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Antifibrotic strategies in *in vitro* and *in vivo* models of inflammatory bowel disease

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Dottoranda

Serena Artone

Coordinatore del corso

Prof. Claudio Ferri

Relatore

Prof. Giovanni Latella

Co-Relatrici

Prof.ssa Theresa T. Pizarro

Prof.ssa Benedetta Cinque

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ABSTRACT

The underlying mechanism of intestinal fibrosis, a major complication of inflammatory bowel disease (IBD), remains incompletely understood, and no therapies currently exist to prevent or reverse this condition. Intestinal fibrosis develops due to ongoing tissue damage and is closely linked to chronic inflammation. It is, in fact, a frequent complication in patients with Crohn's Disease (CD), with more than 70% of patients experiencing complications such as stenosis and fistulae due to the progression of fibrosis. While there has been significant progress in understanding the mechanisms of IBD-related inflammation and potential treatment options, the development of effective anti-fibrotic therapies remains limited. The partial effectiveness of existing treatments highlights the urgent need to explore new strategies for addressing fibrosis in IBD and improving patient outcomes. In this study, the ability of various drugs commonly used in IBD to counteract key processes involved in intestinal fibrosis was assessed. Specifically, the differentiation of intestinal fibroblasts to activated myofibroblasts was examined using CCD-18Co cells, and the epithelial-to-mesenchymal transition (EMT) of intestinal epithelial cells was examined using Caco-2 cells (IEC), with both processes being induced by transforming growth factor-β1 (TGF-β1). The tested drugs included mesalamine, azathioprine, methotrexate, prednisone, methylprednisolone, budesonide, infliximab, and adalimumab. Fibrosis and EMT markers (collagen-I, α-smooth muscle actin (α-SMA), pSmad2/3, occludin) were evaluated through Western blot analysis and immunofluorescence. Among the drugs tested, prednisone, methylprednisolone, budesonide, and adalimumab were the only ones that effectively counteracted the pro-fibrotic effects induced by TGF-β1 in CCD-18Co cells, reducing the expression of fibrosis markers. Additionally, methylprednisolone, budesonide, and adalimumab were able to significantly reduce the TGF-β1-induced EMT process in Caco-2 IECs, increasing occludin expression while decreasing α-SMA expression.

Moreover, given the involvement of the renin-angiotensin system (RAS) in fibrosis, therapeutic strategies targeting this pathway have gained attention. Losartan, an angiotensin II (ANGII) type 1 receptor blocker (ARB), is commonly used in hypertension management and has demonstrated potential anti-fibrotic effects in other organ systems. Therefore, this study aims to investigate the potential of losartan in reducing intestinal fibrosis both *in vitro* and *in vivo*, as well as its ability to prevent the recurrence of inflammation and fibrosis after steroid treatment. The efficacy of losartan to counteract ANGII-induced profibrotic responses was *in vitro* tested using CCD-18Co performing western blot analysis and immunofluorescence analysis. ANGII promoted myofibroblast differentiation and upregulated key profibrotic genes, including TGFβ, insulin-like growth factor 1 (IGF1), and Collagen type 1 alpha 1 chain (COL1A1). Losartan reversed these effects, restoring the expression of profibrotic genes to near-control levels and inhibiting myofibroblast activation. Further

in vivo investigation was conducted using the SAMP mouse model, known for spontaneous CD-like ileitis, to better reflect human intestinal fibrosis. Quantitative real-time PCR (qPCR) indicates that the RAS signaling components in SAMP mice displayed notable changes in expression over time compared to AKR control mice. Initially, there was an upregulation of *Angiotensinogen* (*Agt)* and *Renin* (*Ren)* and a downregulation of the *Angiotensin-converting enzyme* (*Ace)*. As inflammation and fibrosis progressed, there was a shift towards activating alternative pathways, likely to counteract these pathological changes. Notably, *Ren* remained elevated in SAMP mice with established disease. Next, the efficacy of losartan in modulating intestinal fibrosis was *in vivo* confirmed. Treatment with losartan led to a significant reduction in both inflammation and fibrosis in the distal ileum of SAMP mice, as evidenced by lower scores for inflammation and fibrosis shown by Hematoxylin & Eosin (H&E) and Masson's Trichrome (MTC) staining. This effect was associated with a decrease in angiotensin type 1 receptor (AGTR1) expression showed by immunohistochemistry (IHC), likely leading to the downregulation of profibrotic genes and reduced COL1A1 deposition as evidenced by qPCR analysis and immunofluorescence respectively. Notably, losartan was able to maintain its anti-fibrotic effects even following dexamethasone (DexM) treatment, preventing the rebound of fibrosis and reducing COL1A1 infiltration into deeper tissue layers, likely through AGTR1 reduction.

In conclusion, these findings highlight the direct antifibrotic effects of drugs currently used in IBD, suggesting which drugs may have potential antifibrotic properties. Additionally, they indicate that losartan exerts a targeted anti-fibrotic effect that is independent of its anti-inflammatory action.

1. INTRODUCTION

1.1 Inflammatory bowel disease

Inflammatory bowel disease (IBD) is a collective term for chronic, relapsing inflammatory disorders of the gastrointestinal (GI) tract. The two main types of IBD are ulcerative colitis (UC) and Crohn's disease (CD), which differ in clinical presentation, pathophysiology, and disease complications (Eom *et al*., 2018). The global prevalence of IBD has been steadily rising, particularly in newly industrialized regions, a trend associated with environmental and lifestyle changes, including shifts in diet, increased antibiotic use, and urbanization. Although IBD predominantly affects young adults, it can occur at any age. Approximately 25% of cases are diagnosed in individuals under 20 years old. Within pediatric IBD, the highest incidence occurs during adolescence, though about 20% of cases are present before the age of 10, and roughly 5% are diagnosed before the age of 5. Worldwide, the incidence of IBD, particularly childhood-onset forms, has been increasing (Borowitz, 2023). The etiology of IBD remains poorly understood, but it is generally accepted that a multifactorial process involving genetic susceptibility, intestinal microbiota imbalance, immune dysregulation, and environmental exposures plays a crucial role in initiating and maintaining intestinal inflammation (**Figure 1)** (Yuan *et al*., 2023).

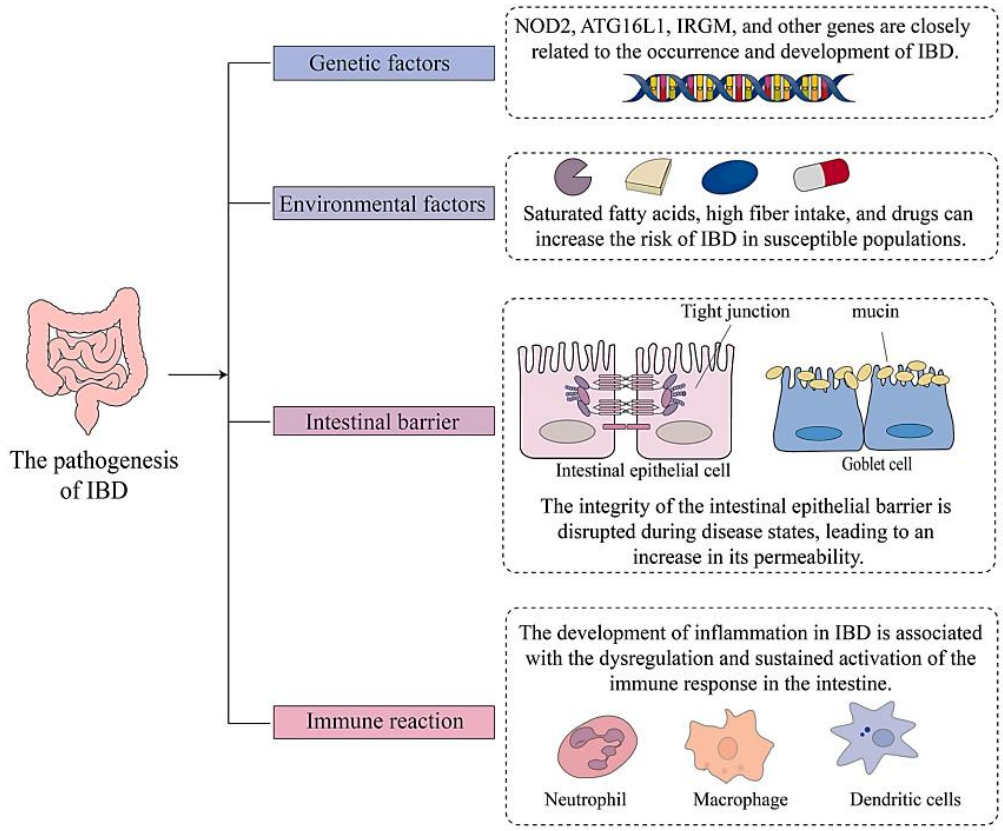


Figure 1. The pathogenesis of IBD. (Yuan *et al*., 2023)

Environmental factors

Environmental factors that contribute to gut dysbiosis and IBD include lifestyle choices like smoking, obesity, lack of physical activity, and dietary habits. Specifically, a Western-type diet, which typically involves high consumption of saturated fats, refined sugars, red and processed meats, and low consumption of fruits, vegetables, fiber, and fish, has been associated with these conditions (Carreras-Torres *et al*., 2020)

- Smoking

Cigarette smoking is a recognized environmental risk factor for IBD, although its mechanisms of action remain unclear. In Western populations, smoking increases the risk of CD while decreasing the risk of UC. In UC, smoking may influence the immune system by reducing TNF-alpha production, increasing IL-10 levels, and altering mucin synthesis and inflammatory responses in the colon. Conversely, in CD, smoking can impair vasodilation in inflamed microvessels, resulting in ischemia and exacerbating ulceration and fibrosis (Abegunde *et al*., 2016). Smoking also impacts oxidative stress: mononuclear cells from smokers with CD, but not UC, exhibit reduced sensitivity to anti-inflammatory protection against oxidative free radical stress due to decreased synthesis of heat shock protein 70 (Bergeron *et al*., 2012). Furthermore, smoking influences the gut microbiota, as smoking cessation is linked to early changes in the microbiome, which may interact with immune responses to affect UC development and progressions (Ananthakrishnan, 2015).

- Diet

Diet plays a critical role in the development of IBD, beginning as early as infancy. Breastfeeding has been shown to confer significant protective effects against IBD, particularly when sustained for at least 12 months. This protective effect may arise from breastmilk's role in supporting a healthy intestinal microbiome, enhancing mucosal integrity, and providing immunoglobulins that guard against enteric infections (L. Xu et al., 2017). As solid foods are introduced, their composition and preparation methods continue to influence microbiome diversity and gut health (Homann *et al*., 2021). For example, Western diets rich in processed foods often include preservatives and emulsifiers, which have been associated with an increased risk of CD. Emulsifiers, in particular, can disrupt intestinal mucus layers, promoting bacterial translocation and inflammation, as demonstrated in both mouse models and ex vivo human microbiota studies (Chassaing *et al*., 2015) (Naimi *et al*., 2021).
High-fat diets, another hallmark of Western dietary patterns, exacerbate IBD risk and severity through multiple mechanisms. Obesity associated with such diets promotes inflammation in mesenteric fat, increases intestinal permeability, and alters microbiota composition. Additionally, high-fat diets elevate levels of proinflammatory adipokines, such as leptin, contributing to both local and systemic

inflammation (Paik *et al*., 2013). However, certain dietary fats like omega-3 polyunsaturated fatty acids may offer protective effects, potentially mitigating colitis severity. Specific nutrients also influence inflammatory processes in IBD. Amino acids such as tryptophan, glutamine, and histidine have shown promise in experimental models, where their supplementation improved colitis outcomes by regulating gut homeostasis and downregulating inflammatory cascades. Similarly, plant polysaccharides and poorly digestible fibers enhance the production of anti-inflammatory short-chain fatty acids, reducing colitis severity (Sugihara *et al*., 2019). Vitamin D supports intestinal mucosal integrity and suppresses proinflammatory responses, acting directly on T cells and macrophages in experimental models (Fletcher *et al*., 2019) (Meeker, 2016). In contrast, dietary iron has pro-inflammatory effects, generating free radicals that damage intestinal cells and altering the microbiome to produce harmful metabolites (Werner *et al*., 2011). These findings underscore the complex interplay between diet, inflammation, and IBD, highlighting opportunities for dietary interventions in disease prevention and management.

- Antibiotics

The gut microbiome, which consists of billions of bacteria, is essential for digesting food, protecting against harmful pathogens, and regulating immune responses. However, antibiotics, particularly broad-spectrum ones, can disrupt the balance of this microbiome by killing both harmful and beneficial bacteria. This disruption can negatively affect microbial diversity and ecology, potentially increasing the risk of certain diseases, including IBD (Meng *et al*., 2021) (Duong *et al*., 2022). Duan *et al*. found that exposure to antibiotics was significantly linked to an increased risk of developing new-onset IBD, including both CD and UC, in both children and adults, based on prescription data. Most antibiotic classes were associated with a heightened risk of IBD, with metronidazole and quinolones showing particularly higher risk estimates. Additionally, a positive nonlinear dose-response relationship was observed, indicating that the risk of IBD increased with the number of antibiotic prescriptions (Duan *et al*., 2024). Additionally, a population-based case-control study involving 736 IBD patients and 1,472 controls found that antibiotic use was more frequent among IBD cases (61.8%) compared to controls (33.6%). Prolonged antibiotic use (≥30 days) was associated with an even higher risk, with the highest risk observed in individuals under 18 years. These findings reinforce the idea that antibiotic use may be a significant factor in the development of both CD and UC (Aniwan *et al*., 2018).

- Stress

Chronic stress affects the gastrointestinal (GI) system, particularly through its influence on intestinal microbes. One notable consequence is the dysregulation of the hypothalamic-pituitary-adrenal (HPA)

axis, which can lead to disruptions in the integrity of the intestinal barrier (Gubert *et al*., 2020). This weakened barrier function increases susceptibility to inflammation and may exacerbate underlying conditions. Humbel *et al*. (2020) suggest that intestinal dysbiosis can initiate oxidative stress, creating a feedback loop that further aggravates microbial imbalances (Humbel *et al*., 2020). For instance, lower microbial diversity has been observed in individuals experiencing heightened stress and anxiety. In contrast, those with minimal daily stress have a reduced risk of developing IBD (Y.-Z. Zhang & Yong-Yu Li, 2014). Beyond stress, mental health conditions such as depression and anxiety can negatively influence disease progression, potentially worsening symptoms and outcomes. Additionally, deterioration in mental well-being has been linked to an increased frequency of disease exacerbations, underscoring the importance of addressing psychological health in the management of IBD (Ananthakrishnan, 2013).

Genetics factors

In recent decades, genetic research, particularly genome-wide association studies (GWAS), has significantly enhanced the understanding of IBD. These studies have identified approximately 200 susceptibility loci, with some loci specifically associated with CD and UC. While many of these genes influence shared pathways, only one subset has been well-characterized as a critical contributor to IBD pathogenesis. These genetic mutations disrupt key processes, including pathogen recognition, immune regulation, and intestinal barrier integrity, which collectively drive the inflammatory processes characteristic of IBD (Younis *et al*., 2020).

<u>Key Genetic variants:</u>

- Autophagy-Related 16-like 1 (ATG16L1) mutations:

A substantial amount of evidence linking this genetic variant to the development of IBD stems from functional studies involving the ATG16L1 T300A variant. This polymorphism is associated with an increased risk of developing CD. ATG16L1 plays a role in regulating the autophagy pathway, which consists of the degradation of lysosomes and the elimination of intracellular bacteria. It is noted that autophagy is a fundamental cellular process, where cytoplasmic materials are broken down within the lysosome. This mechanism enables the cell to recycle damaged components and repurpose degraded materials for energy production and new synthesis. Effective gene-product interactions are crucial for the proper clearance of invading pathogens. When ATG16L1 function is lost, autophagy in intestinal epithelial cells (including Paneth cells) is disrupted, impairing their ability to secrete antimicrobial peptides. This dysfunction fosters bacterial overgrowth and facilitates their invasion through the intestinal epithelium (Jarmakiewicz-Czaja *et al*., 2022). Furthermore, the ATG16L1 gene mutation has an impact on the microbiota in IBD as demonstrated by the study of Lavoie *et al.* that

highlighted alterations in the fecal microbiota composition of knock-in mice expressing the ATG16L1 (T300A) variant compared to wild-type (WT) mice. Notably, there was an increased abundance of Bacteroides, particularly *Bacteroides ovatus* with a decrease in *Firmicutes* (Lavoie *et al*., 2019). Additionally, in patients homozygous for the ATG16L1-T300A allele, the inflamed ileum exhibited a higher abundance of *Fusobacteriaceae*. Conversely, individuals homozygous for the protective ATG16L1-300 allele showed a reduction in *Bacteroidaceae* and *Enterobacteriaceae*, alongside an increase in *Lachnospiraceae* within the inflamed ileal tissue (Sadaghian Sadabad *et al*., 2015).

- Nucleotide Binding Oligomerization Domain Containing 2 (NOD2) mutations:

NOD2, also known as the CARD15 gene, represents the most significant genetic risk factor for IBD, particularly CD. NOD2 is a cytoplasmic protein predominantly expressed in immune and antigen-presenting cells, including lymphocytes, macrophages, dendritic cells, and epithelial cells such as ileal Paneth cells (Knights *et al*., 2013). It plays a crucial role in bacterial recognition by detecting muramyl dipeptide, a component of bacterial cell walls in both Gram-positive and Gram-negative bacteria. Upon binding to its ligand, NOD2 activates signaling pathways, including the nuclear factor NF-κB complex, which induces the production of pro-inflammatory cytokines (e.g., IL1β, TNF-α, IL6, IL8) and antimicrobial molecules like α-defensins. This activation recruits innate immune cells and stimulates the differentiation of adaptive immune cells. Additionally, NOD2 regulates cellular processes such as differentiation, proliferation, and apoptosis through the mitogen-activated protein kinase (MAPK) pathway (Philpott *et al*., 2014). Importantly, NOD2 is integral to autophagy, recruiting ATG16L1 to sites of bacterial uptake, further emphasizing its multifaceted role in maintaining intestinal homeostasis. Loss-of-function mutations in NOD2 impair its ability to recognize and eliminate pathogens, reducing the secretion of antimicrobial peptides like α-defensins from Paneth cells. These mutations weaken immune responses by disrupting NF-κB activation, increasing susceptibility to inflammatory lesions in the intestinal mucosa. The most common mutations associated with IBD occur in the C-terminal region of NOD2, which is crucial for microbial recognition. These include two single nucleotide substitutions, R702W (rs2066844) and G908R (rs2066845), and a frameshift mutation, L1007fs (rs41450053). Individuals with one mutated allele face a 2-4-fold higher risk of developing IBD, while those with two mutated alleles have a significantly higher risk, ranging from 20-40-fold. Furthermore, the interaction between NOD2 and ATG16L1 underscores the synergistic role of these genes in IBD pathogenesis. Mutations in ATG16L1 disrupt autophagy, compounding the effects of NOD2 dysfunction and further increasing the risk of developing IBD (Younis *et al*., 2020).

- Protein tyrosine phosphatase non-receptor type 2 (PTPN2)

PTPN2 is another autophagy-related gene/protein that is expressed in Paneth and lymphoid cells. When mutated, it results in defective autophagosome formation and impaired bacterial clearance. Loss-of-function mutations in PTPN2, particularly in T cells, lead to enhanced differentiation of naïve T helper cells into Th1 and Th17 subsets, while simultaneously reducing the number of T regulatory cells. As a result, elevated levels of INF-γ, IL17, and IL22 are observed in both the serum and intestinal mucosal layers of patients carrying PTPN2 variants (Spalinger, 2016) (Spalinger *et al*., 2015).

Intestinal barrier

Intestinal barrier dysfunction is increasingly recognized as a pivotal factor in the development and progression of inflammatory bowel disease (IBD). The intestinal barrier functions as a critical interface between the external environment of the gut lumen and the host's internal milieu, providing a selective shield against luminal antigens, toxins, and microorganisms. When this barrier is compromised, increased intestinal permeability allows the translocation of luminal contents into the lamina propria, triggering inappropriate immune activation. This aberrant immune response contributes to the chronic intestinal inflammation characteristic of IBD (Dunleavy *et al*., 2023). The intestinal barrier is a multilayered system comprising the mucus layer, the intestinal epithelium, and underlying immune cells. The mucus layer serves as a physical and biochemical barrier, preventing direct contact between the epithelium and the gut microbiota. It contains mucins, such as MUC2, along with antimicrobial peptides and immunoglobulins. The intestinal epithelium, a single layer of specialized cells, includes enterocytes, which facilitate nutrient absorption; goblet cells, responsible for producing cross-linked mucin proteins that form the protective mucus layer covering the epithelium; Paneth cells, which secrete antimicrobial peptides to maintain microbial balance; Microfold (M) cells, which sample luminal antigens to initiate microbe-specific immune responses; tuft cells, rare secretory cells involved in regulating type 2 immunity; and enteroendocrine cells, which produce hormones to coordinate gut functions. The intestinal epithelium is supported by immune cells, such as intraepithelial lymphocytes and dendritic cells, contributing to barrier function and immune surveillance. The structural integrity and selective permeability of the epithelium are maintained by three types of junctional complexes: tight junctions (TJ), which regulate paracellular transport; adherents junctions, which provide mechanical cohesion; and desmosomes, which strengthen intercellular connections (Michielan & D'Incà, 2015) (Iyer & Corr, 2021). Studies in both animal models and humans emphasize the crucial role of epithelial barrier integrity in the pathogenesis of IBD. A seminal study by Van Der Sluis *et al*. demonstrated that Muc2-knockout mice, which lack the primary mucin responsible for forming the protective mucus layer, spontaneously

develop colitis (Van Der Sluis *et al*., 2006). This condition is accompanied by a compensatory upregulation of protective yet potentially inflammatory factors, such as Relm-β (expressed predominantly by goblet cells) and Reg3γ (primarily expressed by Paneth cells). While Relm-β and Reg3γ aim to mitigate microbial invasion, their overexpression exacerbates dysbiosis and amplifies the inflammatory response, worsening colitis. Notably, further experiments have shown that the genetic deletion of Relm-β reduces disease severity, highlighting its dual role as both a barrier-associated defense molecule and a contributor to pathogenic inflammation (Morampudi *et al*., 2016). Beyond mucus and antimicrobial peptides, tight junction abnormalities are a major contributor to barrier dysfunction in IBD. In IBD patients, reduced expression and redistribution of proteins such as occludins, claudins, and junctional adhesion molecules are frequently observed. Tumor necrosis factor-α (TNF-α), a central mediator of inflammation in IBD, exacerbates permeability by modulating the transcription of TJ proteins, inducing apoptosis of enterocytes, and hindering the resealing of epithelial gaps. Anti-TNF-α therapies have shown potential in improving barrier function by counteracting these effects. Goblet cell dysfunction in IBD is also a key feature. In UC, a reduction in goblet cell numbers (Singh *et al*., 2022), along with a thinner mucus layer (Gersemann *et al*., 2012) and altered composition (e.g., mucins, phosphatidylcholine, glycosylation) (Braun *et al*., 2009), have been observed. Paneth cell dysfunction, marked by an altered distribution and reduced defensin secretion, also plays a role, particularly in CD, where Paneth cell antimicrobial functions are diminished, contributing to disease pathogenesis (T.-C. Liu et al., 2016). In UC, however, Paneth cells may increase defensin production as a response to inflammation (Wehkamp *et al*., 2003). Additionally, human studies provide support for the idea that barrier dysfunction could be a predictor of IBD onset. A study by Turpin *et al*. found that elevated intestinal permeability in asymptomatic first-degree relatives of Crohn's disease patients significantly predicted future diagnoses of Crohn's, even up to three years before clinical symptoms appeared (Turpin *et al*., 2019).

1.1.1 Ulcerative colitis

UC is characterized by widespread inflammation of the colonic mucosa and as shown in **Figure 2,** it can involve the rectum (proctitis), can extend into the sigmoid colon (proctosigmoiditis), beyond the sigmoid (distal ulcerative colitis), involve the majority of the colon but spare the cecum (subtotal colitis), or affect the entire colon up to the cecum (pancolitis).

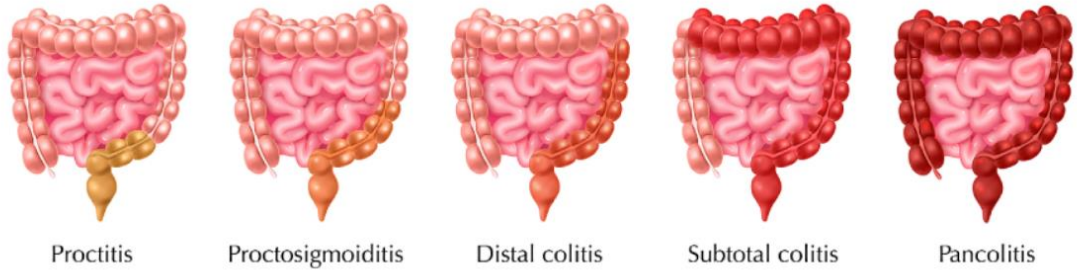


Figure 2. Types of ulcerative colitis. (Fundamentals Of Ulcerative Colitis, 2023)

Clinically, UC presents with characteristic symptoms such as rectal bleeding, diarrhea, an urgent need to defecate, and abdominal cramping. Patients with proctitis or proctosigmoiditis may experience tenesmus and rectal pain. At the same time, pancolitis is frequently associated with systemic manifestations such as fever, malaise, fatigue, and unintended weight loss, indicative of severe disease activity and systemic inflammation. (Ungaro *et al*., 2017). Notably, both subtotal colitis and pancolitis carry a significantly higher risk of developing colorectal cancer (CRC) compared to more localized forms of UC. This elevated risk is attributed to the cumulative effects of chronic inflammation, oxidative stress, and genomic instability in the colonic epithelium and the development can be considered a multistep process characterized by increasing degrees of molecular, histologic, and endoscopic changes, progressing from no dysplasia to low-grade dysplasia to high-grade dysplasia and ultimately cancer (Fantini & Guadagni, 2021). CRC was initially identified as a complication of UC by Crohn and Rosenberg in 1925. Since then, numerous epidemiological studies have confirmed this increased risk. The first retrospective study of the occurrence and progression of CRC was published in 1988. In that study, the incidence of CRC in UC was reported to be 4.25% (Lopez *et al*., 2018). In recent years, data from relevant worldwide studies have shown that the standardized incidence ratio (SIR) of CRC associated with UC may be related to the course of the patient's disease and regional differences. Furthermore, a meta-analysis conducted by Wang *et al*. with a total of 11 studies involving 26,765 patients with UC confirms that it is one of the risk factors for CRC and found that geographical location also had an impact on the transition from UC to CRC (Y. Wang *et al*., 2021). Like sporadic CRC, UC-associated CRC develops through molecular events, including alterations in p53, adenomatous polyposis coli (APC), and K-ras. However, the order of these events differs between sporadic CRC and UC-CRC. Specifically, the loss of APC function is an early event in adenoma formation in sporadic CRC but occurs later in the pathogenesis of UC-CRC, while p53 is the opposite. Furthermore, UC-CRC follows an inflammatory-dysplasia-carcinoma sequence rather than the typical adenoma-carcinoma sequence, and the activation of nuclear factor-κB (NF-κB) and signal transducer and activator of transcription 3 (STAT3), induced by cytokines, are

prominent contributors to its development (Y. Wang *et al*., 2021). It is relevant to note that, although CRC in UC accounts for 5% of all CRC cases seen in the general population, it represents a significant complication of the disease, accounting for one-sixth of all deaths in UC patients (Eaden *et al*., 2001).

1.1.2 Chron's disease

The CD is characterized by discontinuous skip lesions that can involve any part of the GI tract, from the upper segments to the anus (**Figure 3)**. Inflammation is typically transmural, with granulomas often observed on biopsies - a hallmark aiding in diagnosis. Symptoms vary widely and may include diarrhea, abdominal pain, weight loss, nausea, vomiting, and occasionally fevers or chills (Cheifetz, 2013)**.** Once thought to primarily affect young adults, CD is now recognized in individuals of all ages. Approximately one-quarter of cases are diagnosed in patients under 20 years old. While the highest incidence in children occurs during adolescence, around 20% of pediatric cases are diagnosed before age 10, with about 5% identified in children younger than 5 years (da Silva Júnior *et al*., 2023). A classification system considering three primary features - age at diagnosis, disease location, and clinical behavior - was initially introduced during the World Congress of Gastroenterology held in Vienna in 1998 (Gasche *et al*., 2000). This framework was later refined and updated at the Montreal conference in 2005 (Silverberg *et al*., 2005). Based on disease behavior, CD is classified into inflammatory (non-stricturing and non-penetrating), stricturing, or penetrating forms, which may also involve perianal regions (Louis *et al*., 2003). The inflammatory CD is characterized by inflammation of the gastrointestinal tract with no evidence of stricturing or fistulizing disease. This inflammation can eventually lead to fibrosis and luminal narrowing, transitioning these patients to a classification of stricturing disease. Once fibrostenotic changes occur, they are irreversible without surgical intervention. Ongoing transmural inflammation can also result in the development of a sinus or fistulous tract characteristic of fistulizing CD. Fistulas may form between the bowel and nearby organs, such as the bladder, vagina, or other segments of the bowel. If the sinus tract does not fully connect to an adjacent organ, an intra-abdominal abscess may develop (Feuerstein & Cheifetz, 2017).

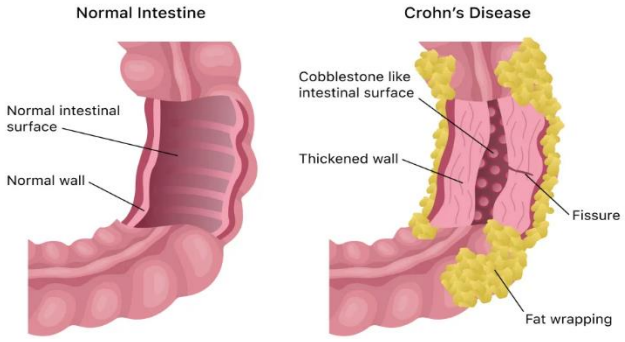


Figure 3. Overview of Crohn's disease (AL-Kefaei, 2024)

In CD, the progression can be divided into active and remission stages, each with distinct characteristics and treatment goals. During the active phase, patients experience acute inflammatory symptoms and different types of complications as mentioned above. The treatment aims to suppress acute inflammation and induce remission, which often involves anti-inflammatory medications, immunosuppressants, antibiotics, and sometimes biologic therapy targeting specific cytokines. During the remission stage, symptoms may improve or even disappear completely. However, there is still a risk of recurrence, so ongoing monitoring is important. The goal during remission is to maintain it, prevent complications, and ensure a good quality of life. This may involve maintenance medications, lifestyle changes such as a balanced diet, and regular check-ups to catch any signs of recurrence early.

1.1.3 *In vivo* model to study IBD

Currently, animal models, with a predominant focus on elucidating the interplay between the mucosal epithelium, intestinal microorganisms, and immunity, play a central role in studying the pathogenesis, drug efficacy, and pharmacological mechanisms of IBD. Rodents, non-rodent mammals, and some non-mammals are used for IBD modeling, and rats and mice are the most commonly used (Wen *et al*., 2024). Various *in vivo* models have been developed to study IBD, each offering distinct advantages and limitations. This section will specifically focus on the models designed to replicate IBD in mice.

Chemically induced models

- Dextran sulfate sodium-induced colitis (DSS)

A common method to induce ulcer formation and inflammation in animal models involves administering DSS, a water-soluble polysaccharide derived from dextran. Strictly speaking, DSS does not directly induce inflammation in the intestines. Instead, it chemically damages the intestinal epithelium, compromising the integrity of the mucosal barrier. This damage exposes the underlying lamina propria and submucosal compartments to luminal antigens and gut microbiota, which subsequently trigger an inflammatory response. The severity and reproducibility of DSS-induced colitis are influenced by multiple factors, such as the concentration of DSS (commonly 1%–5%), the duration of exposure (acute or chronic administration), the specific batch or manufacturer of DSS, the animal strain used (e.g., C3H/HeJ and BALB/c mice are more susceptible), the sex of the animals (male mice are typically more vulnerable), and their microbial environment (e.g., germ-free versus specific pathogemild weight loss, diarrhea, and bloody stools, reflecting mild epithelial damage and inflammation. These modest effects progress to increasingly severe symptoms, such as severe bleeding, hunched posture, and further weight loss. Increased intestinal permeability occurs, which

can lead to bacterial translocation and systemic inflammation. With prolonged exposure or high doses, DSS can result in severe colonic inflammation and mortality. Acute histological changes in the colon can be induced by relatively short exposure (4-7 days) to a high dose of DSS. These include ulcerations, loss of goblet cells, and immune cell infiltration, which resemble acute lesions seen in UC in humans (Eichele & Kharbanda, 2017). Chronic (advanced) lesions can be induced by continuous treatment with low-dose DSS or via cyclical DSS application, leading to fibrosis, crypt abscesses, and mucosal ulceration (Chassaing *et al*., 2014). These chronic changes mirror chronic human IBD.

- 2,4,6-Trinitrobenzene sulfonic acid (TNBS)-induced colitis model

TNBS is a small haptenizing molecule that is not inherently antigenic. However, upon binding to host proteins, it triggers an immune response. TNBS is commonly used in preclinical studies as a mouse model to mimic aspects of CD. This model triggers a Th1-mediated immune response, marked by the infiltration of CD4+ T-cells, neutrophils, and macrophages. Granulomas with infiltration of inflammatory cells in all layers are seen in the intestine of this model. The isolated macrophages produce large amounts of Interleukin (IL)-12, and lymphocytes produce large quantities of Interferon (IFN)-γ and IL-2. (Hibi *et al*., 2002) The resulting inflammation leads to transmural colitis, replicating key features of CD pathology (Baydi *et al*., 2021). The SJL/J mouse strain is susceptible to TNBS-induced colitis, making it a preferred choice for such studies. Other strains, such as BALB/c and C57BL/6, are also frequently used. TNBS-induced colitis model can be obtained with TNBS administrations, with an average dose of 1.2 mg/mouse (with 20 mg of body weight), in a volume between 100 μL to 150 μL, in 50% ethanol. Alcohol is considered a solvent or vehicle solution in biochemical reactions and helps produce intestinal inflammation by breaking the mucosal barrier (Baydi *et al*., 2021). Mice treated with TNBS develop clinical symptoms such as weight loss, diarrhea, and rectal bleeding, reflecting the severity of intestinal damage (Silva *et al*., 2019). Histological analysis reveals key pathological features, including mucosal erosion, crypt abscesses, ulceration, and immune cell infiltration, closely mimicking human CD. Furthermore, TNBS treatment induces significant changes in the distal colon's circular muscle layer, affecting its morphology, mechanical properties, and pharmacological responses (Ellrichmann *et al*., 2014). The TNBS model can be used to study both acute and chronic colitis. Acute colitis is induced by a single administration of TNBS, providing insights into the initial inflammatory response, while chronic colitis is modeled through repeated or prolonged administration, replicating the progression and fibrosis seen in long-term disease. These changes align with features observed in human UC, highlighting the versatility of the TNBS model in studying inflammatory bowel disease.

- Oxazolone-Induced Colitis Model

Oxazolone, a haptenizing agent, is frequently employed to induce colitis in mice, serving as a tool to study the pathological mechanisms underlying the persistence of UC. This colitis model elicits an immune response primarily driven by Th2 cells and closely resembles human UC, as demonstrated by inflammation of the mucosal lining, epithelial microulcerations, and histological alterations in the distal colon (Kojima *et al*., 2004). Different mouse strains, such as C57BL/6J, BALB/CJ, and SJL/J, are utilized to assess immunological responses. While C57BL/6J (C57/BL6 or C57/BL10) and SJL/J mice show a predisposition for Th1-mediated responses, BALB/CJ mice exhibit a strong inclination toward Th2 immune responses (Charles *et al*., 1999). Oxazolone-induced colitis differs from TNBS colitis in terms of immune response and cytokine production. The inflammation in oxazolone colitis is driven by the secretion of IL-13 from lamina propria CD4+ natural killer T (NKT) cells, rather than conventional CD4+ T-cells producing IFNγ. Blocking IL-13 with the fusion protein IL-13Ra2-Fc has been shown to prevent colitis, indicating a critical role for IL-13 and NKT cells in this model. Notably, oxazolone fails to induce colitis in mice lacking NKT cells (Heller *et al*., 2002). The C57BL/6J strain requires presensitization (to sensitize the immune system and create a predisposition for an exaggerated immune response upon subsequent exposure) to induce colitis with oxazolone due to its natural resistance, whereas the SJL/J strain is less commonly used due to a high risk of autoimmune disease, elevated mortality rates, and aggressive behavior that complicates housing multiple mice together. Colitis is typically induced through a two-step process. First, the shaved abdominal skin is presensitized with 3% oxazolone dissolved in 100% ethanol, using a 50–150 µL volume. Five to eight days after presensitization, the mice are rechallenged via intrarectal administration of 70–150 µL of 0.75–1% oxazolone in a 45–50% ethanol solution. This procedure triggers the development of colitis. The severity of colitis is then assessed by sacrificing the animals 7–12 days after the initial presensitization (Randhawa *et al*., 2014).

Genetically engineered models

Recent advancements in genetic engineering have provided valuable tools to investigate how the overexpression or deficiency of specific genes impacts the progression of IBD. Murine models of IBD, developed through various gene manipulation strategies, are categorized into nine distinct groups. These include conventional transgenic (Tg) and knockout (KO) models, dominant-negative Tg models, cell-specific Tg or KO models, inducible KO models, knock-in models, innate models, and mutagen-induced models (**Figure 4)** (E. Mizoguchi *et al*., 2020).

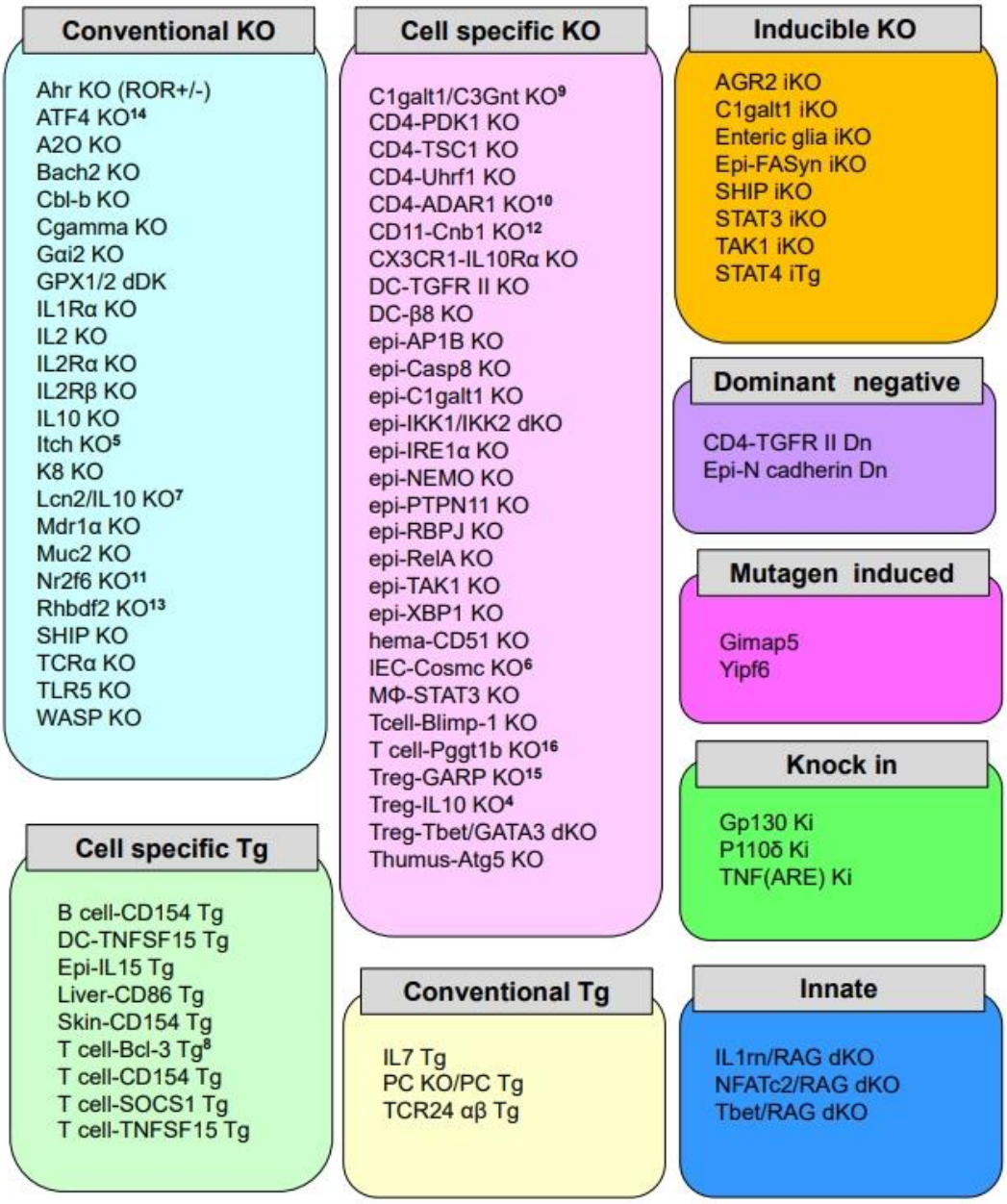


Figure 4. Mouse models of IBD with gene targeting strategies (E. Mizoguchi *et al*., 2020)**.**

Conventional knockout (KO) and transgenic (tg) models are designed to overexpress or eliminate a specific gene across all cell types. Cell-specific or Tg models are tailored to overexpress or delete a target gene exclusively in certain cell types. Inducible knockout (iKO) models offer the flexibility to delete a specific gene at any point during adulthood. Dominant-negative transgenic (Dn) models involve the expression of a non-functional protein that disrupts the normal activity of the target protein by competing with its ligand or receptor. Knock-in (KI) models introduce a mutation in the gene of interest, replicating polymorphisms linked to IBD susceptibility. Innate models lack a particular gene on an immunodeficient background; these models typically require additional modifications, such as crossing with RAG2 KO mice deficient in T and B cells, to induce intestinal

inflammation. Finally, mutagen-induced models harbor functional gene mutations introduced randomly through chemical treatments like N-ethyl-N-nitrosourea (A. Mizoguchi *et al*., 2016).

Spontaneous mutation model

- SAMP1/YitFc (SAMP) Colitis

The SAMP mouse model of CD-like ileitis is particularly useful for studying the pathogenetic mechanisms of experimental CD. This model develops spontaneous ileitis without the need for genetic, chemical, or immunological interventions, offering a natural progression that facilitates research into the pre-disease, early, and chronic stages of ileitis. SAMP mice exhibit striking similarities to human CD, including disease localization, histological characteristics, and extra-intestinal complications (Pizarro *et al*., 2011). The SAMP mouse strain was derived from selective brother-sister mating of AKR/J mice (Higuchi, 1997), leading, after 24 generations, to the development of senescence-prone lines (SAMP 1 - 10) exhibiting accelerated aging, skin lesions, and autoimmune issues. The SAMP1/Yit substrain was then created through continued selective mating based on the presence of skin lesions and ileitis (Matsumoto *et al.,* 1998*)*. By the 20th generation, the strain lost its senescence-accelerated phenotype and consistently developed both acute and chronic small intestinal inflammation, with 100% penetrance by 30 - 40 weeks and persistence up to 80 weeks. At the University of Virginia, continuous inbreeding of the SAMP1/Yit colony led to the creation of the SAMP1/YitFc substrain, which exhibited a distinct phenotype. This included earlier onset of ileitis by 10 weeks, increased intestinal IFNγ production by 4 weeks, and the development of perianal fistulae in 5%–10% of the colony. Additionally, nearly 50% of mice over 40 weeks developed intestinal strictures (Kosiewicz *et al*., 2001) (Rivera-Nieves *et al*., 2003). SAMP1/YitFc ileitis is characterized by areas of discontinuous cobble-stoning with transmural inflammation dispersed between areas of normal gut mucosa within the terminal ileum and cecum, similar to the “patchy” nature of CD (Cominelli *et al*., 2017). The inflammatory infiltrate consists of lymphocytes, macrophages, and neutrophils, and is accompanied by progressive disruption of the epithelium, tissue atrophy, and crypt elongation (Matsumoto *et al*., 1998). The initial stage of ileitis in SAMP mice, occurring between 4 to 7 weeks of age, is characterized by strong mucosal Th1 immune activation, even before any histological lesions are visible. This period, known as the inductive phase, involves elevated levels of IFNγ and TNF production from mesenteric lymph node (MLN) cells, signaling mucosal dysregulation. Th1 polarization intensifies before the onset of ileitis, with increased IFNγ and TNF expression. Blocking IFNγ at this stage reduces the severity of ileitis. Furthermore, the onset of inflammatory lesions in the terminal ileum of SAMP mice at 9 - 10 weeks marks a significant shift in the mucosal cytokine environment, entering the second phase of SAMP ileitis, known as the

chronic inflammatory phase. During this phase, the existing Th1 response is accompanied by a notable increase in Th2 effector pathways. Specifically, between weeks 9 and 16, the mucosal expression of IL-13 and IL-5 rises more than 25-fold compared to baseline levels in younger mice, with this increase maintained throughout the phase (Bamias *et al*., 2005). The presence of both Th1 and Th2 pathways in SAMP ileitis presents an "immunological paradox," as IL-4 is typically regarded as an anti-inflammatory cytokine, and Th1 and Th2 responses are considered mutually exclusive.

1.2 Intestinal fibrosis

Intestinal fibrosis is characterized by the abnormal accumulation of extracellular matrix (ECM) proteins and muscle hypertrophy, typically resulting from prolonged tissue injury. This condition is strongly associated with chronic inflammation and is particularly prevalent in individuals with IBD, especially CD. As noted earlier, CD often leads to complications such as strictures, stenoses, and fistulae, which affect a significant proportion of patients, with these complications occurring in more than 70% of cases (Bamias *et al*., 2022) (Dolinger *et al*., 2024). The development of a fibrostenosing phenotype, marked by persistent narrowing of the intestinal lumen, is a key feature of fibrosis in CD. This narrowing can lead to severe complications, such as bowel obstruction, which often necessitate surgical intervention (Rieder *et al*., 2013). Although strictures can form anywhere in the GI tract, they are most commonly found in the small intestine, consistent with the typical distribution of inflammation seen in CD (Thia *et al*., 2010). Furthermore, ileal CD tends to present with strictures more frequently, whereas fistulas are more commonly observed in patients with colonic or ileocolonic involvement (Louis *et al*., 2001). As mentioned, the process of fibrogenesis is characterized by an abnormal accumulation of ECM primarily composed of collagen. Different cell types, such as smooth muscle cells, myofibroblasts, and fibroblasts drive this process. The activation of these cells is influenced by a range of factors, including genetic and epigenetic changes, microbiota alterations, cytokines, and other soluble factors released during inflammatory responses (Mignini *et al*., 2024). Additionally, the dynamic regulation of ECM is heavily influenced by the balance between matrix metalloproteinases (MMPs), which degrade ECM components, and tissue inhibitors of metalloproteinases (TIMPs), which counteract MMP activity. Dysregulation of the MMP/TIMP axis in intestinal fibrosis contributes to the excessive ECM deposition characteristic of fibrotic tissue. Overexpression of TIMPs or insufficient MMP activity can lead to reduced ECM degradation, promoting fibrosis and further narrowing of the intestinal lumen (Biel *et al*., 2024).

1.2.1 Cellular components of intestinal fibrosis

Recurrent, or persistent, epithelial injury is crucial for initiating and sustaining intestinal inflammation and fibrogenesis. Epithelial and endothelial damage releases chemotactic factors promoting the recruitment and activation of innate and adaptive immune cells, as well as mesenchymal cell precursors of the activated myofibroblasts (J. Wang *et al*., 2021).

Mesenchymal cells

Mesenchymal cells, originating from mesenchymal stem cells, are characterized as non-epithelial, non-endothelial, and non-hematopoietic. Within the intestine, these cells are present across all anatomical compartments and play an essential role in intestinal fibrosis. Notable types include subepithelial myofibroblasts, lamina propria fibroblasts, pericytes, bone marrow-derived mesenchymal stromal or stem cells, and smooth muscle cells (SMCs). Moreover, interstitial cells of Cajal and fibrocytes, often classified as non-conventional mesenchymal cells, also contribute to this process (**Figure 5).**

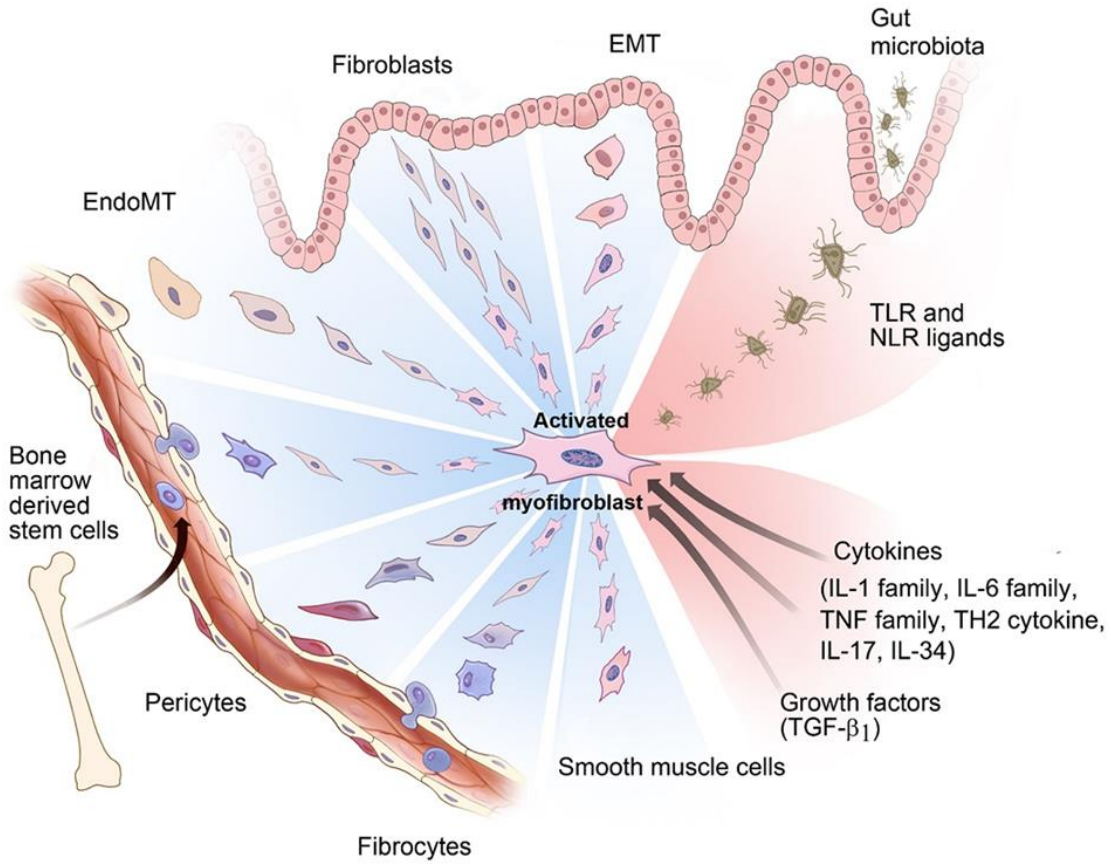


Figure 5. Main cell types and cell differentiation mechanisms involved in ECM secretion and fibrosis in the intestine (J. Wang *et al*., 2021)**.**

- Fibroblast, smooth muscle cells, and myofibroblast

Fibroblasts are specialized cells found in connective tissues, playing a pivotal role in maintaining structural integrity by producing and remodeling ECM. These cells synthesize critical ECM components, such as collagen, elastin, and glycosaminoglycans, which provide structural support and maintain tissue homeostasis. In a healthy state, fibroblasts contribute to normal wound healing by migrating to sites of injury, proliferating, and depositing ECM to repair damaged tissue (Roman, 2023). In pathological conditions, particularly fibrosis, fibroblasts become hyperactivated, transitioning into a myofibroblast phenotype under the influence of profibrotic factors like transforming growth factor-beta (TGF-β). Fibroblasts also interact closely with immune cells, amplifying inflammatory and fibrotic responses through cytokine and growth factor signaling (Antar *et al*., 2023). Smooth muscle cells (SMCs) can also differentiate into myofibroblasts under certain conditions. A previous study found that SMCs isolated from CD ileum presented alterations in morphology and contractile activity (Severi *et al*., 2014). Interestingly, myofibroblasts can revert to smooth muscle cells in response to pro-inflammatory cytokines. This process may contribute to smooth muscle cell hyperplasia and hypertrophy, resulting in a thickened muscularis propria. Such changes are associated with the development of strictures and intestinal obstruction. These myofibroblasts exhibit enhanced ECM production and contractile activity, leading to excessive tissue stiffness and scarring (Chen *et al*., 2017). According to the current marker-based classification system, intestinal fibroblasts express the surface marker CD90 and vimentin but not desmin, whereas SMCs are CD90 (-), vimentin (low), α-smooth muscle actin (α-SMA) (+), and desmin (+). Myofibroblasts are CD90 (+), vimentin (+), α-SMA (+), and desmin (- or low), giving them an intermediate phenotype between fibroblasts and SMCs (Koliaraki *et al*., 2017).

- Fibrocytes

Fibrocytes, derived from bone marrow, are mesenchymal progenitors that exhibit features of both hematopoietic markers (CD34) and fibroblast markers (collagen-I). These cells play a crucial role in the progression of fibrotic diseases. Previous research has shown that fibrocytes can be activated by various inflammatory cytokines (Sahebally *et al*., 2013). Within four days of injury, activated fibrocytes typically migrate to the damaged site, contributing to fibrotic processes directly by producing ECM proteins and fibrogenic cytokines or indirectly by differentiating into myofibroblasts (Sazuka *et al*., 2014) (Grieb *et al*., 2011). Sazuka *et al.* reported that bone marrow-derived fibrocytes are involved in intestinal fibrosis, consistent with findings that the frequency of circulating fibrocytes is elevated in fibrostenotic CD patients compared to healthy individuals (Sazuka *et al*., 2014). Recent studies have revealed that fibrocytes deposited in inflamed colonic tissue produce tissue inhibitors of metalloproteinases, inhibiting collagen degradation (Kuroda *et al*., 2019).

- Bone marrow stem cells

Bone marrow-derived fibrocytes are cells that arise from multipotent bone marrow mesenchymal stem cells (MSCs), and under certain conditions, these MSCs can differentiate into fibrocytes. These fibrocytes then travel through the bloodstream to affected tissues. Fibrocytes are characterized by markers of hematopoietic origin, such as CD45, CD34, CD11, CD13, CD14, CD80, and CD86. They also express collagen I and α-SMA, which are indicative of their role in ECM production and fibrosis. Under normal conditions, fibrocytes mature before entering tissues, where they typically differentiate into tissue-resident macrophages or dendritic cells. However, during inflammation, fibrocytes migrate into inflamed tissues through a C-C chemokine receptor type 2 (CCR2) -mediated mechanism, responding to chemokines like C-C motif chemokine ligand 2 (CCL2) that is involved in the recruitment of various kinds of cells to sites of injury or inflammation. Once they arrive at the site of injury, fibrocytes can differentiate into various cell types, including epithelial, endothelial, neuronal, and mesenchymal cells, contributing to tissue repair and the development of fibrosis (Kuwana *et al*., 2003).

- Epithelial-mesenchymal transition

Another source of myofibroblasts arises from epithelial-mesenchymal transition (EMT). This process involves the transformation of epithelial cells, which lose their characteristic features and functions while acquiring a mesenchymal cell phenotype, specifically, during EMT, epithelial cells progressively lose epithelial markers such as E-cadherin and occludin, coupled with an increased expression of mesenchymal markers like α-SMA and vimentin (Artone *et al*., 2024). Multiple studies have highlighted the role of EMT in the development of intestinal fibrosis (Flier et al., 2010) (Scharl *et al*., 2015). Experimental studies with transgenic mouse models showed that about one-third of fibroblast-specific protein 1 $(FSP1)^{+}$ fibroblasts derived from intestinal epithelial cells (Flier et al., 2010). Furthermore, Ortiz-Masià *et al.* identified fibrosis and the presence of EMT markers in tissue samples from CD patients, regardless of their clinical behavior, whether stricturing (Montreal classification: B2) or penetrating (Montreal classification: B3) (Ortiz-Masià *et al*., 2020).

- Endothelial-mesenchymal transition

Endothelial-mesenchymal transition (EndoMT) can be viewed as EMT occurring in endothelial cells. Similarly, to epithelial cells, during the process of EndoMT, endothelial cells lose the expression of typical endothelial markers and acquire fibroblast-like morphology and gene expression profile (Dejana *et al*., 2017). In vivo evidence of EndoMT has been observed in human colonic tissues from patients with both CD and UC patients (Rieder *et al*., 2011).

Innate and adaptive immune cells

Innate immune cells such as monocytes, neutrophils, mast cells, eosinophils, and basophils secrete various pro-inflammatory and pro-fibrotic molecules, including ILs, TNFα, transforming growth factor-β1 (TGF-β1), and platelet-derived growth factor (PDGF). These immune signaling pathways, through their role in triggering cellular activation and promoting fibrosis, play a key role in driving the transdifferentiation of cells into myofibroblasts (Speca *et al*., 2012) (B. Yang *et al*., 2020). Monocyte-derived cells, including macrophages and dendritic cells, play a significant role in both inflammation and fibrosis. These cells influence the activation of myofibroblasts and their progenitors, particularly through their direct effects on the extracellular matrix (Wynn & Vannella, 2016). The recruitment of specific macrophage subsets and their relative abundance during injury can dictate whether the inflammatory response promotes tissue repair or leads to fibrosis. M1 macrophages, activated by interferon (IFN)-γ, TNFα, or bacterial components, contribute to myofibroblast activation and fibrosis through both cytokine-dependent and independent mechanisms. They also generate reactive oxygen species (ROS), which cause tissue damage and promote myofibroblast resistance to apoptosis. Conversely, M2a macrophages, induced by IL-4 or -13 signaling through the common IL-4 receptor α and STAT6 activation, produce key pro-fibrotic factors such as TGF-β1, connective tissue growth factor (CTGF), PDGF, fibroblast growth factor (FGF), and insulin-like growth factor (IGF). M2c/regulatory macrophages, which arise following IL-10 exposure, have anti-fibrotic properties and exert their effects partly by inhibiting STAT1 and NF-kB. These regulatory macrophages suppress both M1 and M2a macrophages through local production of IL-10 and/or Arginase-1. Additionally, macrophages can alter their phenotype and function as tissue repair or fibrosis progresses, although the precise factors governing these transitions *in vivo* remain unclear (Lawrance *et al*., 2015) (X. Li *et al*., 2024). Neutrophils, which are rapidly recruited after epithelial injury, help clear debris and eliminate pathogens. However, if inflammatory neutrophils persist, they may exacerbate tissue damage and further activate the extracellular matrix and myofibroblasts through the release of pro-fibrotic cytokines, chemokines, and reactive oxygen and nitrogen species. In the adaptive immune response, T helper cell subsets (Th1, Th2, Th17), regulatory T cells (Tregs), and B cells play significant roles in the development of fibrosis. The Th17 response is pro-inflammatory and pro-fibrotic, driven by cytokines such as IL-17, IL-21, and IL-22, which promote inflammation and exacerbate fibrosis by stimulating the production of pro-fibrotic factors like TGF-β. Th2 immunity, characterized by the production of IL-4, IL-5, and IL-13, is strongly fibrogenic, with IL-13 being the dominant mediator in driving fibrosis. IL-13 contributes to the activation of myofibroblasts, collagen deposition, and extracellular matrix remodeling, all key features of fibrotic tissue formation. On the other hand, Th1 immunity, driven by IFN-γ, is generally

associated with anti-fibrotic effects. IFN-γ inhibits fibrosis by counteracting the pro-fibrotic effects of Th2 and Th17 cells and promoting tissue repair mechanisms. However, under specific conditions, Th1 cells can exhibit pro-fibrotic effects. This occurs primarily through their interactions with other immune cells and the production of additional cytokines and molecules in response to chronic inflammation. For example, prolonged or dysregulated Th1 activation can lead to the release of TGF-β, either directly or through the activation of other cells, such as macrophages or fibroblasts, within the fibrotic environment. The role of regulatory T cells (Tregs) in fibrogenesis is not yet fully understood. While they are generally thought to suppress fibrosis by inhibiting Th17 and Th2-driven responses, in certain chronic fibrotic conditions, Tregs can become dysregulated or shift their function, contributing to fibrotic remodeling. This shift may occur when Tregs produce TGF-β in the local tissue environment, and at high levels, TGF-β can promote fibrosis by activating myofibroblasts and increasing extracellular matrix production. Overall, the balance between these T helper cell subsets and their associated cytokines plays a critical role in determining the extent of fibrosis in conditions like IBD (**Figure 6**) (Lawrance *et al*., 2015) (Huang *et al*., 2020).

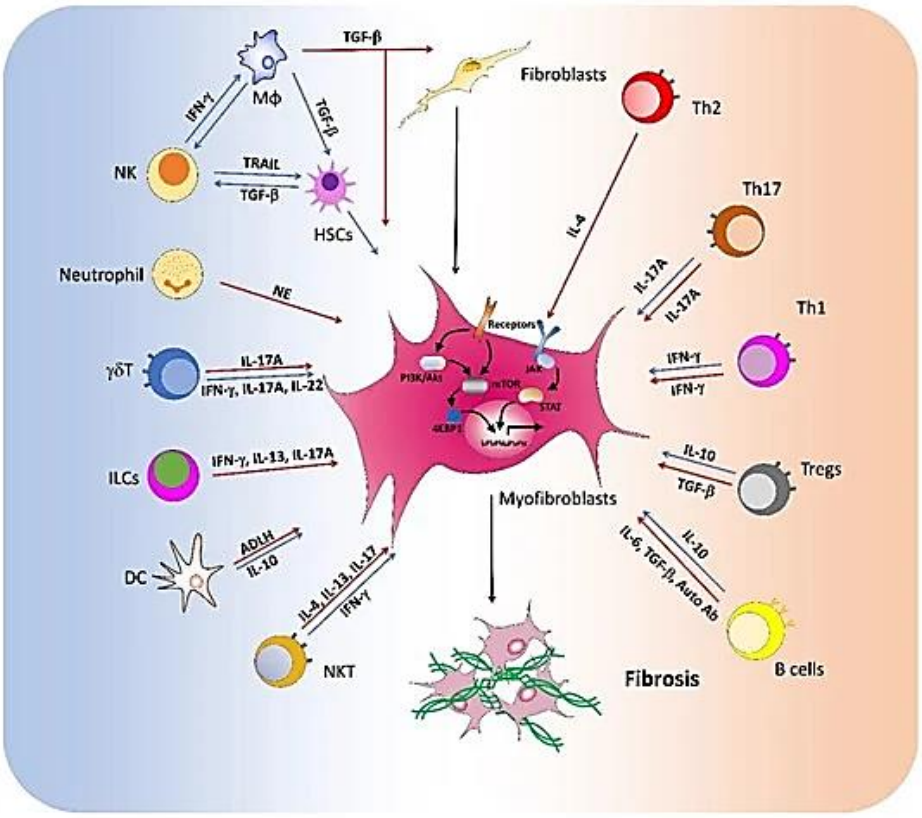


Figure 6. Immune cell contributions to fibrosis pathogenesis. (Huang *et al*., 2020).

1.2.2 Key molecules involved in intestinal fibrosis

In IBD, fibroblasts and myofibroblasts in the mucosa are subjected to a highly dynamic and complex microenvironment characterized by numerous cytokines produced by various cell types. Immune

cells residing in the lamina propria are considered the primary sources of these cytokines, significantly influencing the behavior of fibroblasts and myofibroblasts. Research utilizing both human tissues and animal models has demonstrated that the persistent release of pro-inflammatory cytokines during chronic inflammation triggers the onset of fibrosis and actively contributes to its progression (Friedrich *et al*., 2019).

- TGF-β

Transforming Growth Factor β (TGF-β) is a multifunctional growth factor that plays a pivotal role in both physiological processes and pathological tissue responses. It is secreted by various cell types, including platelets, monocytes/macrophages, T cells, epithelial cells, and fibroblasts. TGF-β promotes fibroblast activation and induces the production of ECM proteins. Recognized as a central regulator of fibrotic responses, TGF-β is critical in driving tissue remodeling and fibrosis (Györfi *et al*., 2018). In mammals, TGF-β exists as three isoforms: TGF-β1, TGF-β2, and TGF-β3, which exhibit similar biological functions. While various cell types produce and respond to TGF-β, fibrosis is primarily linked to TGF-β1. The main cellular sources of TGF-β1 are circulating monocytes and tissue macrophages. In macrophages, the regulation of TGF-β1 activity occurs not through control of its mRNA expression, but rather through the secretion and activation of latent TGF-β1. In its inactive form, TGF-β1 is stored as a disulfide-bonded homodimer, bound to a latency-associated protein (LAP). For TGF-β1 to be activated, the LAP must dissociate, a process facilitated by various factors, including cathepsins, plasmin, calpain, thrombospondin, integrin-αvβ6, and matrix metalloproteinases. Once activated, TGF-β1 binds to its receptors, initiating intracellular signaling through Smad proteins, that mediate canonical TGF-β1 signaling, which regulates the transcription of key genes involved in fibrosis (Wynn, 2008) (Dees *et al*., 2021). In addition to the canonical pathway, including Wnt/β-catenin, TGF-β1 can also activate several alternative pathways that contribute to fibrosis. These comprise the mitogen-activated protein kinase (MAPK) pathway, involving extracellular signal-regulated kinase (ERK), p38, and JUN N-terminal kinase (JNK), as well as the RHO-associated kinase (ROCK) and RAC-α serine/threonine protein kinase (AKT) pathways. These non-Smad pathways work in conjunction with the canonical Smad signaling, amplifying the fibrotic response by influencing cell migration, survival, and ECM production (Derynck & Zhang, 2003). Several studies have highlighted the involvement of TGF-β1 in the development of intestinal fibrosis. Del Zotto *et al*. observed that lamina propria lymphocytes from the inflamed mucosa of UC patients exhibited significantly higher TGF-β1 production compared to controls. This increase was particularly evident upon stimulation with CD2 and CD28, suggesting a heightened TGF-β1 response in the inflammatory environment (Del Zotto *et al*., 2003). Furthermore, the association between TGF-β1 levels and intestinal strictures in CD was demonstrated by Di

Sabatino *et al*., who reported a significant elevation of TGF-β1 expression in the intestinal mucosa overlying strictures. This increase was partially attributed to enhanced TGF-β production by myofibroblasts near the strictured areas (Di Sabatino *et al*., 2009). Similarly, Li *et al*. found that the level of active TGF-β1 was markedly higher in strictured intestinal muscle obtained from surgically resected ilea of CD patients compared to adjacent normal intestinal tissue *(C. Li et al*., 2013). Serum TGF-β levels have also been investigated in inflammatory bowel disease (IBD) patients. Sambuelli *et al*. found that serum TGF-β1 levels were significantly elevated in treatment-naïve patients with active UC compared to healthy controls (Sambuelli *et al*., 2000). In summary, TGF-β plays a pivotal role in the pathogenesis of fibrosis in IBD, and elevated levels of TGF-β, both locally in the intestinal tissue and systemically in the serum, are closely associated with inflammation, fibrosis, and stricture formation.

- IL-1 family members

IL-1 family consists of 11 cytokine members, and in addition to their well-established roles in inflammation, some members, such as IL-1, IL-33, and IL-36, are increasingly recognized for their involvement in intestinal fibrosis.

- IL-1α and IL-1β

IL-1α is typically expressed in epithelial cells but can also be induced in other cell types, including macrophages, monocytes, and endothelial cells, following activation. When released from damaged intestinal epithelial cells (IECs), IL-1α acts as a damage-associated molecular pattern (DAMP), playing a crucial role in initiating and sustaining chronic intestinal inflammation (Valatas *et al*., 2017). In a DSS-induced colitis mouse model, IL-1α-deficient mice exhibited less severe disease symptoms and showed faster recovery. Additionally, neutralizing IL-1α in wild-type mice during acute colitis alleviated both clinical symptoms and histological damage, suggesting that IL-1α contributes significantly to the inflammatory response in the gut. On the other hand, IL-1β, primarily produced by mononuclear phagocytes, is a key pro-inflammatory cytokine in the development of intestinal inflammation (Friedrich *et al*., 2019). IL-1β is involved in the early stages of intestinal inflammation. Stimulating the secretion of pro-inflammatory cytokines, chemokines, and other mediators such as IL-8, monocyte chemoattractant protein (MCP)-1, and MMPs, particularly MMP-1, from intestinal myofibroblasts and fibroblasts. This helps recruit more immune cells to the site of injury and drives further inflammatory responses. Additionally, the pro-inflammatory effects of IL-1β can contribute to the activation of myofibroblasts (Okuno *et al*., 2002). In contrast, some studies have shown that IL-1β may also inhibit collagen synthesis and promote the production of collagenases and TIMP-1 in intestinal smooth muscle cells. These findings suggest a more complex role of IL-1β in fibrogenesis,

where it may either promote or inhibit fibrosis depending on the context. The precise role of IL-1β in intestinal fibrosis remains a subject of ongoing research, with studies indicating both pro-fibrotic and anti-fibrotic effects depending on the stage and type of intestinal injury (J. Wang *et al*., 2021).

- IL-36

IL-36 is a pro-inflammatory cytokine that plays a significant role in the pathogenesis of various inflammatory diseases, including intestinal fibrosis. It is part of the IL-1 family of cytokines, consisting of IL-36α, IL-36β, IL-36γ, and the IL-36 receptor antagonist (IL-36Ra). In the intestine, IL-36 is produced by a variety of cell types, including fibroblasts, myofibroblasts, epithelial cells, goblet cells, and macrophages. In the context of intestinal fibrosis, IL-36 appears to contribute to fibrosis through several mechanisms, primarily by influencing the activation and function of fibroblasts and myofibroblasts. Boutet *et al*. have shown that IL-36α and IL-36γ are upregulated in endoscopic biopsies from patients with active CD, compared to unaffected regions from the same patients (Boutet *et al*., 2016). Additionally, IL-36α and IL-36γ expression is elevated in the intestinal mucosa of UC patients compared to controls (Russell *et al*., 2016). Furthermore, increased expression of IL-36α has been observed in tissues from fibrostenotic CD patients, with elevated numbers of activated myofibroblasts. Both human and mouse fibroblasts show that activation of the IL-36 receptor (IL-36R) leads to upregulation of genes involved in fibrosis and tissue remodeling, including an increase in collagen VI expression. Studies using IL-36R-deficient mice or mice treated with anti-IL-36R antibodies have shown reduced severity of both colitis and fibrosis in models of DSS or TNBS-induced colitis (Scheibe *et al*., 2019). These findings suggest that inhibiting IL-36R signaling could be an effective strategy for preventing and treating intestinal fibrosis in IBD patients.

- IL-33

IL-33 is a potent alarmin, released from damaged or stressed cells, that can activate immune responses through its receptor, ST2. IL-33 is widely expressed across various cell types and organs throughout the body. It is particularly present in cells of hematopoietic origin, including specific populations of professional antigen-presenting cells like macrophages and dendritic cells. Additionally, IL-33 is expressed in a range of non-hematopoietic cells, such as fibroblasts, adipocytes, smooth muscle cells, endothelial cells, and both bronchial and intestinal epithelial cells. IL-33 is a strong activator of the Th2 immune response, which is marked by the production of cytokines such as IL-4, IL-5, and IL-13. In the context of intestinal fibrosis, IL-33 promotes the activation and proliferation of fibroblasts and myofibroblasts, (Hodzic *et al*., 2017) leading to excessive production of ECM components and contributing to fibrosis progression as demonstrated by several studies. Interestingly, Kobori *et al*. found increased expression levels of IL-33 and its receptor, ST2, in IBD patients, particularly in UC (Kobori *et al*., 2010). Immunohistochemistry experiments have shown intense IL-33 staining in the

lamina propria inflammatory infiltrates, primarily localized in cells morphologically resembling macrophages and B cells/plasma cells (Pastorelli *et al*., 2010). Furthermore, research on IL-33 expression in human IBD has been mirrored in the SAMP mouse model. This model is immunologically characterized by an initial Th1 response, followed by a mixed Th1/Th2 phenotype, both of which contribute significantly to disease severity. In the study of Pastorelli *et al*., SAMP mice exhibited elevated levels of IL-33 both in the serum and the intestinal mucosa. IL-33 was predominantly localized to intestinal epithelial cells and macrophages in the lamina propria. Nonetheless, the expression of IL-33 in the mucosa was found to correlate positively with the severity of enteritis in these mice (Pastorelli *et al*., 2010).

- IL-6 family members

Current studies suggest IL-6 and IL-11 may have a role in intestinal fibrosis.

- IL-6

IL-6 can be produced by a variety of cells in the intestine, including mononuclear cells, epithelial cells, and mesenchymal cells, particularly in response to inflammatory cytokines (Friedrich *et al*., 2019). IL-6 is highly expressed in intestinal mucosa and contributes to chronic inflammation. It promotes the survival and proliferation of immune cells, such as T cells, and enhances the production of other pro-inflammatory cytokines, sustaining the inflammatory cascade. Beyond its role in inflammation, IL-6 also contributes to fibrosis, particularly in chronic inflammatory conditions like CD. When stimulated by TGF-β1, intestinal mesenchymal cells become activated, resulting in increased autocrine secretion of IL-6, along with heightened production of ECM components such as collagen I. In stricturing Crohn's disease (Montreal classification: B2), α-SMA (+) muscle cells exhibit higher levels of immunoreactive IL-6 compared to the unaffected resection margins (C. Li & Kuemmerle, 2014).

- IL-11

IL-11 has been identified as a profibrotic cytokine released by myofibroblasts, injured epithelial cells, and SMCs (Lim *et al*., 2020). Its receptor, IL-11RA, is predominantly expressed on stromal cells, such as fibroblasts, smooth muscle cells, adipocytes, and hepatic or pancreatic stellate cells (pericytes), as well as on epithelial or polarized cells, including hepatocytes, alveolar epithelial cells, and kidney tubular epithelial cells (W.-D. Xu *et al*., 2019). Furthermore, transgenic mice (Il11SMC) engineered for smooth muscle cell-specific overexpression of murine IL-11 exhibit symptoms such as loose stools, progressive bleeding, and rectal prolapse. These mice also display an inflamed, fibrotic, and thickened bowel wall, which is associated with the activation of ERK and STAT3 signaling pathways (Lim *et al*., 2020). Further research employing a loss-of-function approach is

required to better understand the role of IL-11 in intestinal fibrosis. This could involve studies using IL-11-deficient mice or IL-11-neutralizing antibodies to clarify its specific contributions to the fibrotic process.

- Th17-related cytokines

Th17-related cytokines, including IL-17, IL-21, and IL-22, are increasingly implicated in the development of intestinal fibrosis. These cytokines, primarily produced by Th17 cells, play a significant role in promoting inflammation and immune responses.

- IL-17, IL-21 and IL-22

IL-17 cytokines, mainly produced by Th17 cells, consist of six related proteins: IL-17A (commonly referred to as IL-17), IL-17B, IL-17C, IL-17D, IL-17E (also known as IL-25), and IL-17F. These cytokines signal through a complex of five receptor subunits, namely IL-17RA through IL-17RE, which mediate their diverse biological effects (Ramani & Biswas, 2019). Numerous clinical and experimental studies have highlighted a potential pathogenic role for members of the IL-17 cytokine family in intestinal fibrosis. For instance, research has shown upregulation of IL-17 in the strictured gut of CD patients. Furthermore, patients with active CD have elevated levels of fecal IL-17 and an increased frequency of IL-17-producing cells in the lamina propria (Biancheri *et al*., 2013). Additionally, studies suggest that IL-17 interacts directly with colonic myofibroblasts expressing IL-17 receptors, playing a significant role in the development of strictures in CD (Hata *et al*., 2002) (Yagi *et al*., 2007). Accordingly, in response to IL-17 stimulation, gut-resident fibroblasts display reduced migration capacities and produce elevated levels of collagen and TIMP1. This altered behavior contributes to the fibrotic process by promoting the accumulation of extracellular matrix components and inhibiting ECM remodeling, ultimately enhancing tissue stiffness and fibrosis in the intestinal tract. Furthermore, the occurrence of EMT and high levels of IL-17A are observed in the intestinal mucosa of CD patients, and IL-17A may induce EMT in intestinal epithelial cells. Interestingly, anti-IL-17A treatment alleviated intestinal fibrosis by reducing EMT in mouse intestines(H.-J. Zhang *et al*., 2018). In addition to IL-17A-F, Th17 cells also produce IL-21, and IL-22 which are significantly increased in active IBD (W. Xu *et al*., 2017). IL-21 plays a crucial role in promoting the differentiation of Th17 cells and enhances the production of other pro-inflammatory cytokines, thus contributing to the chronic inflammation characteristic of IBD. It also influences the activation of fibroblasts, contributing to the fibrotic processes observed in IBD. Consistently, IL-22 is involved in tissue repair and protection of the intestinal epithelium under normal conditions, but its overproduction in the context of IBD can promote both inflammation and fibrosis.

- Th2-related cytokines

The Th2 immune response is characterized by producing key cytokines, particularly IL-4, and IL-13, which promote inflammation and tissue remodeling.

- IL-4 and IL-13

Originally, it was thought that Th1 cells were primarily involved in CD, while Th2 or Th2-like cells were linked to UC. However, more recent research has shown that Th2 immune responses are also present in the later stages of both experimental and human CD (J. Li *et al*., 2016) (Kugathasan *et al*., 2007). Previous research on fibroblastic synovial cell lines has suggested that IL-4 may act as a pro-fibrogenic cytokine, promoting the synthesis of collagen and fibronectin during both normal healing and pathological fibrosis. Additionally, when human hepatic fibroblasts are treated with IL-4, collagen production significantly increases (Aoudjehane *et al*., 2008). IL-13 signals through IL-13Rα2 to activate the TGF-β1 promoter and the prevention of IL-13Rα2 expression reduced production of TGF-β1 in oxazolone- or TNBS-induced colitis (J. Wang *et al*., 2021). Furthermore, IL-4 and IL-13 drive M2 polarization: both cytokines promote the differentiation of macrophages into the M2 phenotype, which supports fibrosis by secreting TGF-β1 and other fibrogenic factors amplifying the fibrotic response (Barron & Wynn, 2011).

- TNF-α family members

Two members of the TNF-α family, TNF-α and TNF-like ligand 1A (TL1A), are well-known for their involvement in the pathogenesis of IBD. Growing evidence indicates that these cytokines may also directly influence myofibroblasts or EMT, linking them to fibrosis in both the intestine and other organs *(*Friedrich *et al*., 2019) (Curciarello *et al*., 2017).

- TNF-α

Anti-TNF monoclonal antibodies are widely recognized for their effectiveness in treating IBD (Frei et al., 2019). TNF-α has been shown to increase the secretion of IL-8, MCP-1, and MMP-1, as well as alter the release of type I and IV collagen in subepithelial myofibroblasts, though it does not affect their proliferation (Okuno *et al*., 2002). Additionally, TNF-α upregulates the expression of TGF-β1 and TIMP-1 in colonic epithelial cells. Furthermore, conditioned medium from epithelial cells treated with TNF-α can induce the production of MMP-9 in colonic subepithelial myofibroblasts (Drygiannakis *et al*., 2013). A study investigating the potential impact of anti-TNF-α antibodies on small bowel stenosis found no progression of pre-existing stenoses, no development of new stenoses, and complete regression in 1 out of 22 cases following the induction phase (Pallotta *et al*., 2008). However, a separate study involving pediatric CD patients revealed that those who received early anti-TNF-α therapy were less likely to develop penetrating complications, though this benefit did not

extend to stricturing complications (Kugathasan *et al*., 2017). Overall, it is widely believed that anti-TNF antibodies do not effectively prevent the formation of strictures in CD.

- TL1A

TL1A and its receptor DR3 (TNFRSF25) are key members of the TNF/TNFR superfamilies, playing critical roles in chronic intestinal inflammation as universal costimulators of immune responses and regulators of Treg function (Siakavellas & Bamias, 2015). Recently, their involvement in intestinal fibrosis has also been highlighted. The TL1A/DR3 interaction enhances various immunological pathways, including Th2/IL-13 and Th17/IL-17A-dependent mechanisms, which, when persistently activated, may contribute to fibrosis. TL1A expression is found in human intestinal myofibroblasts and is upregulated in CD patients. This expression is further stimulated by CD-associated pro-inflammatory cytokines such as TNF, and IL-1α, as well as by supernatants from intestinal tissue cultures of IBD patients or prestimulated epithelial cells (Bamias *et al*., 2017). Notably, significant increases in both TL1A and DR3 expression have been observed in the ileitis- and fibrosis-prone SAMP mouse strain (Bamias *et al*., 2006). Notably, in a phase 2 clinical trial (NCT02840721), treatment with the anti-TL1A antibody (PF-06480605) resulted in a reduction of fibrotic-related gene expression and helped alleviate ECM remodeling (Hassan-Zahraee *et al*., 2022).

- Angiotensin II

Angiotensin II (ANG II) is a hormone that plays a crucial role in regulating blood pressure and fluid balance, and it is part of the REN-angiotensin system (RAS). ANGII is produced from angiotensin I, which is converted by the enzyme angiotensin-converting enzyme (ACE). Inokuchi *et al.* have provided valuable insights into the role of RAS in the development and regulation of chronic intestinal inflammation and associated fibrosis. The direct impact of ANG II on the pathogenesis of immune-mediated colitis was investigated using mice genetically deficient in angiotensinogen (Atg-/-). In this study, TNBS-induced acute colitis was found to be less severe in Atg-/- mice compared to wild-type (Atg+/+) mice (Inokuchi *et al*., 2005). A recent study revealed a link between mucosal fibrosis, evaluated through MTC staining, and the expression of ANGII, as indicated by IHC staining intensity, in endoscopic biopsies from the colon. Additionally, the study showed that ANGII could stimulate myofibroblast proliferation and collagen production via AGTR1 after PDGF and TGF-β1 activation in colonic myofibroblasts (Garg *et al*., 2020). Both ANG II and TGF-β1 are overexpressed in intestinal fibrosis and stenosis, especially in CD. The production of TGF-β1 is significantly enhanced by the local activation of ANG II. Furthermore, an increased expression of ANG II in the colon of rats and mice with experimentally induced colitis further supports the involvement of the RAS in intestinal inflammation and fibrosis (Wengrower *et al*., 2004) (Katada *et al*., 2008). ANG II promotes

the recruitment of infiltrating inflammatory cells to tissues by stimulating the production of specific cytokines and chemokines. For instance, Ang II has been shown to induce the production of MCP-1, a potent monocyte chemoattractant, in cultured monocytes (Dai *et al*., 2007). The pro-inflammatory effects of ANG II are also mediated through the activation of dendritic cells (DCs), which are specialized antigen-presenting cells (APCs) essential for immune defense and inflammatory responses. Cultured DCs express both ANG II receptors, and ANG II promotes their migration, maturation, and antigen-presenting capacity (Lapteva *et al*., 2001).

1.2.3 Canonical and non-canonical pathways driving intestinal fibrosis

As mentioned earlier, TGF-β1 is inactive as a disulfide-bonded homodimer, attached to a LAP. Activation of TGF-β1 requires the dissociation of the LAP, a process that several factors, including cathepsins, plasmin, calpain, thrombospondin, integrin-αvβ6, and matrix metalloproteinases can facilitate. Once activated, TGF-β1 binds to its receptors, initiating signaling through two main pathways: the canonical (Smad-dependent) pathway (**Figure 7)** and the non-canonical (Smad-independent) pathway (**Figure 8**).

- Canonical pathway

- Smad-dependent pathway

The 'canonical' TGF-β1 signaling pathway is characterized by activating Smad proteins, which act as transcriptional regulators. Upon TGF-β1 binding to its receptor, TGF-β receptor type I (TβRI) and TGF-β receptor type II (TβRII), the receptors phosphorylate the Receptor-activated Smads (R-Smads), specifically Smad2 and Smad3. These phosphorylated Smads then associate with Smad4, a common mediator, and together they translocate to the nucleus where they interact with other transcription factors and co-factors to regulate the expression of target genes (Leask & Abraham, 2004). These R-Smads are central to the fibrotic response, particularly in the activation of myofibroblasts, which are responsible for collagen and other ECM components that contribute to tissue scarring. On the other hand, Smad7, classified as an inhibitory Smad (I-Smad), acts as a negative regulator of TGF-β1 signaling. By binding to the TGF-β1 receptor, Smad7 prevents the phosphorylation of Smad2 and Smad3, inhibiting their activation and preventing excessive ECM accumulation. Smad7 also facilitates the degradation of the TGF-β receptor complex, further limiting the duration and intensity of the signaling (Yun *et al*., 2019).

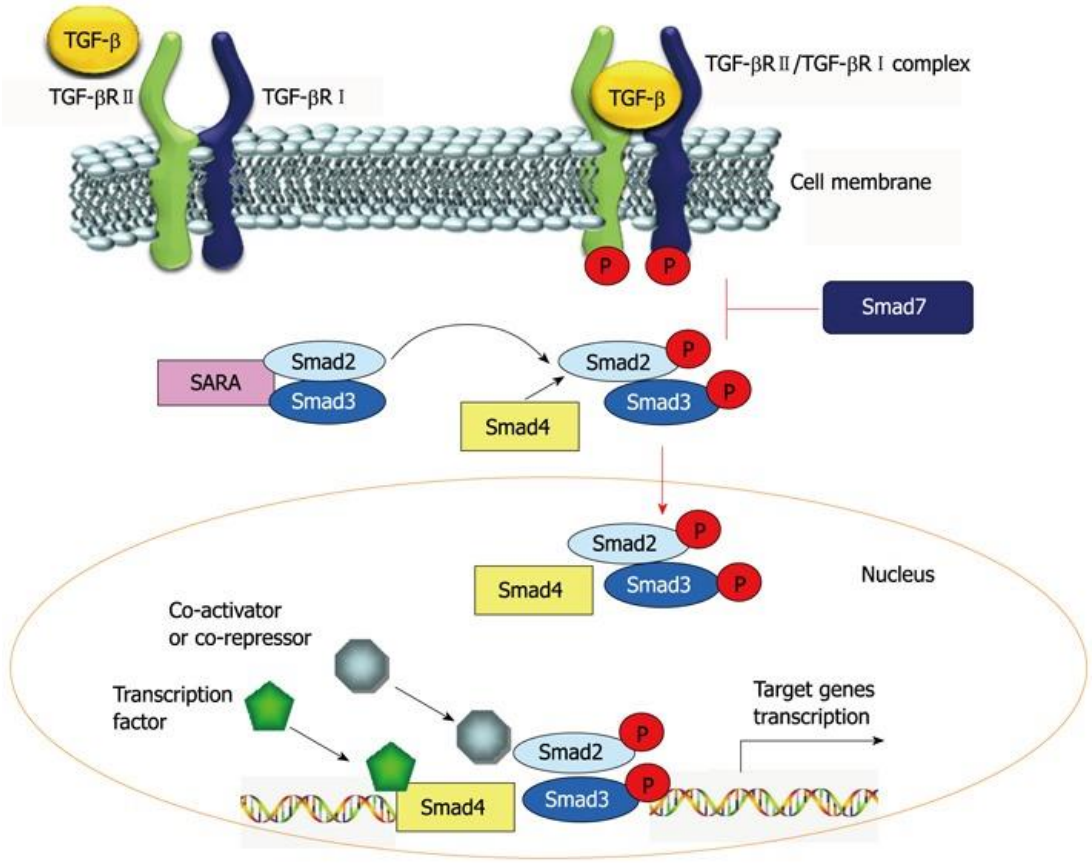


Figure 7. TGF-β1 activated canonical signaling pathway in intestinal fibrosis. (Speca *et al*., 2012).

Xu *et al*. have demonstrated the involvement of TGF-β signaling in inducing EMT. When TGF-β is introduced to epithelial cell cultures, it leads to a decrease in epithelial markers and an increase in mesenchymal markers, which is a hallmark of EMT. The activation of Smad2 and Smad3 correlates with the promotion of EMT. Furthermore, knockout of Smad3 prevents the initiation of EMT in response to TGF-β stimulation, underscoring the essential role of Smads in mediating TGF-β-induced EMT (J. Xu *et al*., 2009). Additionally, in human intestinal organoids, which contain both epithelial and mesenchymal cells, including myofibroblasts, TGF-β stimulation increased the production of a series of pro-fibrotic factors, such as collagen I, α-SMA, and fibronectin (Rodansky *et al*., 2015). Nonetheless, knockdown of Smad7, obtained with a specific antisense oligonucleotide, attenuated colitis, and colitis-driven colonic fibrosis in the TNBS mice model (Izzo *et al*., 2018).

- Wnt/β-catenin pathway

In the Wnt/β-catenin pathway, Wnt ligands binding to the Frizzled receptor and LRP5/6 co-receptor, inhibit the β-catenin destruction complex, and lead to β-catenin accumulation and nuclear translocation. There, β-catenin interacts with transcription factors TCF/LEF to regulate genes that promote fibroblast activation, EMT, and extracellular matrix production. Smad-dependent pathways

and Wnt/β-catenin pathway can interact, as TGF-β1 can enhance Wnt signaling by upregulating Wnt ligands or stabilizing β-catenin. At the same time, β-catenin amplifies TGF-β1 activity by supporting Smad signaling. This crosstalk exacerbates fibroblast activation, EMT, and matrix deposition, creating a feedback loop that perpetuates fibrosis in the intestinal microenvironment (Vallée *et al*., 2017). Interestingly, Ye *et al*. showed that TGF-β1 can upregulate β-catenin and Wnt signaling through the inhibition of the kinase Glycogen Synthase Kinase 3 beta (GSK3-β), thus enhancing the EMT process (Ye *et al*., 2013). The role of the Wnt/β-catenin pathway in EMT induction is well established. In normal conditions, the inactive β-catenin forms a complex with E-cadherin at the epithelial barrier. This complex is involved in maintaining the epithelial stability and in keeping silent the β-catenin signaling. In pathological conditions, such as fibrosis, the Wnt signaling is active, the complex is disrupted, the β-catenin is free to translocate into the nucleus, and the E-cadherin is degraded thus enhancing the loss of the epithelial phenotype. Moreover, the epithelial phenotype is restored by inhibiting the complex disruption, suggesting an essential role for the Wnt-mediated EMT in fibrosis (Di Gregorio *et al*., 2020).

- Non-canonical pathway

- PI3K-Akt pathway

PI3K-Akt signaling is key in regulating various cellular processes such as survival, growth, and metabolism. TGF-β was shown to activate Akt through PI3K via both Smad-dependent and Smad-independent pathways. Additionally, the p85 regulatory subunit of PI3K is found to be continuously bound to TβRII, while its interaction with TβRI is observed only when TGF-β is present (Yi *et al*., 2005). Evidence increasingly supports the role of the PI3K/Akt pathway in the development of fibrosis across various tissues. For instance, studies have reported that human skin systemic sclerosis (SSc) fibroblasts exhibit higher levels of Akt phosphorylation compared to healthy fibroblasts both *in vitro* and *in vivo* (Finnson *et al*., 2020). Although these studies did not assess whether this effect is directly linked to enhanced TGF-β signaling, a proteomic analysis comparing SSc and normal fibroblasts treated with TGF-β showed increased activation of the PI3K/Akt pathway in primary human SSc fibroblasts *in vitro* (Chaigne *et al*., 2019). This finding suggests that TGF-β may induce PI3K/Akt signaling more robustly in SSc fibroblasts compared to their normal counterparts. Furthermore, TGF-β has been shown to activate the PI3K-AKT-mTOR pathway, also enhancing the EMT process (Lamouille *et al*., 2012). Notably, inhibition of this pathway has been found to mitigate both EMT and fibrotic processes. Additionally, *in vitro* studies have demonstrated that TGF-β rapidly induces TORC2 (mTOR complex 2) activity via AKT phosphorylation, and this activation is essential for TGF-β-induced EMT. TORC2 plays a critical role in regulating various aspects of EMT, including

gene expression and cytoskeletal reorganization, and serves as a positive feedback modulator of TGF-β signaling (Cai *et al*., 2017).

- ERK/JNK/P38 MAPK pathway

As a dual-specificity kinase, TβRI can phosphorylate its tyrosine residues to activate ERK signaling in response to TGF-β stimulation. In this process, TβRI's tyrosine kinase activity phosphorylates the adaptor protein sarcoma (SRC) homology and collagen A (SHCA). This phosphorylation event leads to the formation of a complex involving SHCA, growth factor receptor-bound protein 2 (GRB2), and son of seven less homolog (SOS). The resulting SHCA-GRB2-SOS complex triggers the canonical MAPK signaling cascade, which progresses through the activation of RAS, the MAPKKK RAS-associated factor (RAF), the MAPKK MAPK/ERK kinase (MEK), and ultimately the ERK MAPK. Once activated, ERK regulates a wide range of biological processes, including cell survival, proliferation, differentiation, adhesion, migration, and metabolism, and is associated with various diseases, such as developmental disorders, chronic inflammation, neurodegeneration, obesity, and cancer (Z. Deng et al., 2024). Furthermore, TGF-β can activate the signaling of another two MAPKs known as p38 and JNK through a receptor kinase-independent mechanism distinct from ERK signaling. The TGF-β-activated TβR complex recruits TRAF4 and TRAF6, triggering their K63-linked polyubiquitination. Polyubiquitinated TRAF activates TAK1, a MAPKKK, which then phosphorylates MAPKKs. These MAPKKs activate p38 and JNK, with MKK3 and MKK6 specifically targeting p38, while MKK4 activates both p38 and JNK. Additionally, TGF-β-activated Rho GTPases, such as RHOA, RAC1, and CDC42, contribute to p38 and JNK activation (Z. Deng et al., 2024). Several studies have demonstrated increased JNK activation in the intestines of patients with IBD. Waetzig *et al*. were the first to report elevated activation of JNK, along with ERK and p38 MAPK, in colonic tissue from 27 patients with moderate to severe CD or UC (Waetzig *et al*., 2002). Similarly, Hommes *et al*. observed heightened activation of p38 and JNK in their study involving 12 patients. These findings were later corroborated by Mitsuyama *et al*., who investigated whether JNK phosphorylation was more pronounced in areas of active inflammation compared to unaffected intestinal regions in IBD patients (Mitsuyama *et al.*, 2006). Using ELISA and immunostaining, they confirmed significant JNK activation in inflamed colonic tissues, with phospho-JNK localized primarily in the nuclei of intestinal cells, macrophages, and lymphocytes. This *in vivo* evidence aligns with earlier *in vitro* cell culture studies. Notably, increased JNK activation has also been associated with steroid-resistant cases of IBD (Bantel *et al*., 2002).

- Hippo pathway

The Hippo signaling pathway plays a crucial role in regulating organ size, cellular regeneration, and self-renewal. It involves multiple components, including serine/threonine kinases MST1/2, Salvador

(SAV), MOB kinase activator 1 (MOB1), and serine/threonine kinases LATS1/2. Yes-associated protein 1 (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) function as transcriptional coactivators within this pathway (Piersma *et al*., 2015). When Hippo signaling is active, YAP and TAZ are phosphorylated, leading to their cytoplasmic retention or degradation via β-TrCP proteins. Phosphorylation of YAP through active Hippo signaling correlates with reduced β-catenin levels. F-actin polymerization inhibits MST1/2 activity during fibrosis, which inactivates the Hippo pathway. This deactivation allows YAP and TAZ to translocate into the nucleus, interacting with transcription factors like Runt-related transcription factor (RunX) and TEA domain (TEAD) family members to promote gene expression. YAP and TAZ have been implicated in fibrosis progression by activating myofibroblasts. Elevated levels of these proteins have been observed in biopsies from patients with idiopathic pulmonary fibrosis, where they contribute to fibroblast activation and subsequent fibrosis. Studies in lung fibroblasts and mouse liver models have shown that silencing YAP and TAZ decreases the expression of procollagen, α-SMA, and plasminogen activator inhibitor 1 (PAI-1), all of which are associated with myofibroblast differentiation (Vallée & Lecarpentier, 2019).

- PPARγ

PPARγ, a transcription factor within the nuclear hormone receptor superfamily, forms heterodimers with the retinoid X receptor to bind specific DNA sequences known as PPAR response elements (PPREs). This factor is expressed in various cell types, including adipose tissue, muscles, brain, and the immune system. By regulating numerous target genes, PPARγ plays a critical role in maintaining glucose homeostasis, enhancing insulin sensitivity, modulating lipid metabolism, and influencing immune responses and inflammation (Lehrke & Lazar, 2005). PPARγ is also present in fibroblasts, where it influences connective tissue dynamics, mesenchymal cell activation, differentiation, and survival. It serves as a link between cellular metabolism and fibrogenesis (Wei, 2012). Dysregulated levels of PPARγ are associated with several pathological conditions, such as obesity, cancer, diabetes, atherosclerosis, and multiple sclerosis. In the context of fibrosis and skin repair, PPARγ plays a vital role. Following skin injury, resident fibroblasts experience mechanical stress and respond to TGF-β1 released by immune cells and platelets at the wound site. This response drives the migration of dermal fibroblasts from surrounding tissues into the injury area, where they differentiate into myofibroblasts. PPARγ helps mitigate fibrosis by counteracting TGF-β1 activity. Agonists of PPARγ are known to reduce TGF-β-induced collagen deposition and inhibit myofibroblast differentiation (Burgess *et al*., 2005). The expression of PPARγ is suppressed by various cytokines, chemokines, and intracellular signaling pathways, including TGF-β1, the canonical WNT/β-catenin pathway, TNF-α, and hypoxia. Conversely, transcription factors such as C/EBP, ELF proteins, and nuclear factor erythroid 2-related

factor 2 (Nrf2), associated with NF-E2, are known to positively regulate PPARγ expression (Abdelkarim *et al*., 2010).

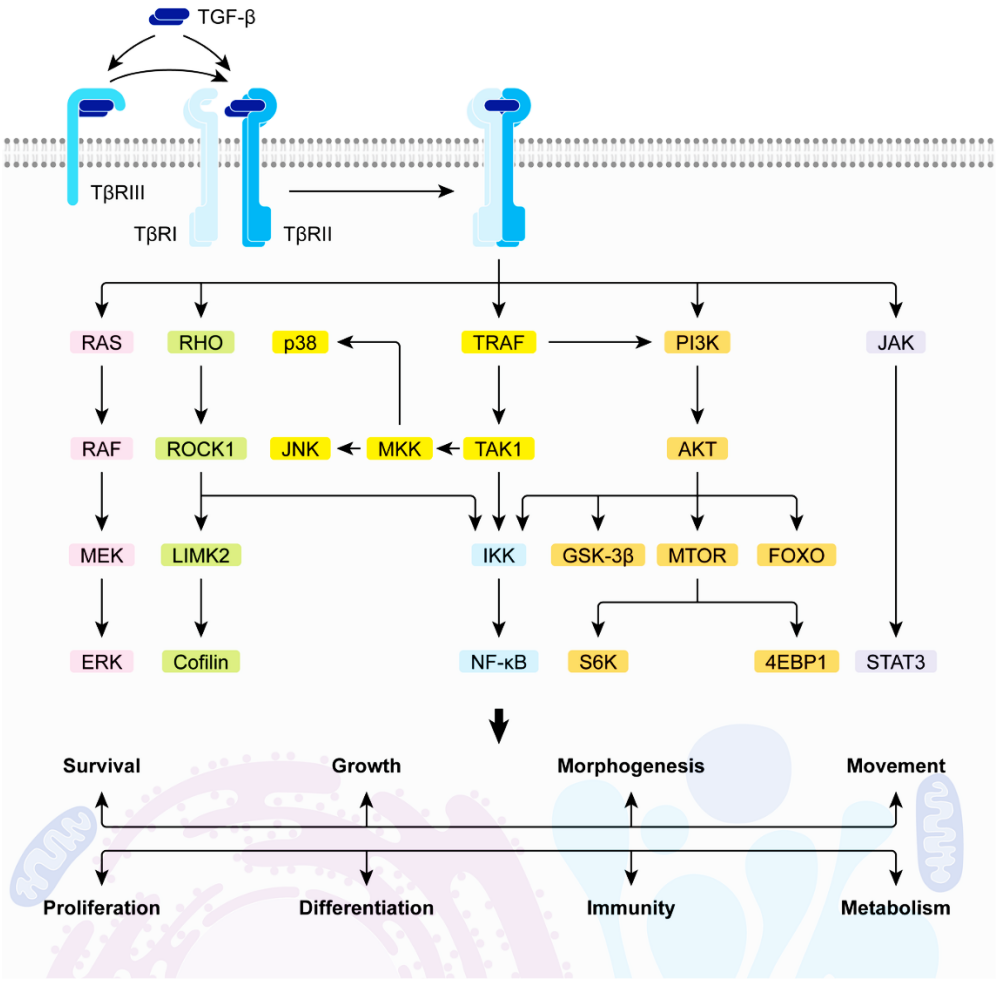


Figure 8. TGF-β -activated non-canonical signaling pathway (Z. Deng et al., 2024)**.**

Following the discussion of the canonical and non-canonical TGF-β pathways, which play a central role in the fibrotic process, it is essential to consider additional signaling systems that further modulate fibrosis in the intestine. One such mechanism is the RAS, which has been extensively shown to contribute to fibrosis development across multiple organs (Bataller et al., 2003) (J. Wang et al., 2015) (Hedayatyanfard *et al*., 2020).

1.2.3.1 Emerging role of RAS in inflammation and fibrosis.

RAS is a complex network of enzymes and peptides that plays a critical role in regulating blood pressure, fluid balance, and various inflammatory processes. Angiotensinogen (AGT), primarily synthesized in the liver, serves as the precursor for all RAS components (Salmenkari *et al*., 2021). The RAS is now recognized as a "ubiquitous" system, locally expressed in various tissues, where it

exerts numerous paracrine and autocrine effects crucial for tissue physiology and homeostasis. Beyond its classical roles, RAS is actively involved in regulating cellular processes such as growth, proliferation, differentiation, migration, and apoptosis. Additionally, it contributes to ECM remodeling and modulates inflammatory responses, highlighting its multifaceted roles in maintaining tissue integrity and function. Recent developments in understanding the RAS have revealed two complimentary pathways, classical and alternative (**Figure 9**), that mediate opposing effects on inflammation, fibrosis, and cell proliferation. It is the balance of signaling from these pathways that determines tissue homeostasis (Nehme *et al*., 2019).

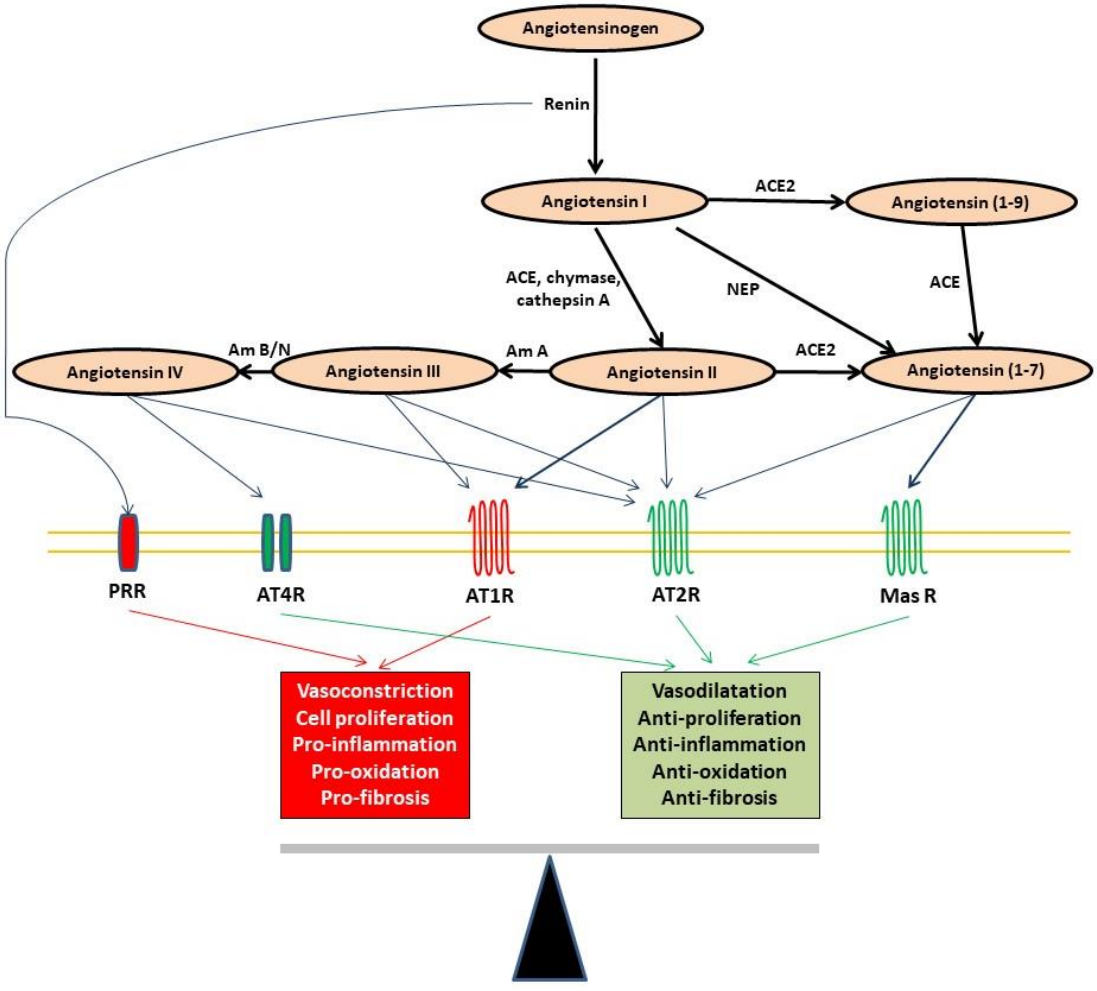


Figure 9. The balance between the classical and the alternative RAS pathway (Garg *et al*., 2020)**.**

1.2.3.1.1 Classical and alternative RAS pathways

In its classical view, RAS involves a series of enzymatic reactions that convert the "inactive" substrate AGT into the active peptide ANG II, which binds to specific membrane receptors to mediate cellular effects. AGT, a glycoprotein primarily produced by the liver, is also expressed in various tissues, including the heart, blood vessels, kidneys, GI tract, and adipose tissue. Its production can be

stimulated by factors such as inflammation, insulin, estrogen, glucocorticoids, thyroid hormone, and ANG II. Within plasma, AGT is converted into the decapeptide angiotensin I (ANG I) through the action of REN (REN), an enzyme tightly regulated by the juxtaglomerular cells (JG) in the kidneys. This conversion is considered the rate-limiting step in the release of ANG II. Ren is initially synthesized as an inactive precursor, prorenin, which is either secreted in its inactive form or processed by various proteases into active REN stored in JG cell granules. Upon specific stimuli, active REN is released into circulation via an exocytic mechanism, with ANG II providing negative feedback regulation. ANG I is subsequently converted into the vasoactive octapeptide ANG II by ACE, a membrane-bound exopeptidase (Nehme *et al*., 2019). The effects exerted by ANG II are mediated through its interaction with two G protein-coupled receptors (GPCRs), AGTR1 and angiotensin type 2 receptor (AGTR2) (**Figure 10**).

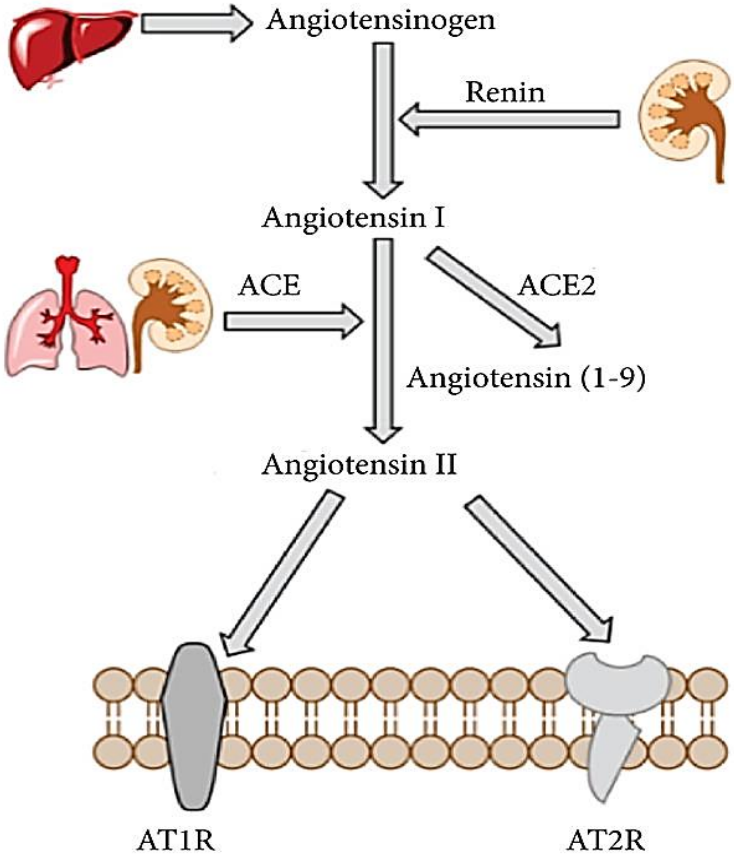


Figure 10. Components of the classical RAS pathway (Feng *et al*., 2020).

The majority of ANG II's functions are attributed to its binding to AGTR1, which triggers the activation of phospholipases C, A2, and D while inhibiting adenylate cyclase activity. Additionally, AGTR1 engagement by ANG II promotes tyrosine kinase phosphorylation and phospholipase C-γ activation, initiating downstream signaling cascades, including Janus kinases and MAPKs. These

pathways ultimately modulate gene transcription and various cellular responses. ANG II binding to AGTR1 also facilitates the opening of calcium channels, allowing extracellular Ca^{2+} influx into the cell. Furthermore, ANG II activates the Smad signaling pathway, similar to TGF-β, by phosphorylating R-Smads such as Smad2 and Smad3. Notably, this activation occurs even in the presence of TGF-β antagonists, contributing to pro-fibrotic effects downstream (AlQudah *et al*., 2020).

The alternative RAS pathway is primarily composed of ACE2, the heptapeptide Ang-(1–7), and the Ang-(1–7) Mas receptor (MasR/MAS1). Unlike the classical pathway, the alternative pathway often counteracts its effects. ACE2, which shares 42% similarity in the catalytic domain with ACE, cleaves ANG I to produce Ang-(1–9) and cleaves ANG II to form Ang-(1–7). ANG-(1–7) can also be generated from ANG-(1–9) through ACE activity or directly from ANG I via neutral endopeptidase or prolyl endopeptidase. ANG-(1–7) primarily exerts its effects by binding to MasR, while ANG-(1–9) interacts with AGTR2 to mediate various physiological and pathological processes. MasR, a G protein-coupled receptor with oncogenic properties, is expressed ubiquitously (AlQudah *et al*., 2020). An identified RAS component, Ala1-ANG-(1–7), or alamandine, is produced either by the decarboxylation of ANG-(1–7) or through ANG II decarboxylation to form angiotensin A, which is then converted into alamandine by ACE2. Alamandine exerts anti-hypertensive and anti-fibrotic effects through its interaction with the Mas-related GPCR, member D (MrgD) (Lautner *et al*., 2013).

The ANG II/AGTR2 axis typically opposes the effects mediated by the ANG II/AGTR1 axis while ANG II/AGTR1 promotes phosphorylation and protein synthesis, ANG II/AGTR2 dephosphorylates signaling molecules and inhibits protein synthesis. However, the actions of these two axes are highly dependent on the specific dose and tissue location. The activities of ACE and ACE2 are crucial for maintaining the balance between these RAS pathways, as they regulate local shifts in their equilibrium and modulate their physiological and pathological effects (Crackower *et al*., 2002).

1.2.3.1.2 RAS functions across intestinal and extraintestinal environments in healthy and disease

ANG-II was initially recognized as a circulating hormone responsible for regulating blood pressure and electrolyte balance through its effects on vascular contraction, aldosterone secretion, renal sodium handling, sympathetic activity, and vasopressin release (Ribeiro-Oliveira *et al*., 2008). However, molecular research has revealed that activation of AGTR1 can also induce long-term genetic effects, alongside its rapid, short-term cellular impacts (Nehme & Zibara, 2017). In addition to its role as a circulating hormone, RAS is increasingly recognized as a complex and expanded

system that operates locally in tissues. Different enzymatic pathways within the RAS can generate various angiotensin peptides, each with distinct effects that are dependent on the specific tissue type and environmental conditions. This dual nature of RAS allows it to act both systemically (as a circulating hormone) and locally (as an autocrine, paracrine, or intracrine regulator), playing important roles in processes such as blood pressure regulation, inflammation, fibrosis, and tissue homeostasis (Ferrario *et al*., 2014). The initial evidence for a local RAS was provided in 1971 when REN-like activity was detected in the brains of dogs, separate from the REN found in the kidneys and plasma (Husain *et al*., 1983). Since that discovery, the existence and physiological significance of local angiotensin pathways have been identified in various tissues, including the heart, blood vessels, GI tract, kidneys, brain, adipose tissue, adrenal glands, pancreas, liver, reproductive system, lymphatic tissue, placenta, and eyes (Nehme *et al*., 2019) (Garg *et al*., 2012). Within these tissues, local RAS operates independently of the systemic RAS, functioning in a paracrine and autocrine manner. However, it can still interact with the systemic RAS to produce endocrine effects.

RAS outside the GI tract

Outside the GI tract, the RAS has distinct functions across various organs. In the kidney, local AGT is converted into ANG I and subsequently into ANG II by tubular ACE, which facilitates sodium and fluid absorption through luminal AGTR1, potentially influencing blood pressure independently of systemic ANG II. Components such as REN, ACE, AGT, AGTR1, and AGTR2 regulate myocyte proliferation and cardiac remodeling in the heart. In the brain, RAS elements, including REN, AGT, ANG II derivatives, and their receptors, influence blood pressure, electrolyte balance, thirst, blood-brain barrier maintenance, and neuronal development, including memory and learning. The liver expresses REN, AGT, ANGII, ACE, AGTR1, ANG (1–7), ACE2, and mas receptors, all upregulated in the diseased liver. In the pancreas, ANG II inhibits glucose-stimulated insulin secretion and, through AGTR1 and AGTR2, modulates exocrine enzyme release and microcirculation (Garg *et al*., 2012). As previously reported, RAS plays a crucial role in maintaining physiological balance across various organs; however, dysregulation of this system can lead to pathological processes, contributing to the development and progression of diseases, including fibrosis. In line with this consideration Yoshiji *et al*., demonstrated that RAS is frequently activated in patients with chronic liver diseases and ANG II has been suggested to play an important role in liver fibrogenesis since it induces hepatic stellate cell (HSC) proliferation and up-regulates TGF-β1 expression via ATGR1 *in vitro* (Yoshiji *et al*., 2001). Furthermore, Bataller *et al*., showed that quiescent hepatic stellate cells barely express the RAS components AGT, REN, and ACE and do not secrete ANG II. In contrast, both *in vivo*-activated hepatic stellate cells and culture-activated hepatic stellate cells highly express active REN and ACE

and secrete ANGII to the culture media (Bataller *et al*., 2003). Wang *et al*. found that RenTgMK mice (transgenic mouse model that produces active REN in their liver) spontaneously develop progressive pulmonary fibrosis that impairs lung function, and the development of lung fibrosis depends upon the REN-ANG II-AGTR1 cascade but is independent of high blood pressure. In this transgenic model, ANG II stimulates lung fibroblast activation with increasing levels of fibronectin and collagen I and III (J. Wang *et al*., 2015). Additionally, injury of the skin induces time-dependent expression changes in various RAS components. In human skin, ACE and both AGTR1 and AGTR2 are up-regulated 24 hours after cutaneous injury, with higher expression of AGTR2 in the latter stages of the healing process. Inhibition of ACE is correlated with a reduction in TGFβ and tissue necrosis factor α suggesting that ACE is involved in skin healing (AlQudah *et al*., 2020). Nonetheless, Ang II is a key mediator of chronic kidney disease, in which EMT is a critical process mediated by the TGFβ/Smad signaling pathway. The F. Yang *et al*., study demonstrated that ANG II through the receptor of AGTR1, not AGTR2, activates Smad2/3 and induces EMT in a normal rat tubular epithelial cell line probably by degradation of an inhibitory Smad7 (F. Yang *et al*., 2010).

RAS in the GI tract

The small intestine has received significant attention in studies of RAS, with key components such as ACE, ACE2, neutral endopeptidase (NEP), and angiotensin receptors playing critical roles. ACE is abundantly present on the brush border of epithelial cells and the mesenteric microvascular endothelium in humans. Similarly, ACE2 mRNA and protein are highly expressed in the small intestinal epithelial brush border, muscularis mucosa, and muscularis propria, as well as in microvascular endothelium and vascular smooth muscle cells. Notably, the highest concentrations of ACE and ACE2 mRNA in the human body are found in the terminal ileum, duodenum, and colon. AGTR1 and AGTR2 are distributed across various regions of the small intestine. AGT1R is primarily localized to the epithelial brush border but is also found in the circular and longitudinal muscle layers and the myenteric plexus, whereas AT2R is mostly restricted to the myenteric plexus. The expression of angiotensin peptides in the small intestine has been explored, with ANG II detected in the crypt and crypt-villus junction epithelial cells. In rats, angiotensinogen has been found in various intestinal regions, including the brush border, epithelial cells, lamina propria, muscularis mucosa, and submucosal blood vessels. The presence of the necessary components for local production and action of Ang II in the small intestine is clear, highlighting its potential role in regulating intestinal processes. RAS is implicated in several important functions within the small intestine. These include bicarbonate secretion, which is stimulated by ANG II via both AGTR1 and AGTR2 in the duodenum. Sodium and water absorption in the jejunum and ileum is modulated by ANG II in coordination with the enteric

sympathetic nervous system. Brush border ACE and ACE2 play roles in the digestion and absorption of peptides, with ACE2 enhancing the activity of the neutral amino acid transporter B0AT1. Furthermore, ACE2 is also suggested to play a role in active secretion, and its involvement in the pathogenesis of SARS-CoV infection, which causes watery diarrhea, has been proposed, although the exact mechanism is still unclear. In contrast, the colon has been less extensively studied about RAS components. REN, AGTR1, ACE, and ACE2 are expressed in the colonic wall, with REN being found in the surface epithelium, lamina propria mesenchymal cells, microvascular walls, and muscularis mucosa. AGTR1 expression is observed on surface epithelial cells, crypt bases, lamina propria macrophages, myofibroblasts, and mucosal vessel walls, while AGTR2 shows weak expression in some of these regions. ACE is weakly expressed in surface epithelial cells and more prominently in mesenteric microvascular walls and submucosal mesenchymal cells. ACE2 is mainly localized to the mesenteric microvascular endothelium and is absent from the colonic epithelium. Although fewer functional roles for RAS in the colon have been established, ANG II has been shown to enhance sodium and water reabsorption in rats and plays a role in regulating colonic motility through muscle contraction. Furthermore, higher levels of ANG I and ANG II have been associated with inflammation in patients with active CD, suggesting that the RAS may contribute to the pathology of IBD. In the stomach, components of the RAS have been identified in gastric mucosal biopsy specimens, particularly in mesenchymal cells, vascular endothelial cells, and the basal surface of gastric epithelial cells. The functional roles of the RAS in the stomach are less well understood, but a potential involvement in gastric inflammation has been suggested, especially in patients with Helicobacter pylori infection. The esophagus also expresses ACE, AGTR1, and AGTR2, with these components found in the lamina propria microvascular walls, superficial stratified epithelium, and smooth muscle layers. ANG II has been shown to cause smooth muscle contraction in the esophagus, however, research into the expression and functional roles of other RAS components in the esophagus is limited (Garg *et al*., 2012) (**Figure 11**).

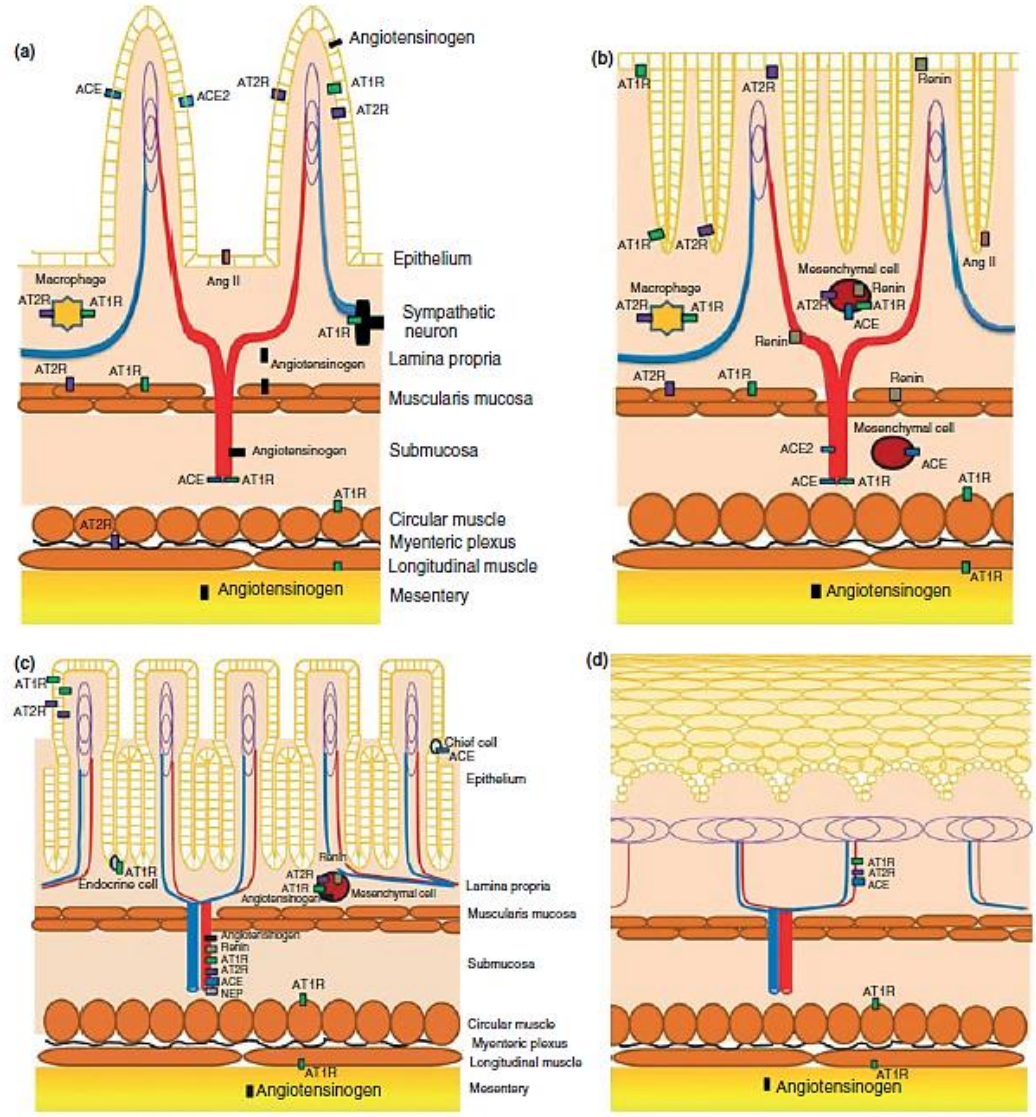


Figure 11. Diagrammatic representation of the distribution of RAS components across different regions of the gastrointestinal tract: (a) small intestine, (b) colon, (c) stomach, and (d) esophagus (Garg *et al*., 2012)**.**

Given the extensive involvement of RAS in processes like inflammation, fibrosis, immune cell recruitment, and apoptosis (increasing reactive oxygen species (ROS) production through activation of NADPH oxidase), disturbances in RAS effectors and the activation of the classical pathway may contribute to the pathogenesis of IBD. Alterations in RAS have been suggested in IBD, with early evidence from a 1990 study by Jaszewski *et al*., who found elevated levels of ANG I and ANG II in the inflamed colonic mucosa of CD patients. These levels were higher compared to healthy controls and UC patients and correlated with endoscopic inflammation scores (Jaszewski *et al*., 1990). Since then, several studies have reported varying changes in RAS component levels in IBD, though the findings have been inconsistent. For example, while one study observed increased *AGT* gene expression in inflamed areas of colonic biopsies from IBD patients, no changes were seen at the peptide level (Garg *et al*., 2020). Another study found a correlation between AGT and inflammation

severity. In contrast, *AGT* gene expression was reduced in ileal samples from CD patients. Additionally, studies on REN expression in colonic lesions have shown conflicting results, with some reporting increased, decreased, or unchanged levels in IBD patients (Nowak *et al*., 2020) (Hume *et al*., 2016). Furthermore, He *et al*. reported increased REN protein levels in the involved (INV) areas compared to non-involved (NON-INV) areas in CD and UC patients. The same study demonstrated elevated *Ren* and *Agtr1* expression levels in RenTgMK mice treated with TNBS compared to wild-type (WT) mice treated with TNBS, along with increased expression of pro-inflammatory cytokines (He *et al*., 2019). Similarly, DSS-treated mice exhibited upregulated *Ang II* expression and elevated Ang II protein levels (Katada *et al*., 2008). The intricate role of RAS in inflammation, fibrosis, immune cell dynamics, and apoptosis underscores its contribution to the pathogenesis of IBD. Despite early evidence and subsequent studies pointing to alterations in RAS components in IBD, the findings are context-dependent, varying across tissue types, disease subtypes, and experimental models. These discrepancies highlight the complexity of RAS dysregulation in IBD and emphasize the need for further research to clarify its role in disease onset and progression, as well as its potential as a therapeutic target.

1.2.4 Current therapies for intestinal fibrosis

A range of drugs and established treatment guidelines exist for managing IBD, with proven effectiveness in reducing inflammation. In the past 20 years, the availability of biologics and advanced small molecules has increased dramatically improving outcomes and quality of life of patients with CD. However, the recurs to surgery has diminished only modestly, suggesting that current therapies are ineffective or insufficiently effective in preventing fibrotic complications (Jeuring *et al*., 2017). To date no anti-fibrotic medication has been approved, even though some anti-inflammatory drugs have been shown to improve symptoms and outcomes in patients with Crohn's associated strictures, most notably adalimumab is the only prospectively agent that demonstrated significant improvement in obstructive symptoms (Bouhnik *et al*., 2018). In severe cases of intestinal fibrosis and stricture, surgery or endoscopic balloon dilation are the primary options. Unfortunately, neither approach is curative: surgery is associated with high recurrence rates, and dilation does not resolve fibrosis, which often progresses and requires repeat interventions. While anti-inflammatory drugs can provide symptomatic relief, their preventive impact on fibrogenesis remains unclear. These limitations emphasize the pressing need for more effective therapeutic strategies.

Different classes of drugs are currently used in the therapy of IBD, including salicylates (5-ASA), corticosteroids (prednisone, methylprednisolone, and budesonide), immunosuppressants (azathioprine, methotrexate, and cyclosporine), and biological drugs (infliximab, adalimumab,

golimumab, certolizumab pegol, vedolizumab, and ustekinumab), all with specific anti-inflammatory actions, but their potential antifibrotic effects are poorly known. Among traditional medications, 5-5-ASA has been used for over five decades and has demonstrated both efficacy and safety in achieving and maintaining clinical and endoscopic remission in UC. However, there is limited evidence regarding its potential antifibrotic effects, and the guidelines from the European Crohn's and Colitis Organisation (ECCO) do not recommend it for the induction and maintenance of remission in CD. (H. Gordon *et al*., 2024). Furthermore, immunosuppressive therapies have been a staple in treating chronic inflammatory conditions, including IBD, but they have not been shown to reduce the likelihood of intestinal fibrostenosis or the subsequent need for intestinal resections. Interestingly, corticosteroids such as prednisone, methylprednisolone, and budesonide are effective in managing intestinal inflammation and are currently employed for inducing clinical remission in patients with moderate to severe CD and UC (Quera *et al*., 2023), though they are not typically used for long-term remission maintenance. Patients with steroid-dependent or refractory IBD may benefit from biological treatments. The biologic therapies currently used for IBD are primarily monoclonal antibodies targeting three key pathways: anti-TNF-α, anti-integrins (specifically α4β7 integrin-receptor antagonists), and anti-IL 12/23. Numerous studies have demonstrated the effectiveness of anti-TNF-α agents, with data showing that about two-thirds of CD patients with stenosis were able to avoid abdominal surgery (Allocca *et al*., 2017). A cohort study following patients for approximately ten years, who had received early anti-TNF-α treatment, revealed a reduced risk of developing bowel stenosis (Frei *et al*., 2019). It is important to note that recent preclinical studies have identified several promising therapeutic targets and approaches that focus on addressing the fibrotic process in the intestines. One potential therapeutic avenue is the regulation of the balance between TIMPs and MMPs, which control ECM turnover. Thalidomide, an anti-inflammatory drug that is emerging as a treatment for refractory CD, has been shown to restore this balance, inhibiting ECM degradation and reducing intestinal fibrosis in animal models (Flier *et al*., 2010). Additionally, targeting the EMT process has gained attention as a therapeutic strategy. Recombinant human bone morphogenic protein-7 (rhBMP-7), known for its ability to counteract TGF-β1's pro-fibrotic effects, has shown promise in animal models by inhibiting EMT and improving fibrosis in the intestine (Santacroce *et al*., 2022). The gut microbiota is also increasingly recognized for its role in the development of fibrosis. Probiotics and prebiotics have been studied for their ability to modulate the microbiota, reduce inflammation, and suppress fibrotic signaling pathways. Probiotic treatments have demonstrated beneficial effects by decreasing pro-inflammatory cytokines and fibrotic markers, improving microbial balance, and attenuating fibrosis in animal models of colitis. Deng *et al*. studied the beneficial effect of a combination of probiotics (*Lactobacillus plantarum*, *L. acidophilus*, *L.*

rhamnosus, and *Bifidobacterium animalis*) on abdominal adhesions in a rat model. The probiotic treatment significantly reduced the levels of pro-inflammatory factors IL-1β, IL-6, and TNF-α in serum and intestinal tissue, and markedly downregulated the inflammatory (TLR4/NF-κB) and fibrotic (TGF-β1/Smad) signaling pathways in intestinal tissue (X. Deng *et al*., 2020). Moreover, a study by Lombardi *et al*. demonstrated that a multi-strain probiotic formulation effectively counteracted the pro-fibrotic effects of TGF-β1 (Lombardi *et al*., 2021). Finally, one of the most promising targets for anti-fibrotic therapy is TGF-β and its associated signaling pathways. TGF-β1 production is strongly stimulated by ANG II which is upregulated in the colonic mucosa of CD patients. As previously described, ANG II is a key effector of RAS, and inhibitors of RAS, such as ACE inhibitors (ACEi) and angiotensin II receptor antagonists (ARBs), have shown potential as effective candidates for improving fibrosis in animal models. Preclinical and clinical data show that ACEi (X. Zhang *et al.*, 2022) and ARBs reduced the progression of liver (Gu *et al*., 2023), and pulmonary (Amirkhosravi *et al*., 2024) fibrosis, supporting the targeting of RAS to modulate fibrogenesis. Furthermore, in studies on intestinal fibrosis, research using animal models has explored the inhibition of AGTR1, the final effector of the RAS pathway, as well as its upstream effector ACE, with mainly positive results (Wengrower *et al.*, 2012) (Okawada *et al*., 2011).

2. AIM OF THE STUDY

Intestinal fibrosis is a common complication of inflammatory bowel disease (IBD), including Crohn's disease (CD), which can affect the entire gastrointestinal tract, and ulcerative colitis (UC), which is limited to the large bowel. Gut fibrosis develops in response to chronic tissue injury, is associated with persistent inflammation, and is characterized by excessive extracellular matrix deposition and muscle hypertrophy. In CD, intestinal fibrosis affects over 70% of patients, often leading to stenosis and fistulae that require invasive interventions such as surgical resection or balloon dilation. Although significant progress has been made in understanding the mechanisms underlying IBD-related inflammation, the development of effective anti-fibrotic therapies remains challenging, as current treatments provide only partial relief. The significant impact on patients' quality of life, coupled with high medical costs, highlights the urgent need for new anti-fibrotic treatments. To shed light on the current anti-fibrotic therapies in use and those potentially applicable, the initial purpose of this work was to evaluate *in vitro* the ability of different classes of drugs currently used in IBD to counteract two pivotal processes of intestinal fibrosis, i.e. the differentiation of intestinal fibroblasts to activated myofibroblasts, using human fibroblast, CCD-18Co cell line, and the epithelial-to-mesenchymal transition (EMT) of intestinal epithelial cells, using Caco-2 cell line (IEC), after TGF-β1 stimulation. The drugs tested included mesalamine (5-aminosalicylic acid (5-ASA)), azathioprine, prednisone, methylprednisolone, budesonide, methotrexate, and anti-TNFα antibodies (infliximab and adalimumab). For this purpose, the expression of fibrosis and EMT markers (collagen-I, α-SMA, pSmad2/3, occludin) as well as the ability of the above-mentioned drugs to modulate the fibrotic phenotype was checked by Western blot analysis and by immunofluorescence (Artone *et al*., 2024).
After evaluating the antifibrotic effects of drugs commonly used in IBD, the critical role of the Renin-Angiotensin System (RAS) pathway in the development of intestinal fibrosis was highlighted. Specifically, RAS contributes to fibrosis through key mediators such as angiotensin II (ANGII) and its receptor, Angiotensin Type 1 receptor, AGTR1, which are involved in regulating the inflammatory and fibrotic processes in the intestine. Among others, given the significant role of RAS in fibrotic processes, the investigation was extended to examine the potential anti-fibrotic effects of losartan, an Angiotensin II Receptor Blocker (ARB), in both an *in vitro* model of fibroblasts CCD-18Co and an *in vivo* model of CD-like ileitis (SAMP mice). These mice spontaneously develop inflammation in the distal ileum, making them an ideal model for studying the progression of the fibrotic phenotype. To investigate the efficacy of losartan in modulating fibrotic pathways *in vitro*, CCD-18Co cells were stimulated with ANGII and treated with losartan or vehicle. The ability of losartan to modulate the profibrogenic genes and proteins was assessed by quantitative real-time PCR (qPCR) analysis and immunofluorescence respectively. The expression of RAS effectors in the terminal ileum of SAMP

mice before the onset of inflammation and fibrosis (4 weeks) and during established disease (30 weeks), was assessed by performing histological and qPCR analysis. To assess the *in vivo* efficacy of losartan in modulating gut inflammation and fibrosis, 10-week-old SAMP mice were treated with losartan (0.6 g/L) or vehicle for 10 weeks as a therapeutic protocol. Additionally, the efficacy of losartan in sustaining remission of inflammation and fibrosis after corticosteroid induction was evaluated by treating 20-week-old SAMP mice with dexamethasone (DexM) (1 mg/kg) for 1 week as a remission protocol. Histological evaluations were conducted to assess total ileal fibrosis and inflammation scores. Moreover, immunohistochemistry (IHC), qPCR, and immunofluorescence were used to evaluate the AGTR1 protein expression, mRNA levels of fibrogenic genes, and the presence of Collagen type 1 alpha 1 chain (COL1A1) and α-SMA respectively (Artone *et al*., manuscript in preparation).

3. MATERIALS AND METHODS

3.1 *In vivo* model

SAMP and AKR/J mice for these experiments were obtained from the main mice colonies kept in Case Western Reserve University's animal facilities, which are certified as pathogen-free. All experimental protocols using animals were fully approved by the IACUC (The Institutional Animal Care and Use Committee, Case Western Reserve University). All studies were performed in a blinded fashion, and mice were subjected to randomization and identified using a numerical code known only to the animal caretaker. The identification code was revealed only at the end of the experiments.

3.1.1 *In vivo* experimental design

Losartan efficacy was tested in two protocols, a therapeutic and a remission one. For the former, 10-wk-old sex and age-matched SAMP mice were treated with vehicle or losartan (0.6 g/L) for 10 weeks. Losartan (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in water, filtered using the END Millipore Stericup 0.22-micron vacuum-assisted filtration system and administered via cage water bottle; the same filtration process was used to prepare the vehicle. Mice were monitored daily, with the drinking solution changed at least twice weekly for 10 weeks before euthanasia for tissue collection and analysis. For the remission protocol, 20-wk-old SAMP mice were treated with DexM, 1mg/kg) or vehicle (aseptically prepared PBS) daily for 1 week by intraperitoneal injections (i.p.); losartan or vehicle were administered for an additional 5 weeks as indicated for the therapeutic protocol. Mice were sacrificed either at the end of the first week of DexM pretreatment and after treatment with vehicle or losartan (at 6 weeks).

3.2 Tissue harvest and histologic assessment of SAMP inflammation and fibrosis

Mice were euthanized by injecting 1 mL of 2.5% tribromoethanol in PBS, followed by cervical dislocation. The terminal ileum was removed, opened longitudinally, rinsed with PBS, and placed on a cork board, pinned from the proximal to distal ends. Two small tissue strips were taken from the ileum edges for RNA extraction and protein isolation. These samples were immediately stored in RNA later solution (Invitrogen, Waltham, MA, USA) for RNA preservation and in RIPA buffer (Thermo Scientific, Logan, UT) for protein analysis. The cork board with the attached ileum was then fixed in Bouin's solution (Picric Acid-Formalin-Acetic Acid Mixture from Ricca Chemical, Texas, USA) for 24 hours, followed by washing three times with 70% ethanol. The ileum was embedded in paraffin and sectioned into 4 μm-thick slices, which were stained using Hematoxylin & Eosin (H&E) and Masson's Trichrome (MTC) methods. A trained pathologist, blinded to the experimental groups, assessed the histological slides. Inflammation severity was evaluated using a semiquantitative scoring system that included active inflammation, chronic inflammation, and villus distortion. Additional

parameters such as mononuclear and transmural inflammation were scored on a 0-4 scale for severity and extent. The cumulative inflammation score was derived from these parameters. A separate fibrosis scoring system was used to assess the H&E and trichrome-stained sections, based on three criteria: depth of fibrosis involvement, percentage of the area affected, and fibrosis density, with each parameter scored from 1 to 4. The depth of fibrosis was graded as: 0) none, 1) mucosal involvement, 2) submucosal involvement, 3) muscularis propria involvement, and 4) extension to subserosal adipose tissue. The affected area was scored as: 0) 0%, 1) 1-25%, 2) 26-50%, 3) 51-75%, and 4) 76-100%. Fibrosis density was rated as: 1) mild, 2) moderate, or 3) severe. The total fibrosis score was the sum of these three criteria, independent of the inflammation score. Muscularis propria thickening was measured using ImageJ (Bethesda, MA, USA).

3.3 *In vitro* models

CCD-18Co cells, a normal human intestinal fibroblast cell line (ATCC CRL-1459) obtained from the American Type Culture Collection (Georgetown, DC, USA), were grown in Eagle's Minimum Essential Medium (EMEM; ATCC) supplemented with 10% fetal bovine serum (FBS), 100 µg/mL streptomycin, and 100 U/mL penicillin (Euro Clone, West York, UK). Once the cells reached 90% confluence, they were harvested using a trypsin solution (Euro Clone) and reseeded into sterile 6-12 or 96-well tissue culture plates (Becton, San Jose, CA, USA) at a density of 5000 or 14000 cells/cm². The human colorectal adenocarcinoma cell line, Caco-2, was sourced from Sigma-Aldrich (St. Louis, MO, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 U/mL penicillin, 1 mM sodium pyruvate, 2 mM L-glutamine, 1% non-essential amino acids, and 100 µg/mL streptomycin (Euro Clone). After reaching 80% confluence, Caco-2 cells were detached using a trypsin solution (Euro Clone) and plated into sterile 12- or 96-well tissue culture plates (Becton) at a density of 60,000 cells/cm². The cells were maintained in culture for 14 days post-confluence to promote differentiation into intestinal epithelial-like cells and then treated as described in subsequent sections. All cell cultures were maintained in a 37°C incubator with a humidified atmosphere of 95% air and 5% CO_2.

3.3.1. Intestinal fibrosis experimental designs

- **TFG-β1 treatment of CCD-18Co cells**

CCD-18Co cells plated at 5000 cells/cm^2 were serum-deprived for 24 h and then treated with 10 ng/mL of human transforming growth factor (hTGF-β1; Cell signaling Technology, Danvers, MA, USA) for 48 h to induce the fibrotic phenotype in the presence or absence of mesalamine (Sigma-Aldrich), azathioprine (Azafor, Sofar, MI, Italy), prednisone (Deltacortene, Bruno Pharmaceuticals, RM, Italy), methylprednisolone (Urbason, Sanofi, MI, Italy), budesonide (Intesticort, Sofar, MI, Italy), methotrexate (Reumaflex, Alfasigma, BO, Italy), infliximab (Flixabi, Biogen, Cambridge,

MA, USA), and adalimumab (Hyrimoz, Sandoz, Princeton, NJ, USA). All drugs were previously dissolved and diluted to reach the tested concentrations in the culture medium. The pellets were incubated with a 0.04% Trypan blue (Euro Clone) solution for 5 min to analyze cell number and viability. Non-treated cells served as controls. The cells were counted by microscopy (Eclipse 50i, Nikon Corporation, Tokyo, Japan).

- **ANG II treatment of CCD-18Co cells**

CCD-18Co cells plated at 14000 cells/cm^2 were treated with 100 ng/mL of ANG II (ENZO, Farmingdale, NY, USA) in the presence of Losartan (Selleckchem, Houston, TX, USA) (10µM) or vehicle (dimethyl sulfoxide, DMSO; Thermo Fisher Scientific, Waltham, MA, USA) for 24h. The pellets were incubated with a 0.04% Trypan blue (Euro Clone) solution for 5 min to analyze cell number and viability. Non-treated cells served as controls. The cells were counted by microscopy (Eclipse 50i, Nikon Corporation, Tokyo, Japan).

3.3.2 Epithelial-Mesenchymal Transition experimental design

To establish an EMT *in vitro* model, Caco-2 IEC cells were stimulated with 20 ng/mL TGF-β1(Cell signaling Technology) in the serum-free medium up to 96 h in the presence or absence of mesalamine (Sigma-Aldrich), azathioprine (Azafor, Sofar), prednisone (Deltacortene, Bruno Pharmaceuticals), methylprednisolone (Urbason, Sanofi), budesonide (Intesticort, Sofar), methotrexate (Reumaflex, Alfasigma), infliximab (Flixabi, Biogen,), and adalimumab (Hyrimoz, Sandoz,). Then, the cells were harvested and centrifuged at 400× g for 10 min. The pellets were incubated with a 0.04% Trypan blue (Euro Clone) solution for 5 min to analyze cell number and viability. Non-treated cells served as controls. The cells were counted by microscopy (Eclipse 50i, Nikon Corporation).

3.4 Cell viability assay

Cell viability was measured using the Cell Counting Kit-8 (CCK-8) (Sigma-Aldrich) according to the manufacturer's instructions. The 96-well-plated CCD-18Co cells were treated for 48 h with different concentrations of the selected drugs: mesalamine (0.1–5 mM), azathioprine (5–20 µM), prednisone (10–100 µM), methylprednisolone (50–200 µM), budesonide (0.1–5 µM), methotrexate (0.1–1 µM), infliximab (10–50 µg/mL), and adalimumab (20–100 µg/mL). Similarly, Caco-2 cells plated in 96 wells were incubated for up to 96 hours with mesalamine (5-50 mM), azathioprine (1-20 µM), prednisone (10-200 µM), methylprednisolone (10-200 µM), budesonide (7-70 µM), methotrexate (1-50 µM), infliximab (25-200 µg/mL), and adalimumab (8-100 µg/mL). After incubation, 10 µL of CCK-8 assay reagent was added to each well. Optical density (OD) values were measured using a spectrophotometer (Bio-Rad, Hercules, CA, USA) at 450 nm.

3.5 Total RNA extraction and quantitative real-time PCR (qPCR)

Total RNA was isolated from CCD-18Co cells and mice distal ilea using the High Pure RNA Isolation Kit (Roche, Basel, Switzerland) and the RNeasy Plus Mini Kit (Qiagen, Germantown, MD), respectively. RNA was then reverse transcribed using the RNA-to-cDNA kit (Applied Biosystems, Forest City, CA), following the manufacturer's instructions. qPCR was performed using target gene primers indicated in **Table 1**. Gene expression for each primer was normalized to β-actin and reported as a relative fold difference among groups, with the baseline/control group set arbitrarily at 1. Samples were run in quadruplicate and the experiments were repeated three times.

Gene	Forward Primer (5'-3')	Reverse Primer (3'-5')	Company
TGFB1	CTC CCG TGG CTT CTA GTG C	GCC TTA GTT TGG ACA GGA TCT G	IDT
IGF1	GCT CCG GAA GCA GCA CT	GAT GCT CTT CAG TTC GTG TGT	IDT
MMP9	CCA GGT CAT TTA GGA AGT CAG G	CGT CCT GTA CTG AAG GAG AAA G	IDT
THSB2	CTC TCT CCG CTT GTT GTG TTA G	GAG ACG GAT GCG TGT GAT ATT	IDT
CCL11	CGA CTA GAG AGC TAC AGG AGA A	GAC TCT GTG CCC TAG ACA TTA TT	IDT
TIMP1	GTC CCT GAT CTC TCT TGC TAT TT	GAC AGG TGA TCA CAC GGT ATT	IDT
ACTB	AGG CAC CAG GGC GTG AT	GCC CAC ATA GGA ATC CTT CTG AC	IDT
Agt	Gene Globe ID (PPM04219E)		Qiagen
Ren	Gene Globe ID (PPM34372A)		Qiagen
Ace	Gene Globe ID (PPM05319A)		Qiagen
Ace2	Gene Globe ID (PPM05301A)		Qiagen
Agtr1	Gene Globe ID (SBH0123663)		Qiagen
Agtr2	Gene Globe ID (PPM04811A)		Qiagen
Col1a1	Gene Globe ID (PPM03845F)		Qiagen
Mmp9	Gene Globe ID (PPM03661C)		Qiagen
Igf1	Gene Globe ID (PPM03387F)		Qiagen
Il13	Gene Globe ID (PPM03021B)		Qiagen
Actb	Gene Globe ID (PPM02945B)		Qiagen

Table 1. Human and mouse primer sequences or IDs used for qPCR analysis.

3.6 Western Blot Analysis

For Western blot analysis, cell pellets were obtained and washed with PBS, followed by lysis in RIPA buffer (Merck KGaA, Darmstadt, Germany) supplemented with a mixture of protease inhibitors (5 µg/mL carboxypeptidase inhibitor, 5 µg/mL trypsin inhibitor, 1 mM PMSF, 10 µg/mL leupeptin, 10 µg/mL aprotinin, and 10 µg/mL pepstatin) from Sigma Aldrich. The protein concentration in the samples was quantified using the DC Protein Assay (BioRad), with BSA serving as the standard. Next, 25 µg of protein was combined with the sample buffer, heated for 5 minutes at 100 °C, and then subjected to 10% SDS-polyacrylamide gel electrophoresis. Proteins were transferred to a 0.45 µm nitrocellulose membrane (BioRad) for 1 hour at 4 °C at 70 V, utilizing a Mini Trans-Blot Cell apparatus (BioRad). Membrane blocking was achieved by immersing the membrane in 5% non-fat dry milk for 1 hour at room temperature. The membranes were incubated overnight at 4 °C with the

following antibodies: rabbit polyclonal anti-COL1A1 (Boster Biological Technology, Pleasanton, CA, USA) at a dilution of 1:1000, rabbit monoclonal anti-human phospho-SMAD2(Ser465/467)/SMAD3(Ser423/425) (Cell Signaling Technology) at 1:1000, rabbit polyclonal anti-OCCLUDIN (OriGene, Rockville, MA, USA) at 1:1000, mouse monoclonal anti-α-actin smooth muscle (α-SMA) (OriGene) at 1:1000, and mouse monoclonal anti-GAPDH (OriGene) at 1:1000. For detection, a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody at 1:2000 was applied for the anti-COL1A1, anti-phospho-SMAD2/3, and anti-occludin antibodies, while a horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG secondary antibody at 1:2000 was used for the anti-α-SMA and anti-GAPDH antibodies (Millipore EMD, Darmstadt, Germany). The visualization of immunoreactive protein bands was carried out using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, Amersham, UK), following the manufacturer's guidelines. Band densities were analyzed with the ALLIANCE (UVITEC, Cambridge, UK) chemiluminescence documentation system and were normalized to the relative GAPDH bands, with values expressed in relative units.

3.7 Immunofluorescence staining

CCD-18Co and IEC Caco-2 cells were cultured on coverslips placed in 12-well plates, with seeding densities of 5000 cells/cm² and 60000 cells/cm², respectively, as previously outlined. After treatment, the coverslips were rinsed with PBS, fixed in 4% formaldehyde for 20 minutes, and permeabilized using 0.1% Triton X-100 (Sigma-Aldrich) for 5 minutes. Following this, the coverslips were blocked with 3% BSA (Sigma-Aldrich) for 20 minutes at room temperature. The cells were then incubated overnight at 4°C with the following primary antibodies: rabbit polyclonal anti-COL1A1 (Boster Biological Technology) at 1:250, mouse monoclonal anti-α-actin smooth muscle (ACTA, α-SMA) (OriGene) at 1:250, and rabbit polyclonal anti-Occludin (OriGene) at 1:50. Subsequently, staining was carried out using FITC-conjugated goat anti-mouse IgG secondary antibody (Bethyl Laboratories, Inc., Montgomery, TX, USA) at 1:1000 or FITC-conjugated goat anti-rabbit IgG secondary antibody (Millipore) at 1:1000 for 1 hour at room temperature. After washing, the cells were further incubated with TRITC-labeled phalloidin (Sigma-Aldrich) for 45 minutes at room temperature. Finally, coverslips were mounted using VECTASHIELD® Antifade Mounting Medium with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA) and observed under a fluorescent microscope (Eclipse 50i, Nikon, Tokyo, Japan) at 40× magnification.

3.8 Immunohistochemistry (IHC) staining

IHC staining was performed using a polyclonal rabbit anti-human AGTR1 IgG at 1:200 (bs-2132R; Bioss USA, Woburn, Massachusetts, USA). Paraffin-embedded tissues were sectioned (3 to 4 mm thick), placed on Superfrost Plus glass slides (Thermo Scientific, Logan, UT), deparaffinized, and

incubated in normal serum for nonspecific blocking. Samples were blocked for endogenous peroxidase activity using 1.75% H2O2, and antigen retrieval was performed on human samples by steamer exposure (twice for 5 minutes), while submerged in an unmasking solution (Vector Labs, Burlingame, CA). After incubation with primary antibodies at 4 C, slides were rinsed in PBS, incubated with appropriate biotinylated secondary antibodies (Vector Labs), rinsed in PBS, and further assayed using Vectastain ABC Kits (Vector Labs). Immunoreactive cells were visualized by the addition of a diaminobenzidine substrate (Vector Labs), counterstained with hematoxylin, and mounted using an 80% glycerol mount. All incubations were conducted at room temperature unless otherwise noted. Negative controls were prepared under identical conditions in the absence of primary antibodies

3.9 ELISA Assay

The levels of released TNF-α were detected in the cell supernatants using an enzyme-linked immunosorbent assay (ELISA) kit (Immunodiagnostic, Bensheim, AG, Germany). Briefly, the CCD-18Co cells were plated at 5000 cells/cm2, grown overnight, and then serum-deprived for 48 h, while the Caco-2 IEC cells were plated at 60,000 cells/cm2, and fourteen days post-confluence, they were cultivated in the serum-free medium up to 96 h. After the media were collected and cleared of cellular debris/dead cells by centrifugation at 600× g for 10 min, the TNF-α concentration was quantified, and the data obtained were normalized for cell number and expressed as pg/10^5 cells.

3.10 Statistical analysis

All data were evaluated by GraphPad Prism (version 6.01 GraphPad Software, San Diego, CA, USA). For the *in vitro* studies one-way ANOVA or two-way ANOVA, followed by Dunnett's or Tukey's post hoc test, was used to compare the mean values among groups. In all the *in vivo* studies parametric and non-parametric tests were used as appropriate for group comparison. For qPCR analysis of SAMP vs AKR mice a two-way ANOVA uncorrected Fisher's LSD was used. Data were expressed as mean ± SD or mean ± SEM. $p < 0.05$ was considered statistically significant.

4.0 RESULTS

4.1 Effect of Selected Drugs on Cell Viability of CCD-18Co and IEC Caco-2

The impact of the selected drugs on the viability of CCD-18Co and IEC Caco-2 cells was assessed using the Cell Counting Kit-8 (CCK-8) assay. In CCD-18Co cells, all tested drugs led to a significant reduction in absorbance after 48 hours, indicating a decrease in cell numbers at the highest dose compared to the control group (**Figure 12A**). Similarly, in IEC Caco-2 cells, a significant reduction in cell numbers was observed only at the highest drug concentrations tested (**Figure 12B**). Based on these findings, the highest drug concentrations were excluded from further experiments.

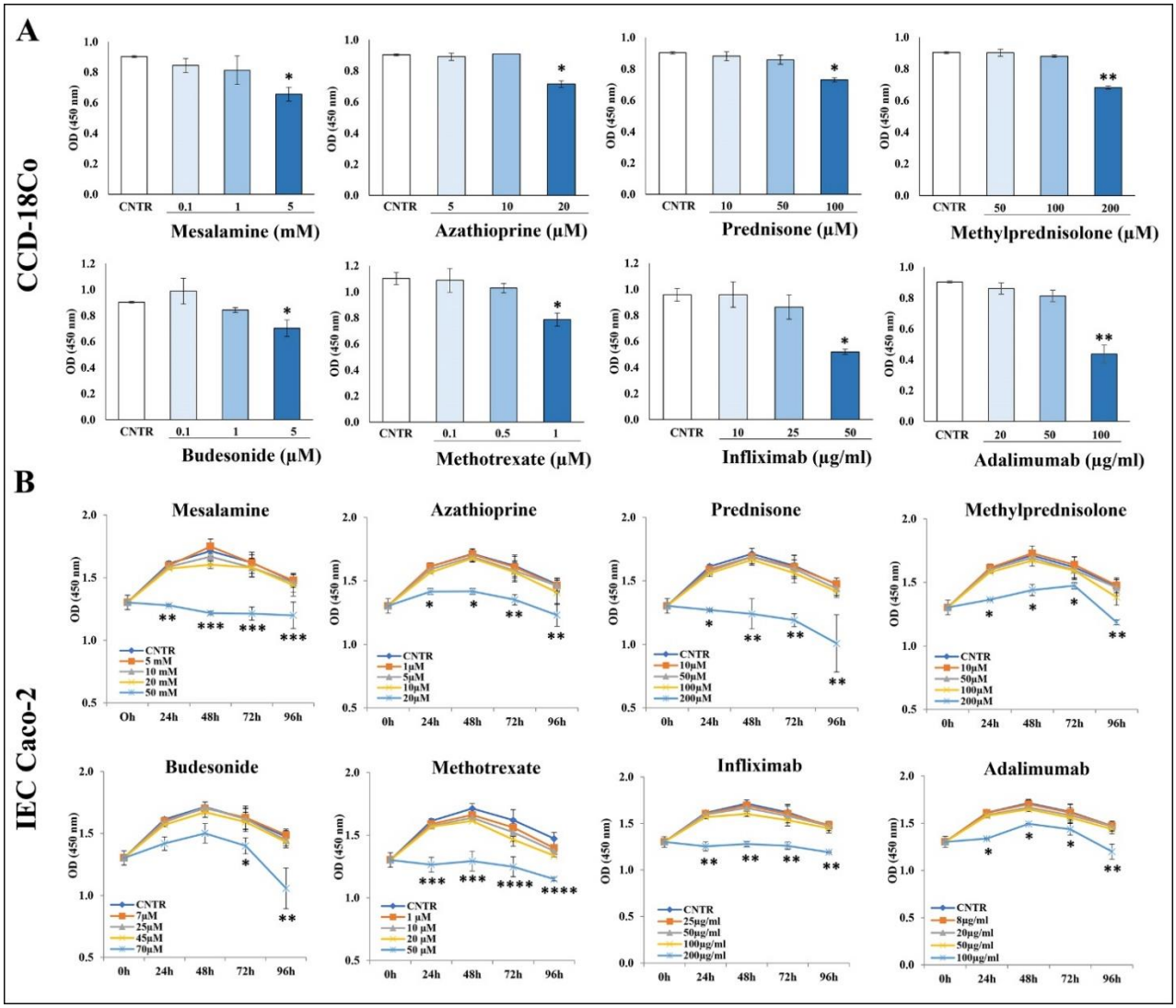


Figure 12. **Cell viability of CCD-18Co and IEC Caco-2.** (**A**) Cell viability of CCD-18Co cells incubated for 48 h with mesalamine (0.1–5 mM), azathioprine (5–20 µM), prednisone (10–100 µM), methylprednisolone (50–200 µM), budesonide (0.1–5 µM), methotrexate (0.1–1 µM), infliximab (10–50 µg/mL), and adalimumab (20–100 µg/mL). (**B**) IEC Caco-2 cells incubated up to 96 h with mesalamine (5–50 mM), azathioprine (1–20 µM), prednisone (10–200 µM), methylprednisolone (10–200 µM), budesonide (7–70 µM), methotrexate (1–50 µM), infliximab (25–200 µg/mL), and adalimumab (8–100 µg/mL). The data are from two independent experiments performed in triplicate, and values are

expressed as mean ± SEM. For comparative analysis of data, a one-way ANOVA for CCD-18Co cells and two-way ANOVA for IEC Caco-2 cells followed by Dunnett's post hoc test were performed. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

4.2 Impact of Selected Drugs on TGF-β1-Induced Expression of Collagen I and α-SMA in CCD-18Co Cells

The differentiation of fibroblasts into myofibroblasts is a key cellular process in intestinal fibrosis, characterized by significant cytoskeletal reorganization and increased expression of fibrotic markers like collagen I and the α-SMA protein. Western blot analysis was conducted to assess the effects of selected drugs on collagen I and α-SMA expression. As demonstrated in **Figure 13**, CCD-18Co cells, deprived of serum for 24 hours and then treated with TGF-β1 (10 ng/mL) for 48 h, exhibited phenotypic changes typical of activated myofibroblasts, marked by a notable increase in collagen I and α-SMA expression when compared to untreated control cells. Among the drugs tested, mesalamine, azathioprine, methotrexate, and infliximab did not mitigate the TGF-β1-induced effects on CCD-18Co cells. However, the steroids prednisone, methylprednisolone, budesonide, and adalimumab showed a moderate ability to reduce the TGF-β1-induced upregulation of collagen I and α-SMA.

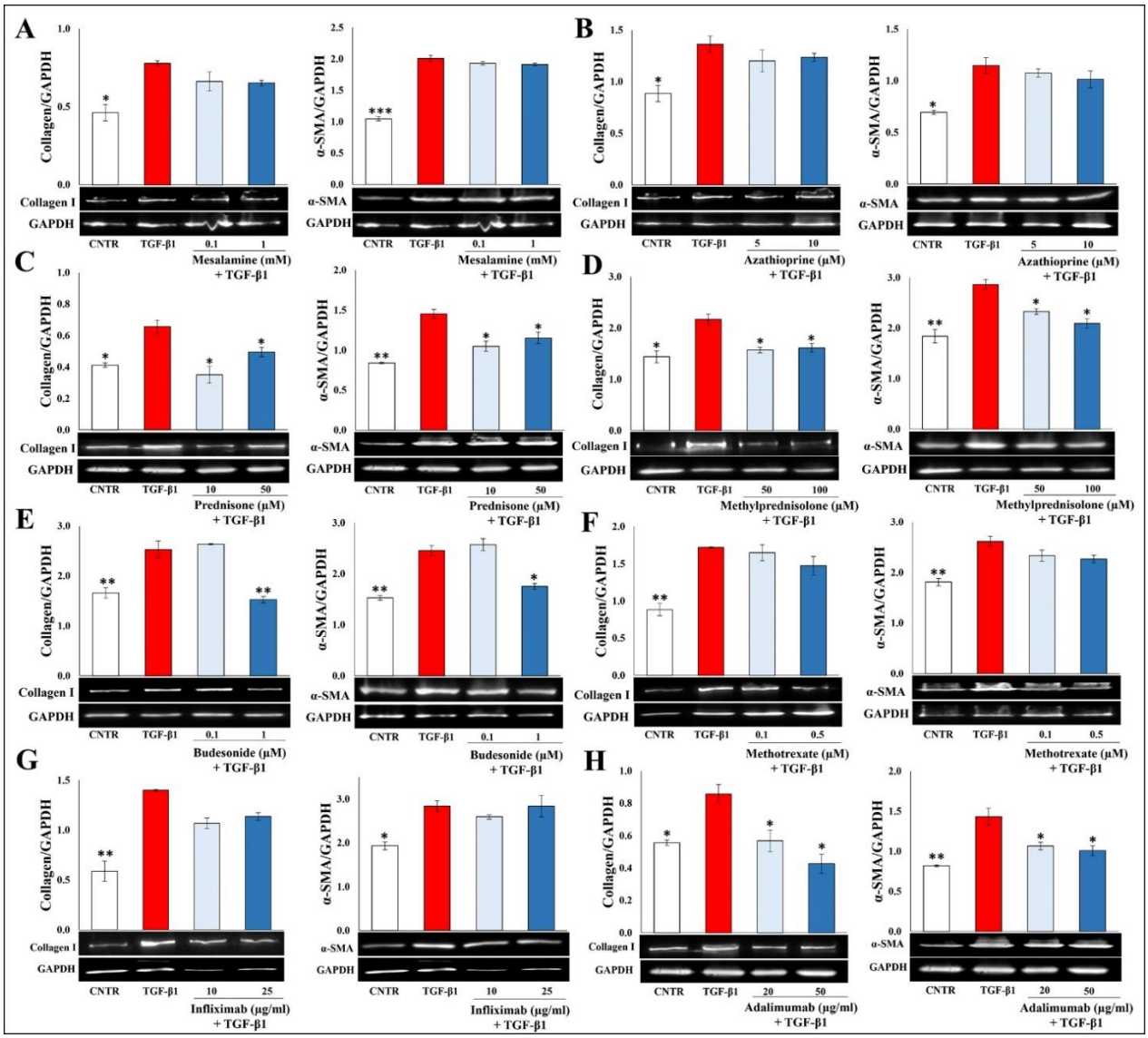


Figure 13. Effect of tested drugs on TGB-β1-induced collagen I and α-SMA expression in CCD-18Co cells. Immunoblotting assay for collagen I and α-SMA was performed on CCD-18Co cells incubated for 48 h with TGF-β1 (10 ng/mL) in the presence or absence of (**A**) mesalamine (0.1–1 mM), (**B**) azathioprine (5–10 μM), (**C**) prednisone (10–50 μM), (**D**) methylprednisolone (50–100 μM), (**E**) budesonide (0.1–1 μM), (**F**) methotrexate (0.1–0.5 μM), (**G**) infliximab (10–25 μg/mL), and (**H**) adalimumab (20–50 μg/mL). Representative images of immunoblotting for collagen I (130 kDa), α-SMA (42 kDa), and GAPDH (37 kDa) are shown. The data are from three independent experiments, and values are expressed as mean ± SEM. A one-way analysis of variance (ANOVA) with Dunnett's post hoc test was used for the comparative analysis of data. Densitometric analysis was performed by normalizing vs. GAPDH. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

To further validate the positive effects of methylprednisolone, prednisone, budesonide, and adalimumab in CCD-18Co cells, immunofluorescence staining was conducted. As anticipated, treatment with TGF-β1 resulted in enhanced collagen I expression, evident from the more pronounced staining in treated cells compared to the untreated controls. Conversely, cells receiving TGF-β1 in combination with prednisone, methylprednisolone, budesonide, and adalimumab displayed reduced

collagen I staining intensity, resembling that of the untreated cells. Additionally, TGF-β1-induced fibrotic activation was marked by significant cytoskeletal reorganization, characterized by the formation of actin stress fibers, which was indicated by strong phalloidin staining. Notably, the addition of the selected drugs diminished the intense fluorescence of phalloidin, returning it to levels comparable to the controls (**Figure 14**).

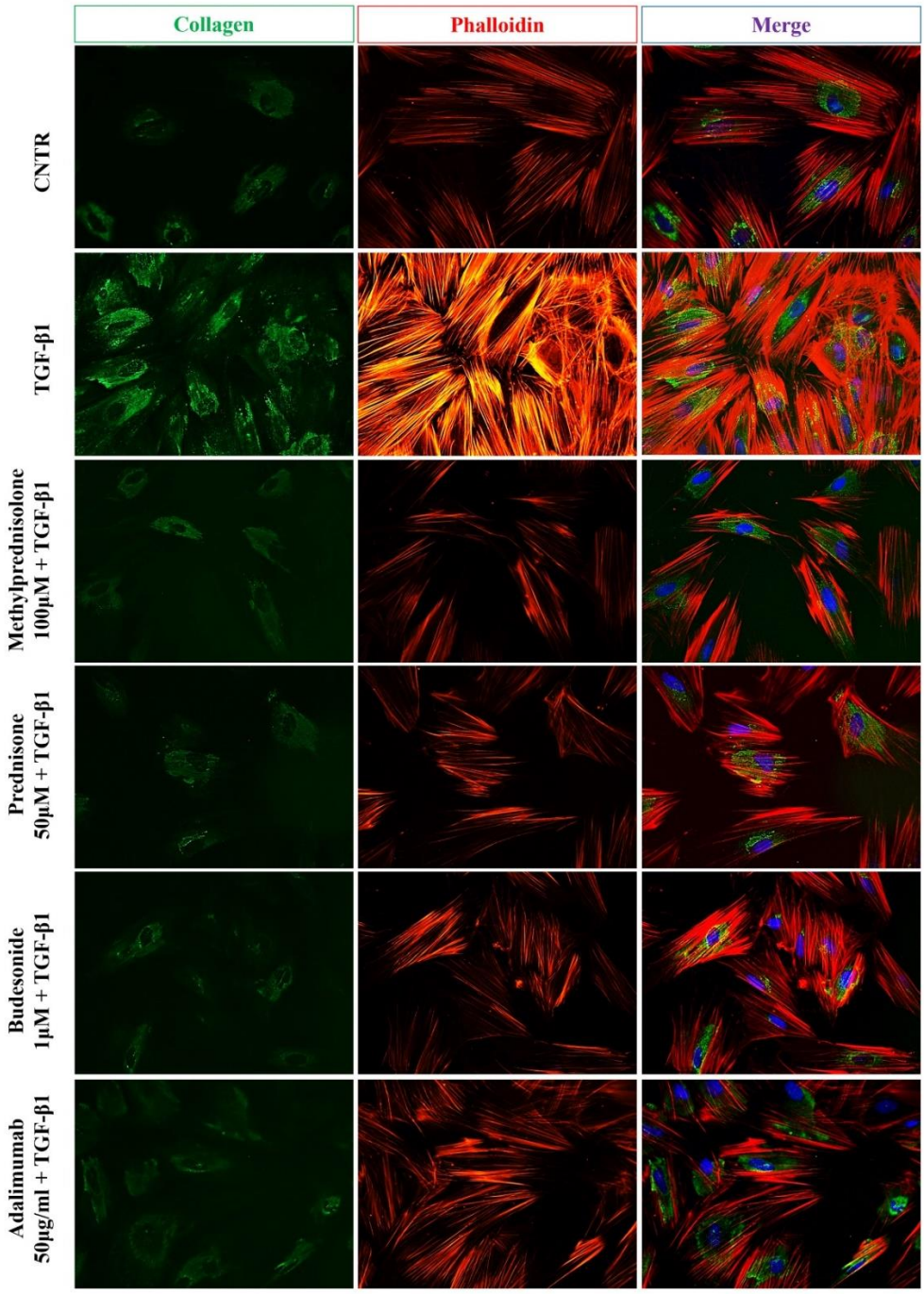


Figure 14. Effect of drugs on TGB-β1-induced collagen I expression in CCD-18Co cells. Representative immunofluorescence images of untreated and treated CCD-18Co cells, as above described, stained with anti-collagen I antibody (green) and TRITC-phalloidin (red) to reveal F-actin. Nuclei were counterstained with DAPI (blue) (magnification 40×). The images are representative of three independent experiments in duplicate.

Additionally, as illustrated in **Figure 15**, immunofluorescence analysis revealed that the green, fluorescent signal associated with α-SMA expression was minimal in untreated control cells but significantly more intense in activated myofibroblasts. This observation confirms the upregulation of α-SMA protein levels following TGF-β1 treatment. Once again, treatment with methylprednisolone, prednisone, budesonide, and adalimumab successfully reversed the phenotype induced by TGF-β1, as evidenced by the reduced and weaker fluorescence of α-SMA.

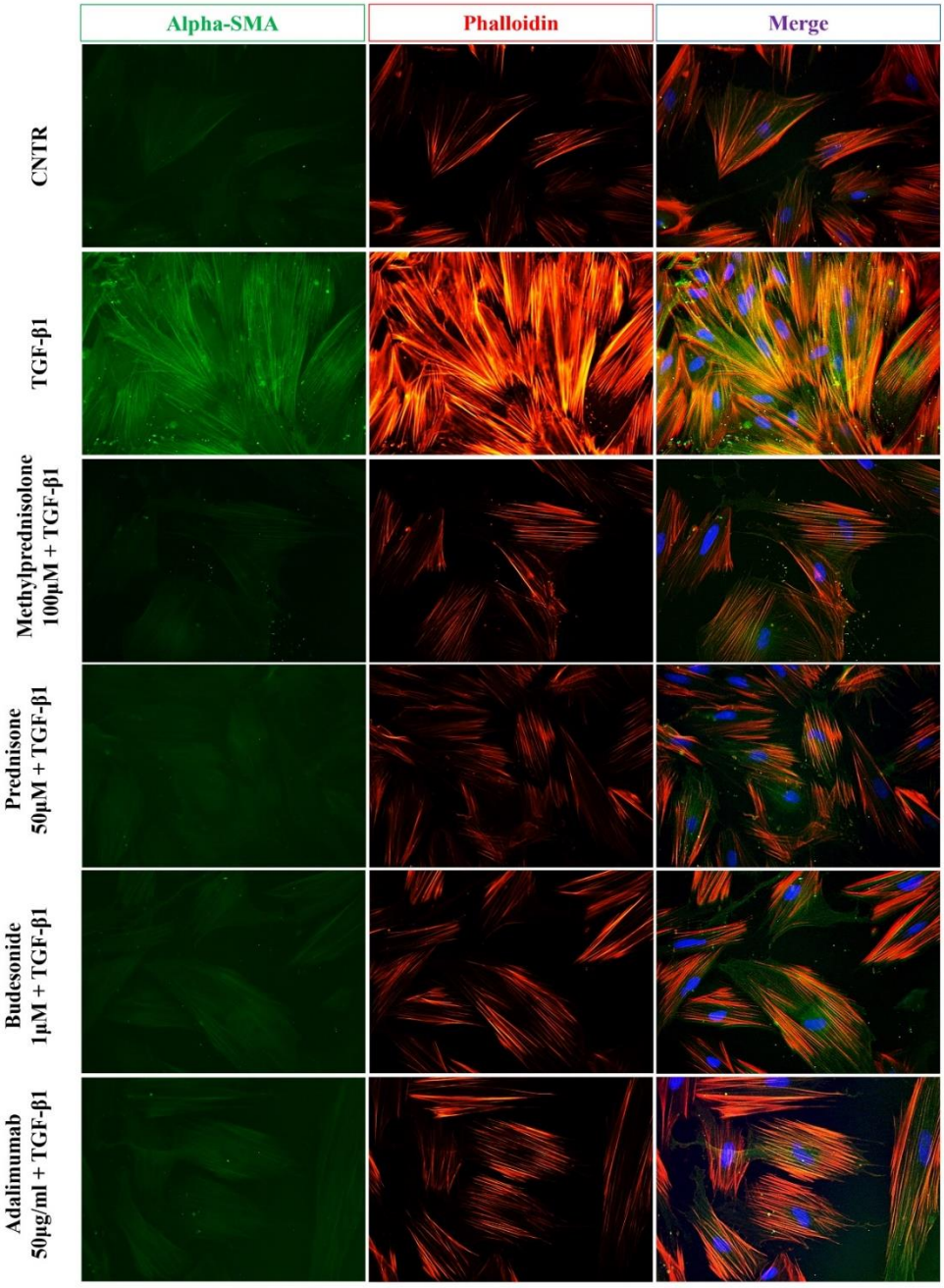


Figure 15. Effect of drugs on TGB-β1-induced α-SMA expression in CCD-18Co cells. As described above, representative immunofluorescence images of untreated and treated CCD-18Co cells were stained with anti-α-SMA antibody (green) and TRITC-phalloidin (red) to reveal F-actin. Nuclei were counterstained with DAPI (blue) (magnification 40×). The images are representative of three independent experiments performed in duplicate.

4.3 Effect of Selected Drugs on TGB-β1-Induced Smad Signaling

TGF-β1 triggers the Smad2/3 signaling pathway to mediate its pro-fibrotic effects. As depicted in **Figure 16**, TGF-β1 stimulation led to a significant increase in the expression of phosphorylated Smad2/3 protein. Notably, only prednisone, methylprednisolone, budesonide, and adalimumab were able to counteract this effect. In contrast, treatments with mesalamine, azathioprine, methotrexate, and infliximab did not influence the phosphorylation of Smad2/3.

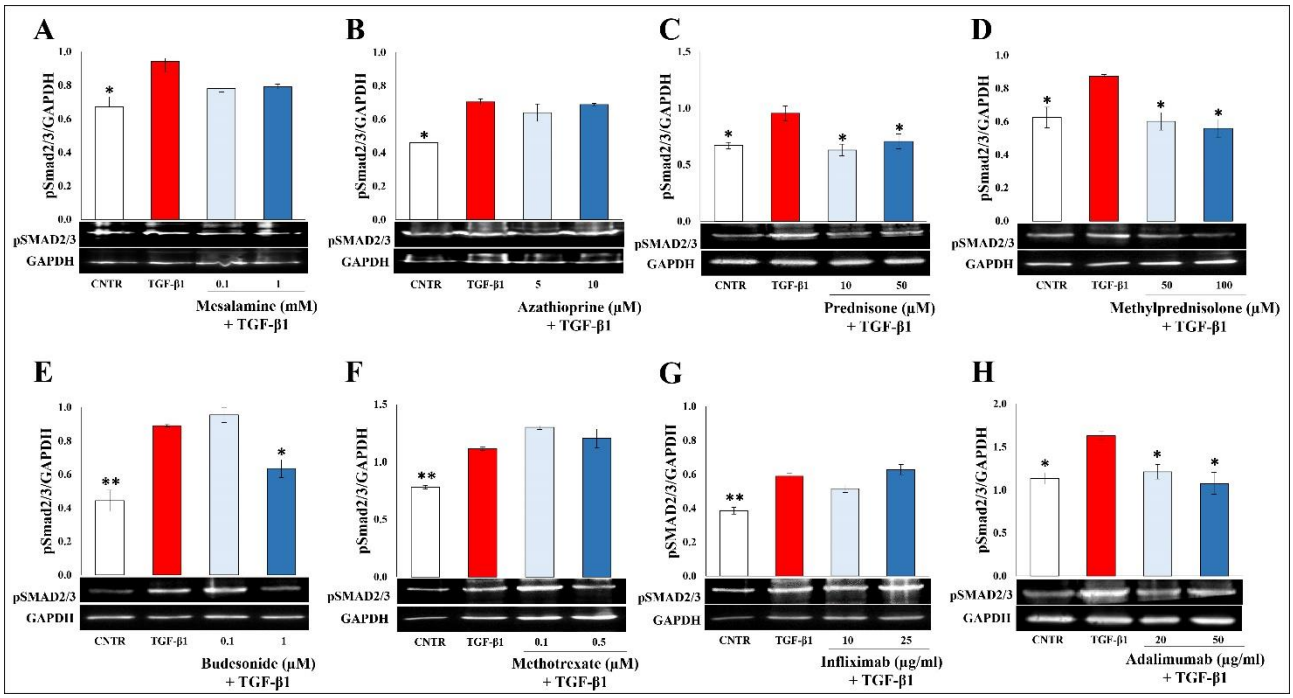


Figure 16. Effect of tested drugs on TGB-β1-induced Smad signaling in CCD-18Co cells. Immunoblotting assay for pSmad 2/3 was performed on CCD-18Co cells incubated for 48 h with TGF-β1 (10 ng/mL) in the presence or absence of (**A**) mesalamine (0.1–1 mM), (**B**) azathioprine (5–10 μM), (**C**) prednisone (10–50 μM), (**D**) methylprednisolone (50–100 μM), (**E**) budesonide (0.1–1 μM), (**F**) methotrexate (0.1–0.5 μM), (**G**) infliximab (10–25 μg/mL), and (**H**) adalimumab (20–50 μg/mL). Representative images of immunoblotting pSmad2/3 (52 kDa) and GAPDH (37 kDa) are shown. The data are from three independent experiments, and values are expressed as mean ± SEM. A one-way analysis of variance (ANOVA) with Dunnett's post hoc test was used for the comparative analysis of data. Densitometric analysis was performed by normalizing vs. GAPDH. * $p < 0.05$, ** $p < 0.01$.

4.4 Impact of Selected Drugs on TGF-β1-Induced Epithelial-to-Mesenchymal Transition in Caco-2 IEC Cells

The EMT, a key aspect of fibrogenesis, is marked by the downregulation of epithelial markers and the upregulation of mesenchymal markers in intestinal epithelial cells. A prominent feature of EMT is the rearrangement of actin as cell-cell junctions are disassembled. To investigate the *in vitro* effects of mesalamine, azathioprine, prednisone, methylprednisolone, budesonide, methotrexate, infliximab,

and adalimumab on EMT, IEC Caco-2 cells were cultured for four days in the presence of 20 ng/mL TGF-β1, with or without the selected drugs. The expression of the epithelial marker occludin, a tight junction transmembrane protein, and the mesenchymal marker α-SMA was assessed via Western blot. As anticipated, treatment with TGF-β1 led to a reduction in the epithelial marker and an increase in the mesenchymal marker. Of the drugs tested, only methylprednisolone, budesonide, and adalimumab were able to significantly counteract the TGF-β1-induced effects (**Figure 17**).

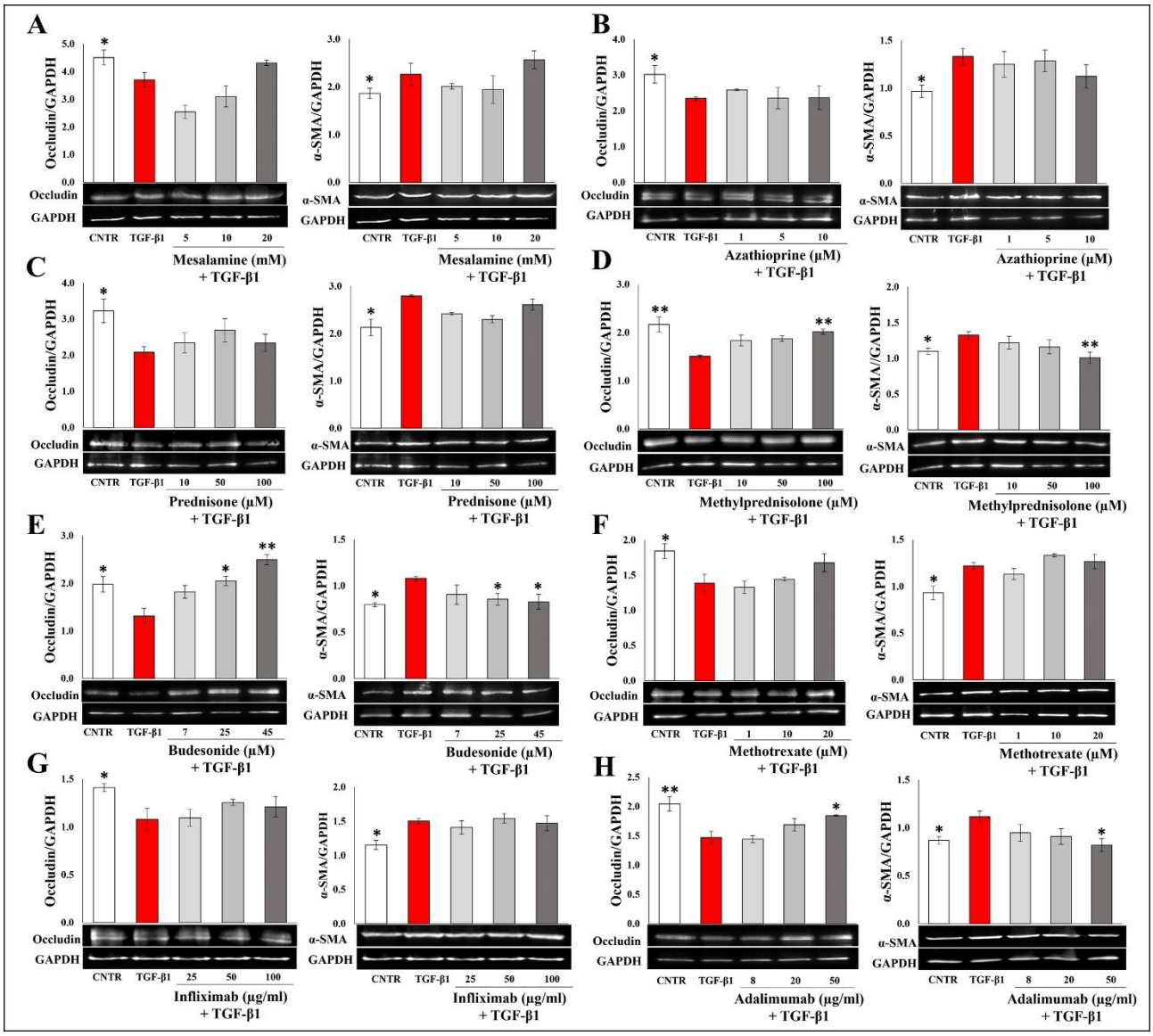


Figure 17. Effect of tested drugs on EMT in Caco-2 IEC cells. Immunoblotting assay for occludin and α-SMA was performed on Caco-2 IEC cells incubated for 96 h with TGF-β1 (20 ng/mL) in the presence or absence of (A) mesalamine (5–20 mM), (B) azathioprine (1–10 μM), (C) prednisone (10–100 μM), (D) methylprednisolone (10–100 μM), (E) budesonide (7–45 μM), (F) methotrexate (1–20 μM), (G) infliximab (25–100 μg/mL), and (H) adalimumab (8–50 μg/mL). Representative images of immunoblotting for occludin (59 kDa), α-SMA (42 kDa), and GAPDH (37 kDa) are shown. The data are from three independent experiments, and values are expressed as mean ± SEM. For comparative

analysis of data, a one-way analysis of variance (ANOVA) with Dunnett's post hoc test was used. Densitometric analysis was performed by normalizing vs. GAPDH. * $p < 0.05$, ** $p < 0.01$.

To further validate the efficacy of methylprednisolone, budesonide, and adalimumab on EMT markers, immunofluorescence analysis was conducted. As depicted in **Figure 18**, treatment of Caco-2 IEC cells with TGF-β1 led to a reduction in occludin levels, indicated by faint staining, and an increase in α-SMA expression, highlighted by intense green fluorescence. Notably, treatment with these drugs successfully counteracted the TGF-β1-induced changes, restoring occludin and α-SMA levels to those observed in untreated cells.

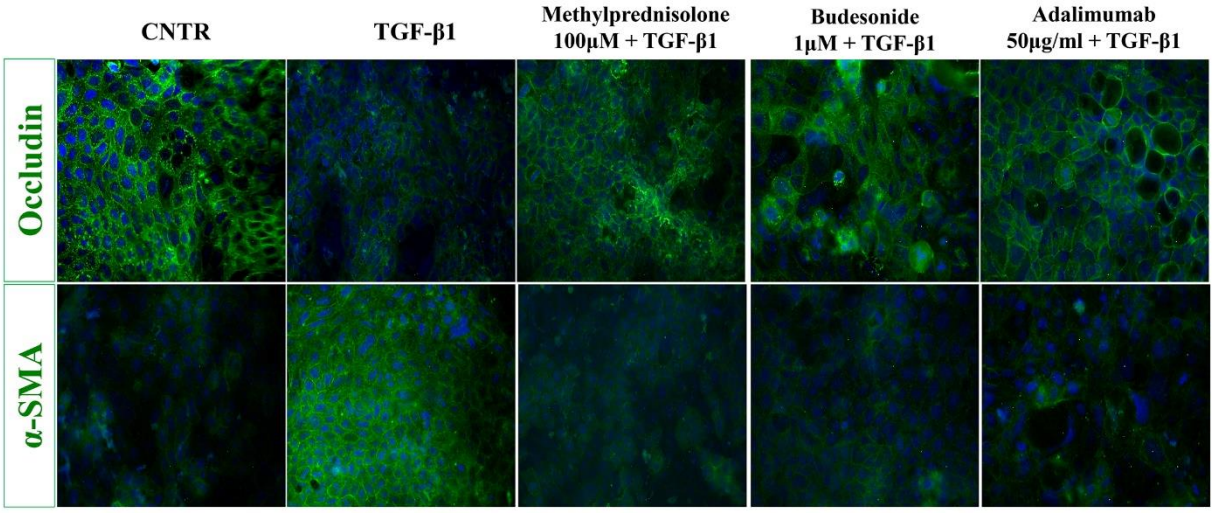


Figure 18. Effect of drugs on EMT markers (occludin and α-SMA expression) in Caco-2 IEC cells. Representative immunofluorescence images of untreated and treated Caco-2 IEC cells, as described above, stained with anti-occludin and anti-α-SMA antibodies (green). Nuclei were counterstained with DAPI (blue) (magnification 40×). The images are representative of three independent experiments in duplicate.

4.5 *In vitro* efficacy of losartan in reducing fibrotic mediators induced by Angiotensin II treatment

After evaluating the antifibrotic effects of drugs commonly used in IBD across two distinct *in vitro* models and recognizing the role of the RAS pathway in the development of intestinal fibrosis, the investigation was extended to examine the potential anti-fibrotic effects of the ARB losartan in an *in vitro* model using CCD-18Co human colon fibroblasts and an *in vivo* model of SAMP mice, which spontaneously develop CD-like ileitis.

To examine the potential of losartan in modulating fibrotic pathways, CCD-18Co cells were stimulated with ANGII and treated with either losartan (10 μM) or vehicle control (DMSO) for 24 h. ANGII stimulation significantly upregulated several profibrogenic genes, including *INSULIN-LIKE GROWTH FACTOR 1 (IGF1)*, *TGF-β1*, *MMP9*, *THROMBOSPONDIN 2 (THSB2)*, *CCL11*, and

COLLAGEN TYPE I ALPHA 1 CHAIN (COL1A1), in vehicle-treated cells compared to untreated controls. However, treatment with losartan effectively reversed this upregulation. (**Figure 19A**). At the protein level, losartan also reduced COL1A1 fluorescence intensity, a marker of fibrosis, alongside a decrease in α-SMA expression (**Figure 19B**). These findings indicate that losartan suppresses profibrogenic pathways at the transcriptional level and mitigates fibroblast-to-myofibroblast differentiation, as evidenced by reduced collagen deposition and cytoskeletal changes in this in *in vitro* model of intestinal fibrosis.

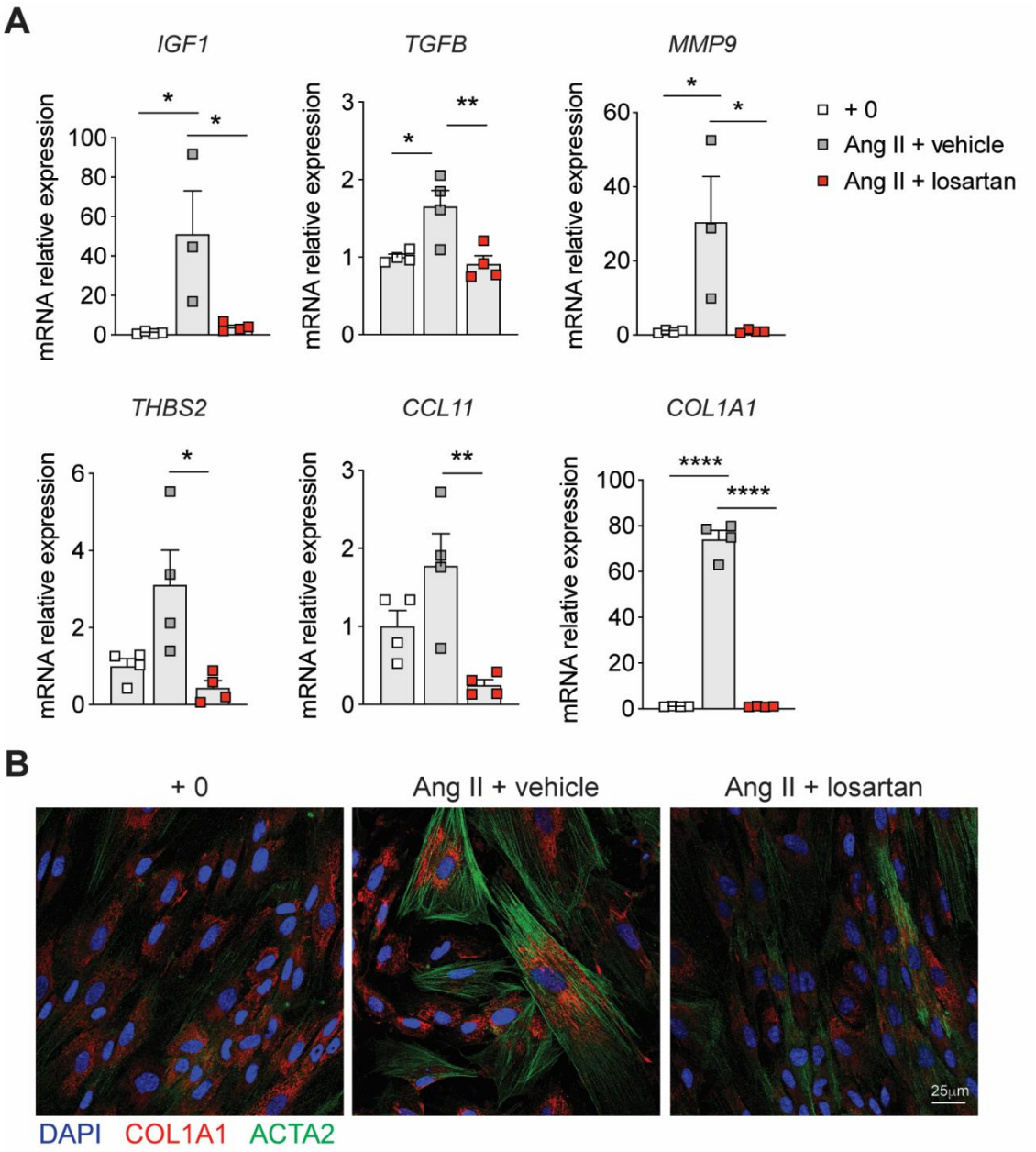


Figure 19. **Losartan reduces profibrogenic gene expression and myofibroblast differentiation induced by ANGII stimulation in CCD-18Co cells.** (**A**) Expression of profibrogenic genes in CCD-18Co cells stimulated with ANGII and treated losartan (10µM) or vehicle (DMSO) for 24h was analyzed by qPCR. Data are representative of three experiments and are expressed as fold difference relative to control cells (+0). Data presented as mean±SEM and *P*-values calculated

by one-way ANOVA followed by Tukey's multiple comparisons test (n=4). *P<0.05; **P<0.01, ****P<0.0001. (**B**) Representative confocal images of control cells and ANGII+vehicle/losartan-treated CCD-18Co cells stained by immunofluorescence for ACTA2 (green) and COL1A1 (red); nuclei were counterstained with DAPI (blue) (n=3). Original magnification: X20.

4.6 Differential modulation in SAMP mice before and after the onset of inflammation and fibrosis compared to age-matched AKR mice

Considering the lack of *in vivo* studies evaluating the effectiveness of ARBs in spontaneous models of intestinal fibrosis, initially the regulation of the RAS pathway in SAMP mice was investigated. The expression of RAS effectors was analyzed in the terminal ileum of SAMP mice at two-time points: before the onset of inflammation and fibrosis (4 weeks) and during advanced disease (30 weeks), in comparison to age-matched AKR parental controls (**Figure 20A**). Histological examination revealed progressive inflammation and fibrosis in 30-week-old SAMP mice compared to both AKR controls and 4-week-old SAMP mice (**Figure 20B**). Notably, the older SAMP mice exhibited increased mucosal inflammatory infiltrates, submucosal collagen deposition extending variably into the muscle layers, and thickening of the muscularis propria (**Figure 20A**).

At the molecular level, 4-week-old SAMP mice showed upregulation of Agt and Ren and a notable reduction in Ace expression compared to AKR mice (**Figure 20C**). However, no significant differences in Ace2, Agtr1, or Agtr2 expression were observed at this early stage. In contrast, 30-week-old SAMP mice with advanced disease displayed reduced Agt expression, while Ren remained significantly upregulated (**Figure 20D**). Interestingly, alternative pathways appeared to be activated in older SAMP mice, potentially as a compensatory mechanism against heightened inflammation and fibrosis. Specifically, Ace2 expression was elevated in 30-week-old SAMP mice compared to AKR controls, with a similar trend observed for Agtr2 that did not reach statistical significance. On the contrary, Ace and Agtr1 were downregulated at 30 weeks. These findings highlight dynamic changes in RAS pathway modulation over time in the SAMP model.

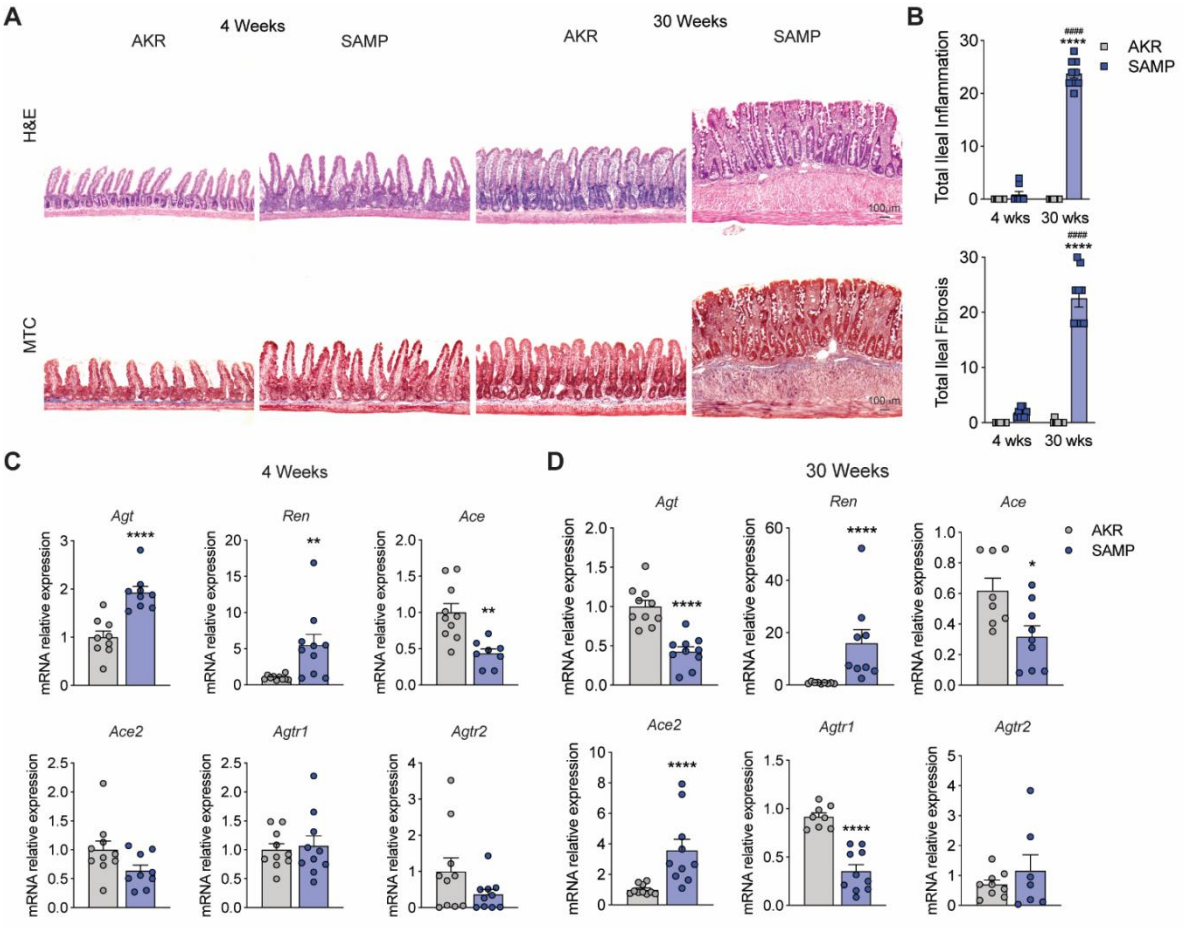


Figure 20. RAS effectors are differentially regulated in SAMP mice before the onset of inflammation/fibrosis and during established disease compared to age-matched AKR mice. (**A, B**) Representative photomicrographs of ileal tissues from 4-/30-wk-old SAMP mice compared to age-matched parental controls mice (AKR), stained for H&E (**A,** upper panel) and MTC (**A,** lower panel), and corresponding inflammatory and fibrotic scores (**B**, upper and lower panels, respectively). Data expressed as mean±SEM and *P*-values calculated by a two-way ANOVA uncorrected Fisher's LSD (n=8). ****P<0.001 SAMP vs. AKR at 30 wks; $^{\#\#\#\#}P$<0.001 SAMP 30 wks vs. 4 wks. Original magnification X5+25. (**C**, **D**) Relative expression of *Agt*, *Ren*, *Ace*, *Ace2*, *Agtr1*, and *Agtr2* in distal ileal tissues from 4-wk-old (**C**) and 30-wk-old (**D**) SAMP and AKR mice by qPCR. Data are representative of three independent experiments and presented as mean±SEM (n≥8). *P*-values calculated by unpaired t-test or Mann-Whitney, *P<0.05, **P<0.01, ****P<0.0001.

4.7 *In vivo* ability of losartan to modulate inflammation and fibrosis using SAMP model

To evaluate the impact of losartan on gut inflammation and fibrosis in vivo, 10-week-old SAMP mice were treated with losartan (0.6 g/L) or vehicle for 10 weeks (**Figure 21A**). Losartan treatment significantly reduced both inflammation and fibrosis compared to vehicle-treated controls (**Figure 21B, C**). Histological analysis revealed a substantial decrease in inflammatory cell infiltration within the lamina propria and improved villus architecture in the distal ileum of losartan-treated mice, as shown by H&E staining (**Figure 21B**, upper panel). This correlated with a reduced inflammatory

score in losartan-treated mice (**Figure 21C**, upper panel). MTC staining demonstrated a notable reduction in collagen I deposition in the submucosa and muscularis propria, as well as decreased muscularis propria thickness in losartan-treated mice (**Figure 21B**, lower panel), consistent with the lower fibrotic score (**Figure 21C**, lower panel). Given the established link between intestinal inflammation and the RAS cascade, we assessed AGTR1 expression, a key RAS effector, through IHC. AGTR1 was prominently localized to the epithelial and muscle compartments in vehicle-treated mice, as indicated by intense brown staining (**Figure 21D**). Losartan treatment significantly reduced AGTR1 expression in both compartments (**Figure 21D**).

To explore the effect of losartan on profibrogenic gene expression, qPCR analysis was performed on ileal tissues. Losartan counteracted the upregulation of all tested profibrogenic genes compared to the vehicle group (**Figure 21E**). Additionally, given the role of the Th2 immune response in fibrosis and collagen synthesis, the cytokine *Il13* was assessed and found to be downregulated in losartan-treated mice (**Figure 21E**). Immunofluorescence staining further confirmed losartan's efficacy, showing a significant reduction in COL1A1 accumulation within the submucosa and muscularis propria, compared to the prominent red staining in vehicle-treated mice (**Figure 21F**). Unlike the *in vitro* findings, losartan did not affect α-SMA expression *in vivo*. Collectively, these results highlight losartan's effectiveness in mitigating intestinal inflammation and fibrosis in SAMP mice.

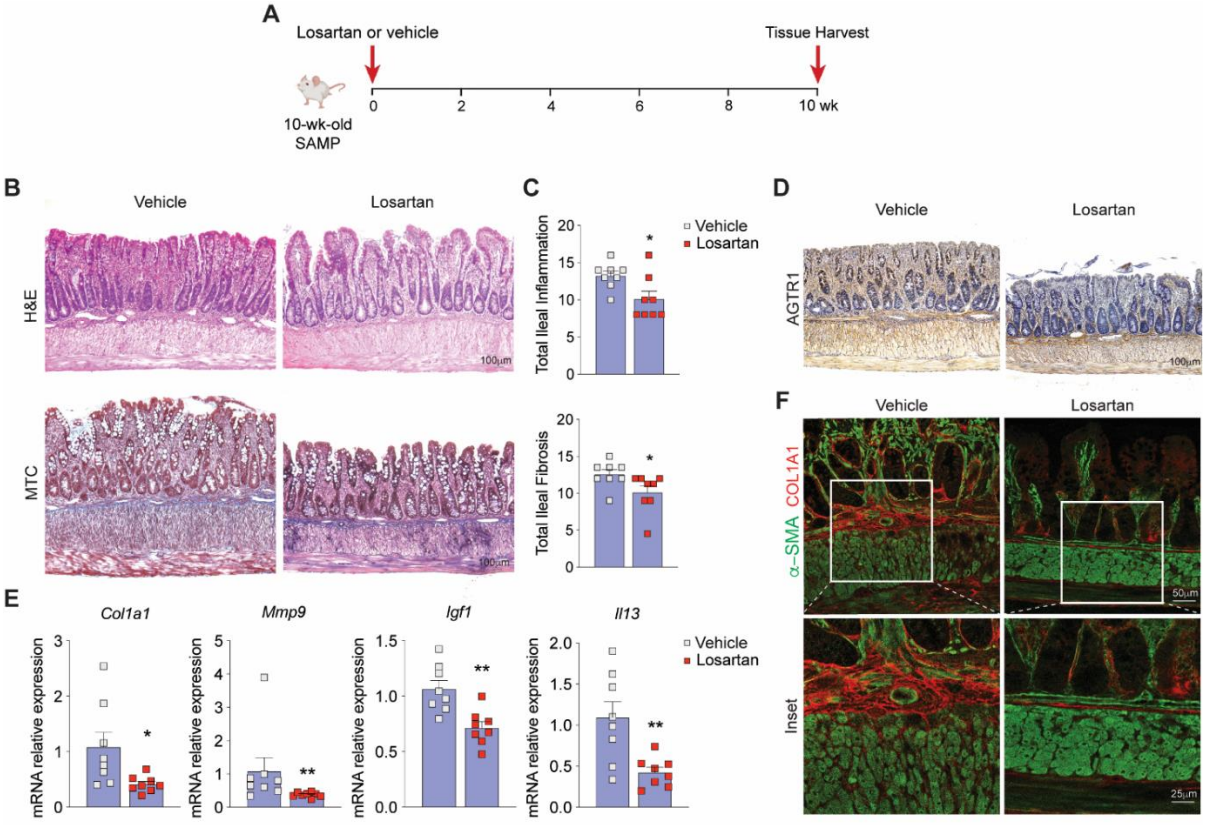


Figure 21. **Losartan shows therapeutic efficacy in reducing inflammation and fibrosis in SAMP mice.** (**A**) Experimental design for the therapeutic protocol using losartan (0.6 g/L) or vehicle (dH_20) in SAMP mice. (**B**) Representative photomicrographs of ileal tissues from losartan-treated SAMP mice compared to the vehicle group, stained for H&E (upper panel) and MTC (lower panel), and corresponding inflammatory and fibrotic scores (**C**, upper and lower panels, respectively). Data expressed as mean±SEM and *P*-values calculated by an unpaired t-test (*n*=8). **P*<0.05. Original magnification X10+1.25. (**D**) Representative IHC for AGTR1 in losartan- compared to vehicle-treated SAMP mice (*n*=3). Original magnification X10+1.25; (**E**) Relative *Col1a1*, *Mmp9*, *Igf1*, and *Il13* measured by qPCR is shown as fold difference vs. vehicle group. Data are representative of three experiments and expressed as mean±SEM (*n*=8). *P*-values are calculated by an unpaired t-test or Mann-Whitney. **P*<0.05, ***P*<0.01. (**F**) Representative confocal images of distal ilea from the vehicle and losartan-treated SAMP mice stained by immunofluorescence for α-SMA (green) and COL1A1 (red); nuclei were counterstained with DAPI (blue) (n=3). Original magnification 20X; inset X40.

4.8 *In vivo* effect of losartan on sustaining the reduction of inflammation and fibrosis in SAMP mice after DexM-induced remission

Given the moderate yet notable effects of losartan in therapeutic protocols, its potential to sustain remission of inflammation and fibrosis following corticosteroid-induced remission was explored. Twenty-week-old SAMP mice received DexM (1 mg/kg) for one week, achieving remission of inflammation and fibrosis, as confirmed by histological assessments (**Figure 22A**, left panel; **Figure 22B**). However, after five weeks of recovery, both inflammation and fibrosis returned (**Figure 22A**, right panel; **Figure 22B**). To evaluate losartan's role in maintenance therapy, we administered either losartan or a vehicle for five weeks post-DexM treatment (**Figure 22C**). Histological analysis revealed that losartan effectively maintained fibrosis remission but did not prevent the recurrence of inflammation, as shown by H&E and MTC staining (**Figure 22D**, upper and middle panels) and histological scores (**Figure 22E**, upper and middle panels). Despite losartan's lack of impact on collagen deposition, consistent with the STAR consensus on histological assessment of intestinal fibrosis (I. O. Gordon *et al*., 2022), muscularis propria thickening was evaluated using MTC-stained ileal sections. Results showed a significant reduction in muscularis propria thickness in losartan-treated mice compared to vehicle-treated controls (**Figure 22E**, lower panels). IHC revealed decreased AGTR1 expression in both the mucosa and muscularis propria compartments during remission maintenance (**Figure 22F**). To further assess fibrosis modulation via AGTR1, immunofluorescence staining for COL1A1 and α-SMA was performed on ileal tissues. The findings demonstrated a notable reduction in COL1A1 signal within the submucosa and diminished infiltration in the muscularis propria in losartan-treated mice compared to controls (Figure 5G). However, α-SMA expression levels remained unchanged in the DexM+losartan group, indicating no effect of losartan on α-SMA expression. Collectively, these results suggest that losartan exhibits a targeted

anti-fibrotic effect, mitigating fibrosis rebound post-DexM-induced remission in SAMP mice, independent of its influence on inflammation.

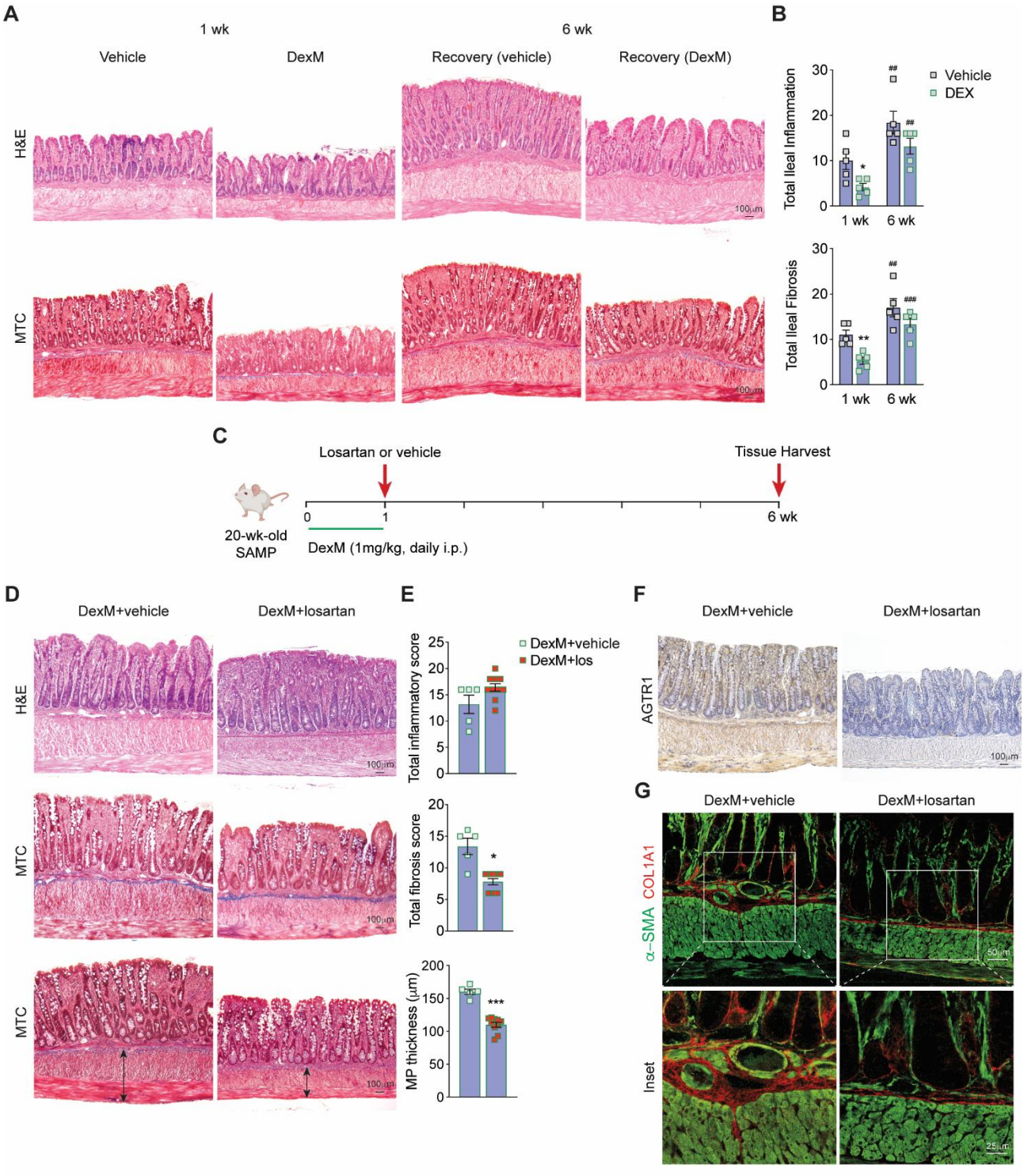


Figure 22. Losartan maintains remission of fibrosis but not inflammation in SAMP mice after DexM-induced remission. (**A, B**) Representative photomicrographs of H&E (upper panel) and MTC (lower panel) staining on ileal tissues from SAMP mice treated with DexM (1mg/kg/day for 7 days by i.p.) or vehicle (PBS) for 1 and 6 weeks (**A**, left and right panels, respectively), scored for inflammation and fibrosis (**B**, upper and lower panels, respectively). Data expressed as mean±SEM; *P*-value calculated by two-way ANOVA test uncorrected Fisher's LSD. **P*<0.05, ***P*<0.01 DexM vs. vehicle at 1 and 6 wk; $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 6 wk vs. 1 wk for vehicle and DexM. (*n*=5). Original magnification

X5+2. (**C**) Experimental design for the remission protocol using losartan (0.6 g/L) or vehicle (dH_20) in SAMP mice after DexM treatment (1mg/kg/day for 7 days by i.p.). (**D**) Representative photomicrographs of H&E (upper panels) and MTC (middle panels) stained ileal tissues from SAMP mice treated with DexM+vehicle and DexM+losartan and scored for inflammation and fibrosis (**E,** upper and lower panels, respectively). Measurements of *muscularis propria* (MP) thickness by ImageJ on MTC-stained ilea from SAMP treated with DexM+vehicle or DexM+losartan (lower panels) (*n*=5). Original magnification X10+1.25. Data expressed as mean±SEM and *P*-value calculated by Mann-Whitney test. **P*<0.05; ****P*<0.001. (**F**) Representative IHC for AGTR1 in DexM+vehicle and DexM+losartan treated mice (*n*=3). Original magnification X10+1.25. (**G**) Representative confocal images of ileal tissues from DexM+vehicle and DexM+los treated mice, stained by immunofluorescence for ACTA2 (green) and COL1A1 (red); nuclei were counterstained with DAPI (blue) (n=3). Original magnification X20 and X40 (insets).

5. DISCUSSION

IBD is marked by chronic, recurring inflammation, often accompanied by fibrosis at inflamed sites, which can result in intestinal stenosis and obstruction. Intestinal fibrosis affects approximately 70% of CD patients and 1–12% of UC patients (Dolinger *et al.*, 2024). This process is multifactorial, progressive, and complex, involving excessive and uncontrolled deposition of ECM components. The resulting tissue stiffening leads to smooth muscle expansion and intestinal obstruction (Lis-López *et al*., 2023) (Alfredsson & Wick, 2020). Various cellular compartments, including mesenchymal cells, fibroblasts, myofibroblasts, and smooth muscle cells drive fibrosis. Myofibroblasts, central to the fibrotic process, can also originate from epithelial and endothelial cells through EMT and EndMT (Flier *et al*., 2010). Evidence increasingly suggests that EMT plays a critical role in intestinal fibrosis, as EMT-related molecules have been identified in fibrotic lesions and fistulas in CD (Z. Wang *et al.*, 2022) (Ortiz-Masiá *et al*., 2020). Activated mesenchymal cells contribute to fibrosis not only by producing ECM components but also by releasing cytokines, chemokines, and growth factors that perpetuate chronic inflammation (Wu *et al*., 2023). TGF-β1 is a key player in this process, promoting EMT and driving fibroblast differentiation into myofibroblasts. These myofibroblasts express α-SMA, which contributes to their contractility, and produce ECM proteins such as collagen and fibronectin (Walton *et al*., 2017). Among these, RAS has emerged as a crucial player in fibrosis and inflammation. RAS components, including ANG II and its receptor AGTR1, have been implicated in promoting fibroblast activation, ECM deposition (Garg *et al*., 2020), and induction of EMT (Balakumar *et al*., 2019). Moreover, RAS activation contributes to the regulation of pro-inflammatory cytokines and chemokines, further exacerbating chronic inflammation and fibrotic progression (Shi *et al*., 2016) (He *et al*., 2019). Furthermore, TGF-β1 is also modulated by RAS activity, thereby linking these pathways to the pathogenesis of IBD-related fibrosis (Garg *et al*., 2020). Despite significant advancements in managing inflammation in IBD, the prevalence of intestinal fibrosis and stricture formation remains notably high. This is likely due to fibrosis becoming a self-sustaining process once initiated, progressing through mechanisms that are independent of inflammation. These characteristics have crucial implications for IBD treatment, potentially explaining why existing therapies, primarily designed to target inflammation, are largely ineffective in preventing fibrosis or reversing it once established (Walton *et al*., 2017). Current IBD treatments include several drug classes such as salicylates (e.g., 5-ASA), corticosteroids (e.g., prednisone, methylprednisolone, budesonide), immunosuppressants (e.g., azathioprine, methotrexate, cyclosporine), and biologics (e.g., infliximab, adalimumab, golimumab, certolizumab pegol, vedolizumab, ustekinumab). While these drugs have well-defined anti-inflammatory properties, their antifibrotic potential remains poorly understood and to date, specific antifibrotic therapies are not yet available.

In this study, we first evaluated, using *in vitro* cellular models, the effectiveness of these different classes of drugs used in IBD to counteract two key processes involved in intestinal fibrosis: the differentiation of fibroblasts into activated myofibroblasts and the EMT both triggered by TGF-β1. As previously reported, TGF-β1 stimulated the differentiation of intestinal CCD-18Co fibroblasts into myofibroblasts and promoted the EMT process in Caco-2 IEC cells. These effects were evident through Western blot and immunofluorescence analyses. In fibroblasts, TGF-β1 induced a marked increase in the expression of collagen I and α-SMA, which are key markers of fibrosis while, in Caco-2 IEC cells, TGF-β1 treatment resulted in a significant reduction in occludin expression, alongside an increase in α-SMA levels, which are indicative of EMT. Notably, the elevated α-SMA expression in activated myofibroblasts is functionally linked to their enhanced contractility and interaction with the ECM during fibrosis progression (Gabbiani, 2003). In our study, CCD-18Co cells treated with TGF-β1 showed a pronounced reorganization of the actin cytoskeleton, as evidenced by stronger phalloidin staining compared to untreated controls. Additionally, there was more intense green fluorescence of collagen I and α-SMA in the TGF-β1-treated fibroblasts, indicating their differentiation into activated myofibroblasts. Consistent with these observations, TGF-β1-treated CCD-18Co cells displayed a significant increase in pSmad2/3 expression. Furthermore, immunofluorescence analysis of Caco-2 IEC cells treated with TGF-β1 revealed weaker occludin staining and stronger α-SMA fluorescence, signaling the onset of EMT. Of the conventional drugs, 5-ASA, widely used for UC, failed to counteract the fibrotic effects induced by TGF-β1 in our *in vitro* models. This result aligns with the ECCO guidelines, which do not recommend the use of 5-ASA in CD due to its lack of antifibrotic properties (Gordon *et al*., 2024). Similarly, azathioprine and methotrexate, commonly used immunosuppressants, did not show any effect on the fibrotic processes induced by TGF-β1 in both fibroblast and IEC models, further supporting ECCO's recommendation for alternative therapies (Gordon *et al*., 2024). Corticosteroids such as prednisone, methylprednisolone, and budesonide are commonly used to control intestinal inflammation, particularly in the induction of clinical remission for moderate to severe cases of CD and UC (Quera *et al*., 2023). However, they are not effective in maintaining remission. While corticosteroids have not been shown to have antifibrotic effects in diseases like idiopathic pulmonary fibrosis or systemic sclerosis, our study found that prednisone and methylprednisolone were able to reduce collagen I and α-SMA expression in CCD-18Co cells treated with TGF-β1. Budesonide was effective at the highest dose tested. Notably, the remodeling of the cytoskeleton was also reverted by these steroids. Consistently, prednisone, methylprednisolone, and budesonide reduced Smad2/3 phosphorylation in CCD-18Co cells. Among the steroids tested, only methylprednisolone and budesonide were able to prevent the EMT process induced by TGF-β1 in Caco-2 IEC cells. Biological therapies, particularly

anti-TNF-α agents, have been widely used to treat steroid-dependent or refractory IBD patients. Anti-TNF-α agents were the first biologics approved for IBD treatment and have shown effectiveness in managing inflammation and preventing surgery in patients with IBD (Allocca *et al*., 2017). In this study, we explored the potential antifibrotic effects of anti-TNF-α agents by evaluating their ability to modify the fibrotic phenotype in cellular models. We confirmed the presence of TNF-α expression in both CCD-18Co and Caco-2 IEC cells, which are relevant to IBD pathology. Our results showed that adalimumab significantly reduced the expression of collagen I, α-SMA, and pSmad2/3 in TGF-β1-treated CCD-18Co cells. Additionally, adalimumab reversed the TGF-β1-induced EMT process in Caco-2 IEC cells by upregulating occludin expression and restoring α-SMA levels to those observed in control cells. These findings suggest that TNF-α may be an important therapeutic target in intestinal fibrosis. Surprisingly, infliximab, despite being structurally similar to adalimumab, showed no significant effects in either of the cell lines tested. The main difference between infliximab and adalimumab is that infliximab is a chimeric monoclonal antibody, while adalimumab is a fully human anti-TNF-α monoclonal antibody. This structural difference could potentially explain the discrepancy in efficacy, although further research is needed to better understand the mechanisms by which TNF-α and its inhibitors influence the fibrotic processes in IBD.

Given the involvement of RAS in the development and progression of intestinal fibrosis, along with the affordability and favorable long-term safety profile of anti-RAS drugs, another aim of this study was to investigate the effect of the ARB losartan in reducing intestinal fibrosis both *in vitro* and *in vivo* and to assess its potential in preventing the rebound of inflammation and fibrosis following steroid treatment *in vivo.* Elevated colonic ANG I and II levels in CD patients have been observed (Jaszewski *et al*., 1990), with studies showing reduced colitis severity in TNBS-treated AGT knockout (KO) (Inokuchi *et al*., 2005) and DSS-treated AGTR1 KO mice (Katada *et al*., 2008). In our *in vitro* model using CCD-18Co cells, ANGII upregulated profibrotic genes (e.g., *TGFB*, *IGF1*, *MMP9*, *THBS2*, *CCL11*, and *COL1A1*) and promoted myofibroblast differentiation, marked by increased α-SMA and COL1A1 expression, even without additional stimuli. Losartan effectively reversed these effects, normalizing gene expression and inhibiting myofibroblast activation. Our findings in CCD-18Co cells provided a rationale for *in vivo* studies. Unlike previous works that used chemically induced models of IBD such as DSS (Salmenkari H *et al*., 2018) or TNBS (Wengrower *et al*., 2012) (Shi *et al*., 2016) (T.-J. Liu *et al*., 2016), we selected the SAMP model, widely regarded as the most suitable for studying intestinal fibrosis (Pizarro *et al*., 2011) (De Salvo *et al*., 2014). SAMP mice naturally develop progressive CD-like ileitis without requiring chemical, genetic, or immunological manipulation. Additionally, they exhibit patchy inflammation and fibrosis localized primarily in the ileum rather than the colon. This pattern closely resembles CD, which is characterized

by patchy lesions and a higher prevalence of fibrosis in the terminal ileum (Lichtenstein *et al*., 2006). Our findings reveal distinct temporal changes in RAS component expression between SAMP and AKR mice. In SAMP mice, early disease stages featured upregulation of *Agt* and *Ren* with *Ace* downregulation, while later stages showed activation of alternative pathways, potentially as a compensatory response to ongoing inflammation and fibrosis. Notably, *Ren* remained elevated in SAMP mice with established disease, aligning with prior observations of high REN levels in plasma and in the inflamed epithelium and stroma of IBD lesions. These findings, coupled with evidence that renin inhibitors alleviate colitis in RenTg mice, highlight REN as a promising target for further investigation into its therapeutic potential. We also demonstrated losartan's efficacy in mitigating intestinal fibrosis *in vivo*. In SAMP mice, losartan significantly reduced inflammation and fibrosis in the distal ileum, as indicated by decreased inflammatory and fibrosis scores and reduced AGTR1 expression. This reduction likely contributed to the downregulation of profibrotic genes and diminished COL1A1 deposition. Furthermore, losartan preserved the anti-fibrotic effects of DexM by preventing fibrosis rebound and limiting COL1A1 infiltration into deeper tissue layers, likely through AGTR1 modulation. These findings underscore losartan’s potential as a targeted anti-fibrotic therapy, distinct from its anti-inflammatory effects, warranting *further* exploration. Furthermore, Th2 cytokines, such as IL4 and IL13, are important in the development of fibrosis (Wynn, 2004) and collagen synthesis (Sugimoto *et al*., 2005). In our study, Il13 was significantly reduced upon treatment with losartan suggesting that losartan’s anti-fibrotic effect could also be mediated by an inhibitory effect on the Th2 cascade in SAMP mice. In summary, our findings confirm RAS activation in involved areas of CD patients and demonstrate losartan therapeutic efficacy in reducing intestinal inflammation and fibrosis *in vitro* and *in vivo*, with a distinct anti-fibrotic effect in an experimental model of CD-like ileitis. However, further human studies are needed to investigate the repurpose of ARBs for intestinal fibrosis.

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