-60 x 10^6 MSC/cm (proportional to fistula size) + F / second dose (1.5x more MSC) if incomplete closure after 8 w	8 w	Healing: 27/33 Incomplete closure: 6/33	-	12 m	No AE	Phase II	2013	Lee *et al.* [150]
Perianal CD fistulas	12	no	Adipose autologous	20 x 10^6	6 m	Healing: 10/12	-	6 m	No AE	Phase I	2017	Dietz *et al.* [151]

TABLE 1. Continued.

Local MSC administration - fistulising CD											
Indication	***n***	**Placebo-controlled**	**Cell type**	**Dosage**	**Evaluation**	**Efficacy**	**Placebo response rates**	**Follow-up**	**Safety**	**Clinical trial**	**Year**	**Study**
CD fistulas (perianal, enterocutaneous)	10	no	Bone marrow autologous	15-30 x 10^6 / monthy (total 2-5x)	at each treatment (monthly) and 3, 6 and 12 months later	Healing: 7/10 Incomplete closure: 3/10	-	12 m (60 m[$])	No AE	Phase I	2011	Ciccocioppo *et al.* [152] [$][153]
Perianal CD fistulas	24 (completed 16)	no	Adipose allogeneic	20 x 10^6 / second dose (40 x 10^6) if incomplete closure after 12 w	12 w and 24 w	Healing: 8/16	-	6 m	5 MSC-related AE	Phase I/IIa	2013	De La Portilla *et al.* [154]
Perianal CD fistulas	6	no	Adipose allogeneic	10 x 10^6 or 30 x 10^6 MSC/ml (proportional to fistula size)	8 w	Healing: 3/6	-	8 m	No AE	Phase I	2015	Park *et al.* [155]
CD fistulas (rectovaginal)	10 (completed 5)	no	Adipose allogeneic	20 x 10^6 / second dose (40 x 10^6) if incomplete closure after 12 w	3 m and 12 m	Healing: 3/5	-	12 m	No AE	Phase I	2015	Garcia-Arranz *et al.* [156]
Perianal CD fistulas	212 (107 MSC)	yes	Adipose allogeneic	120 x 10^6	24 w	Healing: 53/107	Healing: 36/105	12 m	5 MSC-related SAE	Phase III	2016	Panes *et al.* [133] [157]
Perianal CD fistulas	21 (15 MSC)	yes	Bone marrow allogeneic	10 x 10^6, 30 x 10^6 or 90 x 10^6	6 w, 12 w and 24 w	Healing: 9/15	Healing: 2/6	6 m (48 m*)	2 SAE (not MSC-related*)	Phase IIa	2015	Molendijk *et al.* [132] *Barnhoorn *et al.* [134]

F: fibrin glue; AE: Adverse event; SAE: Serious adverse event; d: days; w: weeks, m: months.

TABLE 2. Clincial trials in IBD applying intravenous injection of mesenchymal stromal cells.

Intravenous MSC administration - luminal IBD

Indication	*n*	Placebo-controlled	Cell type	Dosage	Evaluation	Efficacy	Placebo response rates	Follow-up	Safety	Clinical trial	Year	Study
CD	9	no	Bone marrow autologous	2x 1-2 x 10^6 MSC/kg, 7 days apart	6 w and 14 w	no clinical remission, but clinical response in 3/9; though 4/9 disease worsening	-	14 w	No AE	Phase I	2010	Duijvestein *et al.* [158]
CD	12	no	Bone marrow autologous	2 x 10^6, 5 x 10^6 or 10 x 10^6 MSC/kg	2 w	clinical response in 5/11	-	9 w	7 SAE (2 MSC-related)	Phase I	2016	Dhere *et al.* [159]
CD/UC	7 (4 CD / 3 UC)	no	Bone marrow allogeneic (or umbilical cord)	1 x 10^6 MSC/ kg	3 m	clinical remission in 5/7 (CD 2/4; UC 3/3)	-	6-32 m	No AE	Phase I	2012	Liang *et al.* [160]
CD	16 (completed 15)	no	Bone marrow allogeneic	4x 2 x 10^6 MSC/kg once per week	6 w	clinical remission in 8/15 (clincial response 12/15)	-	-	No AE related to MSC	Phase II	2014	Forbes *et al.* [161]
CD	12	no	Placenta allogeneic	2x 2 x 10^8 or 8 x 10^8 once per week	6 m	clinical remission in 3/12 (clinical response 8)	-	24 m	No AE	Phase I	2013	Mayer *et al.* [162]

TABLE 2. Continued.

Intravenous MSC administration - luminal IBD												
Indication	***n***	**Placebo-controlled**	**Cell type**	**Dosage**	**Evaluation**	**Efficacy**	**Placebo response rates**	**Follow-up**	**Safety**	**Clinical trial**	**Year**	**Study**
CD	50 (34 MSC)	yes	Placenta allogeneic	2x 1.5 x 10^8, 6 x 10^8 or (12 x 10^8) once per week	4 w and 6 w	clinical remission in 4/28 (clinical response 10/28)	clinical remission: 0/16 clinical response: 0/16	24 m	10 MSC-related SAEs	Phase Ib/ IIa	2015	Melmed *et al.* [163]
CD	82 (41 MSC)	(yes) - normal treatment	Umbilical cord allogeneic	4x 1 x 10^6 MSC/kg once per week	12 m	no clinical remission, but improved clinical and endoscopic scores	no clinical remission	12 m	No SAE		2018	Zhang *et al.* [164]

AE: Adverse event; SAE: Serious adverse event; d: days; w: weeks, m: months.

MSC-therapy

MSCs are multipotent stromal cells that are able to differentiate, at least *in vitro*, into a variety of cell types and are capable of immunomodulation and tissue regeneration[130]. MSCs can be isolated from different tissues, but are mostly derived from adipose tissue and the bone marrow. In fistulizing CD, treatment with MSCs has been shown to be safe and effective (Table 1). Perianal fistulas, which are abnormal passageways between the colon and skin around the anus, are a serious complication of CD[131]. A study from our group[132] showed that local application of bone marrow-derived MSCs led to fistula healing in 80% (4/5) of the patients. In accordance with these results, a double-blind placebo controlled, multicentre study showed that local treatment with adipose-derived MSCs (Cx601/ darvadstrocel) led to significantly improved fistula closure in MSC-treated patients compared with placebo-treated patients after 24 weeks[133]. Accordingly, darvadstrocel has now been approved as a treatment for refractory CD associated perianal fistulas in Europe. Importantly, the clinical effects of MSCs seem to remain for a longer period of time, as we were recently able to show in our 4-year follow-up study[134]. The treatment of luminal IBD with MSC-therapy has also been investigated in pre-clinical models[135-137] and Phase I/II clinical trials (Table 2). Systemically applied MSCs are able to alleviate experimental colitis in mice[135,136], but in humans no convincing clinical responses upon systemic administration were observed. Therefore we focused on local MSC-therapy for luminal IBD. In pre-clinical experiments local administration of MSCs in the inflamed bowel during endoscopy in DSS-induced colitis in mice showed attenuation of colitis[138] and mucosal injections of colon derived MSCs were more effective in preventing ulcer development compared with intravenously injected MSCs in a colonic wound model[139]. Recently, a phase I clinical trial started in the Leiden University Medical Center (https://www.trialregister.nl/trial/6949; EudraCT number: 2017-003524-75) to determine the safety of local MSC injections in the bowel of patients with refractory ulcerative proctitis.

MSC-therapy could be seen as an approach to normalize the intestinal stroma by the introduction of healthy allogeneic MSCs. Our unpublished data showed, for example, that MSCs express much lower levels of the pathogenic fibroblast marker PDPN, compared with IBD-derived fibroblasts, which demonstrates their 'healthy' phenotype. Like fibroblasts, MSCs are able to modulate local inflammation as well as to support epithelial regeneration. It has been suggested that MSCs are able to suppress immune cell responses through secretion of paracrine factors and by cell-cell contacts[90]. Furthermore, it has been postulated that the therapeutic effects of MSCs in perianal fistulizing CD is partly due to their PD-L1 expression[34]. When focusing on the effects of MSCs on epithelial repair, we showed the ability of MSCs to enhance epithelial proliferation and migration via secreted soluble factors, but also to some extent via MSC-derived exosomes (Barnhoorn et al,

submitted). Next to pro-regenerative and direct immune suppressive functions recently a new hypothesis regarding the workings mechanism has been postulated, in which MSCs upon intravenous injection undergo apoptosis and effect immunosuppression via modulation of the monocytes by which they have been phagocytosed[140,141]. However, there are no data available yet that show that local MSC-therapy works in a comparable manner and our published data show at least the engraftment and survival of locally injected MSCs up to 6 days post-injection[138].

While stromal cell therapy is mainly focused on the use of MSCs, other stromal cells, like fibroblasts may also be capable of stimulating tissue repair and suppressing immune responses. In a Phase II trial spray-applied allogeneic neonatal keratinocytes and fibroblasts successfully treated chronic venous leg ulcers[142]. Furthermore, transplantation of autologous skin fibroblasts and adipose tissue[143,144], including stromal cells, has also been suggested for the treatment of CD perianal fistulas[145].

CONCLUSION

Although unraveling the role of stromal cells in IBD pathogenesis has just started, current research is already showing a considerable role for the different subsets of intestinal stromal cells. In this review we focused on their heterogeneity and the role of stromal subtypes on epithelial repair and immune homeostasis.

There are several challenges investigating and reporting on stromal cells in IBD. One of the difficulties in stromal research is the lack of agreement on the exact and uniform definition of stromal cell subtypes. Although there seems to be agreement on, general fibroblast markers, the use of these markers varies between studies. This makes it difficult to generate a clear overall picture of the recent findings on the various subtypes of stromal cells, as it is unclear whether all studies were actually examining the same cell type. Furthermore, certain subtype definitions do not withstand. For example, the α-SMA^{pos} myofibroblast was always thought to be important for epithelial homeostasis, however several recent studies also showed that α-SMA^{neg} stromal cells surround the epithelial crypt and produce factors important for epithelial homeostasis. Based on the relatively low number of published studies so far it seems there is high heterogeneity between individuals, organs and diseases. In addition, the different isolation and analysis techniques used resulted in the identification of different subtypes. Addressing these problems and setting a stricter definition of stromal cell types, would allow a more accurate and representative subclassification.

Many of the studies discussed in this review have analysed cultured stromal cells, which might have changed their phenotype and functions compared with their *in vivo* counterparts. For example, the immunomodulatory properties of healthy intestinal stromal cells were shown in many studies using cultured fibroblasts. However, in freshly isolated cells *in vivo* only a subpopulation of fibroblasts expressed factors that could potentially affect immune cells[39,82]. In addition, in most studies the effects of medication used by the patient on the function and expression profile of stromal cells has not yet been taken into account. This could have biased results, since for example anti-TNF therapy might also directly influence fibroblasts as indicated above.

Although IBD is mentioned as one disease entity, there are interesting differences between UC and CD, also between stromal cells in CD and UC, which need to be studied in more detail in the future. More generally, it will also be important to unravel which changes in stromal cells subsets are "inflammation"-mediated and which changes are "IBD specific". Data from other inflammatory disease of the gut, like infectious or microscopic colitis, should shed light on this. New technological advances, allowing the analysis of non-cultured fibroblasts and the screening of many samples in depth for both RNA and protein expression profiles, are expected to extend the knowledge of stromal cells in the inflamed and non-inflamed gut. However, in addition the phenotype of stromal cells, also their function needs to be elucidated further, and therefore more advanced three-dimensional culture systems and transgenic rodent systems will be needed to unravel the complex and mutually interactive role of human intestinal stromal cells in contact with immune cells and epithelial cells.

Direct targeting of pathogenic stromal cells in IBD is still difficult, since the specific pathogenic subtypes are not yet well defined. The challenge lies in restoration of the stromal cells that support the epithelial cells, while targeting the stromal cells that attract and aberrantly activate immune cells. For now, the introduction of local MSCs seems to be a safer option in order to modify the stromal component in IBD, since many potential stromal targets would also be targeted for healthy stromal cells in other organs. Furthermore, since stromal cells seems to be involved in anti-TNF resistance, the characterization of stromal cells in inflamed tissue at diagnosis could be helpful in predicting disease course and therapeutic responses. In conclusion, the field of stromal IBD research is developing and will improve knowledge of the pathogenesis of both UC and CD in the coming decade, hopefully providing novel insights and therapeutics approaches.

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Conflict of interest
The authors declare that there are no conflicts of interest.

Author contributions
M.C.B. conducted literature search, wrote the manuscript and designed the figures; S.H. conducted literature search and critically revised the manuscript; R.S.B. conducted literature search, designed the tables and critically revised the manuscript; G.R. critically revised the manuscript; L.J.A.C.H. and M.S. supervised the writing process and critically revised the manuscript. All authors approved the final version of the article.

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