Title
Contributors to Intestinal Fibrosis in the TNFSF15/TL1A Pathway

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Contributors to Intestinal Fibrosis in the TNFSF15/TL1A Pathway

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requirements for the degree Doctor of Philosophy

in Molecular, Cellular, and Integrative Physiology

by

Noam Jacob

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ABSTRACT OF THE DISSERTATION

Contributors to Intestinal Fibrosis in the TNFSF15/TL1A Pathway

by

Noam Jacob

Doctor of Philosophy in Molecular, Cellular and Integrative Physiology

University of California, Los Angeles, 2018

Professor Joseph R. Pisegna, Chair

Tumor necrosis factor-like cytokine 1A (*TL1A, TNFSF15*) is implicated in inflammatory bowel disease (IBD), modulating the location and severity of intestinal inflammation and fibrosis. *TL1A* expression is increased in inflamed gut mucosa and associated with fibrostenosing Crohn's disease. TL1A-overexpression in mice leads to spontaneous ileitis, and exacerbated induced proximal colitis and fibrosis. Studies of the role of TNFSF15/TL1A in fibrogenesis will help define pathogenesis and identify potential therapeutic targets for those most severely affected by IBD. The precise mechanisms and specific contributors to TL1A-driven fibrosis require further investigation. IBD is associated with shifts in the gut microbiome, but the effect of differing microbial populations and their interaction with TL1A on fibrosis has not been determined. Intestinal fibroblasts express the TL1A receptor, DR3, and stimulation with exogenous TL1A induces activation and collagen expression in vitro, but the contribution of direct TL1A-DR3 signaling on fibroblasts to the development of fibrosis in vivo is unknown.

We show that the gut microbiome is required for TL1A-mediated intestinal fibrosis and optimal fibroblast activation into myofibroblasts. Moreover, we provide evidence that the TL1A-mediated intestinal fibrotic phenotype requires cues provided by unique bacterial populations, as opposed to any microbiome *per se*. Our analysis further identifies several candidate organisms that correlate with degree of fibrosis and directly impact fibroblast function. Thus, TL1A-mediated intestinal fibrosis and fibroblast phenotype are dependent on specific microbial populations.

To evaluate for a selective role of direct TL1A-DR3 signaling on fibroblasts in fibrostenosing IBD, TL1A over-expressing naïve T cells (Tl1a-Tg) were transferred into $Rag^{-/-}$ mice; $Rag^{-/-}$ mice lacking DR3 in all cell types ($Rag^{-/-}Dr3^{-/-}$); or $Rag^{-/-}$ mice lacking DR3 only on fibroblasts ($Rag^{-/-}Dr3^{\Delta Col1a2}$). We show that $Rag^{-/-}Dr3^{-/-}$ recipients demonstrate reduced disease activity, inflammation, and an accompanying reduction in fibrosis and fibroblast activation compared with DR3-sufficient $Rag^{-/-}$ recipients. In contrast to pan-DR3-deficient recipients, $Rag^{-/-}Dr3^{\Delta Col1a2}$ recipients exhibit a similar degree of severe inflammatory disease as $Rag^{-/-}$ recipients. Despite the presence of abundant inflammation, however, fibroblast-selective DR3-deficiency significantly reduces intestinal fibrosis and decreases activation of fibroblasts. *Ex vivo*, DR3-deficient fibroblasts isolated from these colitic mice exhibit a significant reduction in gap-closure compared to those from DR3-intact $Rag^{-/-}$ mice. These data demonstrate that direct TL1A-DR3 signaling on fibroblasts *in vivo* significantly contributes to TL1A-mediated intestinal fibrosis, independent of inflammation.

The dissertation of Noam Jacob is approved.

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TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

ACKNOWLEDGMENTS

Chapter 1 is a version of "Cytokine and anti-cytokine therapies in prevention or treatment of fibrosis in IBD." *United European Gastroenterol J.* 2016; 4:531-40.

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Publications/Presentations

1. Jacob N, Kumagai K, Abraham JP, Shimodaira Y, Ye Y, Targan SR, Michelsen KS, Shih DQ. Direct Signaling of TL1A-DR3 on Fibroblasts Induces Intestinal Fibrosis In Vivo. Oral presentation at Digestive Disease Week, 6/2018; Washington, D.C.
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Chapter 1:

Cytokine and Anti-cytokine therapies in prevention or treatment of fibrosis in IBD

Introduction:

The frequency of fibrosing Crohn's disease (CD) is significant, with approximately 40% of CD patients with ileal disease developing clinically apparent strictures throughout their lifetime[1]. Although strictures may be subdivided into fibrotic, inflammatory, or mixed forms, despite immunosuppressive therapy in CD patients in the form of steroids or immunomodulators, the frequency of fibrostenosing complications has still remained significant[2, 3]. A vast number of genetic and epigenetic variables are thought to contribute to fibrostenosing disease, including those that affect cytokine biology, and therefore highlight the complexity of disease, but also shed light on targetable pathways. Exclusively targeting fibrosis may be difficult, however, due to the relatively slow evolution of fibrosis in CD, and the potential adverse effects of inhibiting pathways involved in tissue repair and mucosal healing. Acknowledging these caveats, cytokine-targeted therapy has become the mainstay of treatment for many inflammatory conditions and is being evaluated for fibrotic disorders. The question of whether anti-cytokine therapy will prove useful for intestinal fibrosis is, therefore, acutely relevant. This chapter will highlight current therapeutics targeting cytokines involved in fibrosis and introduce the gene/protein combination of tumor necrosis factor superfamily (TNFSF) member 15/tumor necrosis factor-like (TL) cytokine 1A (referred to as: TNFSF15/TL1A), which has known influence over the severity and expression of inflammation and fibrosis in both animal models and human disease. As TL1A is emerging as an attractive target for both inflammation *and* fibrosis, evaluation of the contributors to intestinal fibrosis in the TL1A pathway is particularly warranted.

"Regulatory" Cytokines

TGFβ

TGFβ is a ubiquitously produced cytokine, and although often considered a "regulatory" molecule, it employs a myriad of functions influencing proliferation, differentiation, inflammation, immunoregulation, wound healing and fibrosis[4]. TGFβ is arguably the most widely studied, targeted, and discussed cytokine contributor to fibrosis. Elevated levels of TGFβ and its receptors have been reported during the development of fibrotic disease of virtually any organ, including heart, lungs, liver, kidney, skin, and intestines; and genetic over-expression or exogenous administration of TGFβ in animals promotes wide-spread fibrotic disease[5]. TGFβ has numerous effects with regards to fibrosis including activation and differentiation of fibroblasts to myofibroblasts with subsequent upregulation in the production of ECM proteins including collagen and fibronectin, expression of adhesive receptors and contractile elements, and inhibition of MMPs and stimulation of TIMP[4, 6, 7]. TGFβ can also induce fibrogenesis via newly-recognized mechanisms of fibrosis including epithelial to mesenchymal transition and endothelial to mesenchymal transition[8]. Role of TGFβ and therapeutic targets in fibrosis is further described below and summarized in Table 1.

The three main isoforms of TGFβ: TGFβ1, TGFβ2, and TGFβ3, are secreted as latent precursor molecules containing a latency associated peptide region (LAP), and complexed with latent TGFβ binding proteins (LTBP). The cytokine is active when LTBP is removed extracellularly via proteolytic cleavage by proteases such as plasmin or thrombin; or by interactions of LAP with other proteins such as thrombospondin-1 or integrins[4]. TGFβ signaling ensues through two receptors, TGFβR1 and TGFβR2, which form transmembrane serine/threonine kinase, hetero- or homo-dimeric complexes that induce phosphorylation of Smad 2 and Smad 3 proteins. Once

phosphorylated, Smad 2 and 3 complexes with Smad 4, translocate to the nucleus, and activate transcription. Regulation of Smad 2/3 occurs via Smad 7, which prevents binding of Smad2/3 to the receptor complex. TGFβ can also signal through other pathways, however, including ERK1/2, c-Jun N terminal kinase, p38 kinases and members of the JAK/STAT family[4, 9].

As mentioned above, in addition to its profibrotic effects, TGFβ is a potent immune modulator central to immune tolerance and development of innate and adaptive immunoregulatory cells. Thus, the global blockade of TGFβ might upset critical balances in immune homeostasis resulting in untoward effects, or perhaps be ineffective due simultaneous blockade of fibrogenic and regulatory functions. Several global TGFβ-blocking agents were either found to be ineffective or led to possible drug associated mortality[10, 11]. While global blockade of TGFβ might be problematic, other strategies have focused on specific pathways in TGFβ signaling, synthesis, activation, or other downstream mediators and effects, with the hope of targeting TGFβ-driven fibrosis while sparing its immunomodulatory effects. Thus in light of this broad complexity of function, antagonizing an individual receptor, rather than the ligand itself, might be more attractive if it proves more efficacious and specific. Accordingly, blockade of TGFβR1 signaling by an injectable inhibitor (SD-208) was evaluated in two experimental animal models of intestinal fibrosis: anaerobic bacteria- and trinitrobenzensulphonic acid-induced colitis (TNBS). SD-208 reduced fibroblast activation, phosphorylation of Smad2 and Smad3 proteins, and intestinal wall collagen deposition in both models[12]. Similarly, more recent studies on blockade of TGFβR1 with oral inhibitors have demonstrated efficacy in animal models of renal fibrosis, carbon tetrachloride- or bile duct ligation-induced cirrhosis,[13,14] pressure-overload-induced cardiac fibrosis[15], and bleomycin-induced pulmonary fibrosis[16]. These agents are

currently being investigated in oncologic trials, with pre-clinical testing ongoing for fibrotic disorders.

With regards to TGFβ synthesis, Pirfenidone (5-methyl-1-phenyl-2-[1H]-pyridone) is a small, orally active molecule that has demonstrated anti-fibrotic effects, in part via inhibiting synthesis of TGFβ. This agent has been efficacious in patients with, and experimental models, of pulmonary and renal fibrosis[17, 18]. Pirfenidone has been evaluated in randomized, double-blind, placebo-controlled clinical trials. Pirfenidone reduced the rate of decline in lung function as measured by changes in forced vital capacity or total lung capacity, as well as improved mortality[19, 20]. It has been approved in Europe and by the FDA for treatment of IPF. Pirfenidone, however, has not been uniformly beneficial in all clinical trials; it had no clinical or histologic benefits in patients with myelofibrosis[21], or primary sclerosing cholangitis, while being associated with increased adverse events[22].

Downregulating or decreasing production of TGFβ without adverse immunological effects has been demonstrated by two classes of medications currently in widespread use in primary care: HMG-CoA reductase inhibitors (statins) and antagonists of Renin-Angiotensin system (RAS). As the primary mediator of the RAS, Angiotensin may contribute to fibrogenesis via induction of TGFβ expression and promotion of collagen production[23]. With regards to intestinal fibrosis, early studies have reported that Angiotensin is increased in the mucosa of CD patients[24]. In TNBS-induced colitis, administration of the ACE inhibitor, captopril, or the angiotensin receptor blocker, losartan, reduced colonic inflammation and fibrosis via reduction in TGFβ1[25, 26]. Like antagonists of the RAS, statins may be of benefit with regards to fibrosis, in part, through decreasing expression of TGFβ. Simvastatin reduces TGFβ1 expression in human fibroblasts by

inhibition of Smad3 phosphorylation[27]. In TNBS-induced colitis, it had anti-fibrotic effects characterized by a dose-dependent decrease in the level of connective tissue growth factor (CTGF) and induction of apoptosis in fibroblasts[28]. Given the safety and ubiquity of RAS antagonists and statins, future prospective investigations will be feasible and determine if they are capable of favorably impacting fibrogenesis.

The activation of TGFβ from its latent precursor state serves as an important regulatory step in TGFβsignaling, which might be exploited as a therapeutic target. Strategies of integrin inhibition, most recently with vedolizumab, have proven effective with regards to inflammation in IBD and may impact fibrosis via their effects on TGFβ activation. As mentioned above, integrins, particularly those of the alphaV (αV)-type, can bind LAP and activate TGFβ. αVβ6 integrin is upregulated in various fibrotic disorders and its blockade has been effective in models of radiation- and bleomycin-induced pulmonary fibrosis, as well as liver fibrosis[29]. Similarly, αVβ3 integrin contributes to excess smooth muscle cell proliferation and hyperplasia in intestinal strictures of CD[30], and Cilengitide, an αVβ3 inhibitor, reduces the development of fibrosis in chronic TNBS-induced colitis[31]. Future studies will demonstrate if these, or the currently used integrin inhibitors, will have favorable effects with regards to fibrosis in IBD.

Another seemingly attractive option is the targeting of specific signaling molecules in the TGFβ cascade. This option might appear favorable, as it may focus on individual mediators of TGFβ signaling rather than broader targets such as TGFβ itself. Two such potential strategies are Smad3 antagonism and Smad7 agonism. Increased Smad3 and decreased Smad7 expression have been observed in intestinal strictures in CD[32]. Furthermore, in multiple animal models, loss of Smad 3 or increase in Smad7 confers resistance to fibrosis in several organs[33-35]. There has been

recent focus on inhibition of Smad7 in IBD via antisense oligonucleotides (and subsequent increase in Smad3 transduction with potential TGFβ-mediated shift towards immune-regulation). This strategy may be troubling with regards to fibrogenesis. An ideal solution might be to clearly identify those patients that would be more prone to develop fibrotic/stricturing disease vs predominantly inflammatory pathology through functional, genetic and epigenetic studies.

IL-10

IL-10 has a well-known role with regards to immune regulation as a prominent product of regulatory T cells and their effects on intestinal inflammation[36]. In contrast to TGFβ, however, IL-10 has been shown to inhibit fibrosis. Mice treated with IL-10 develop less liver and lung fibrosis when administered carbon tetrachloride or bleomycin[37, 38]. Similarly, IL-10 deficiency aggravates kidney inflammation and fibrosis in the unilateral ureteral obstruction mouse model[39]. With regards to human IBD, however, although polymorphisms in the IL-10 locus have been associated with IBD[40], treatment of CD patients with recombinant IL-10 has not been significantly effective (Table 1)[41].

"Inflammatory" Cytokines

TNFα

Like TGFβ, TNFα is a pleiotropic cytokine, classically considered proinflammatory with important immunomodulatory properties. A variety of cell types can elaborate TNFα, including activated macrophages, B cells, T cells, keratinocytes, and fibroblasts. Depending upon the conditions, TNFα can trigger either pro-inflammatory or anti-inflammatory pathways by

engaging one or both of two distinct transmembrane receptors: TNFR1, and TNFR2. In addition to its pro-inflammatory effects, TNFα may potentiate fibrosis via induction of TIMP-1 expression and reduction in matrix metalloproteinases-2 activity and collagen degradation[42]. Treatments targeting TNFα are perhaps some of the most widely used anti-cytokine therapies for inflammatory disorders, but evidence for the role of these agents in preventing fibrosis is somewhat mixed. In some animal models of liver and renal fibrosis, TNF blockade reduced organ inflammation and fibrogenesis[43, 44], but a recent clinical study investigating adalimumab for fibrotic kidney disease (FSGS) failed to meet its primary outcome[45]. An open-label pilot study in 16 systemic sclerosis patients demonstrated improvement in skin scores with reduction in collagen secretion noted from cultured lesional fibroblasts (Table 1)[46-48].

In contrast, there is evidence to suggest that TNFα is a potentially antifibrogenic cytokine and its blockade might consequently promote fibrosis. In some studies, TNFα can exhibit antifibrotic properties by reducing the expression of collagen and connective tissue growth factor in dermal fibroblasts[49], and via suppression of TGFβ signaling through NFKB induction of Smad7 in other cell types[50]. Disparate effects may be cell-specific and segregate at the level of the individual TNF receptors, as globally impaired signaling through TNFR1 accelerates wound-healing, increases collagen deposition, and angiogenesis at wound sites in TNFR1-deficient mice [51]; whereas impaired signaling in TNFR2-deficient intestinal myofibroblasts results in reduced cell proliferation and decreased collagen synthesis [42].

Consequently, with regards to intestinal fibrosis, the evidence for specific use of TNF antagonists as *anti-fibrotic* agents (as opposed to anti-inflammatory agents) has remained vague. In early reports of TNF blockade, obstructive complications were observed in some patients,

with initial concerns that these agents may promote excessive fibrotic changes accompanying mucosal healing. *In vitro* studies, however, showed that TNF blockade decreased myofibroblast collagen production[52] in CD patients treated with infliximab. Later multivariable analyses from the observational TREAT registry and the ACCENT I multicenter trial determined that, rather than TNF-antagonist use, disease duration, severity, location, and new corticosteroid use are factors associated with stricture formation[53]. Positive results have now been seen in a few patients with inflammatory or mixed stenoses[54, 55], as well as small case series reporting intralesional injection of infliximab[56]. Data from population-based cohorts seem to suggest that these agents may reduce the need for surgery in the short term [57] with the rate of surgery ranging between 27-61% within the first 5 years after diagnosis before the introduction of the biologics, and between 25-33% after the introduction of anti-TNF agents[58]. Indeed, anti-TNF agents are recommended to reduce the risk of post-operative recurrence after surgery. Discerning between unique antifibrotic effects in these cases and modification of the fibrotic program due to reduction in inflammation may be difficult.

Th2 Cytokines

The Th2 cytokines, IL-4 and IL-13, have been implicated in fibrogenesis (Table 1). Both are elevated in fibrotic disease and promote fibroblast activation, proliferation, and collagen synthesis[59, 60]. For example, IL-4 is found at increased concentrations in the bronchoalviolar lavage of patients with idiopathic pulmonary fibrosis[61]. IL-4 also increases the expression of collagen in cultured hepatic fibroblasts[62]. Similarly, IL-13 is involved in many Th2-mediated diseases and has a role in fibrosis as well. Deriving from a common receptor subunit (IL-4Ralpha), IL-13 shares overlapping functions with IL-4. IL-13 signals by interacting with a

complex receptor system comprised of IL-4Ralpha and two IL-13 binding proteins, IL-13Rα1 and IL-13Rα2. IL-13 receptors are expressed on a vast array of cells, including human hematopoietic cells, endothelial cells, fibroblasts, multiple epithelial cell types, and smooth muscle cells[63]. Increased IL-13 mRNA was found in intestinal samples of fibrotic CD patients. Fibroblasts from these samples expressed elevated levels of IL13Rα1 and subsequently down-regulated MMP in response to IL-13[64]. Importantly, however, elevated IL-13 production was not detected in UC or strictured CD [65], questioning if anti-IL-13 therapy would be an appropriate strategy in IBD. *In vivo* inhibition of IL-13Rα2 expression reduced production of TGFβ1 in oxazolone-induced colitis and led to marked decrease of collagen deposition in bleomycin-induced lung fibrosis[66]. Similarly, IL-13 blockade reduces experimental hepatic fibrosis[67]. In TNBS-induced colitis, inhibition of IL-13 signaling by administration of small interfering RNA targeting the IL-13Rα2, reduces fibrosis and expression of TGFβ[68]. Given the suggested experimental benefits of IL-13 antagonism, IL-13 antibodies such as lebrikizumab and tralokinumab are currently being evaluated for anti-fibrotic efficacy in pulmonary fibrosis (NCT01872689, NCT01629667). Given the benefit in pre-clinical investigations, clinical studies targeting IL-13 or IL-13 receptor may be envisioned for fibrosis in CD.

Th1 Cytokines

In contrast to pro-fibrotic cytokines produced by Th2 cells, Th1 cells, through production of IFNγ have opposing anti-fibrotic effects. IFNγ has been shown to inhibit fibroblast proliferation and migration[69]. IFNγ signaling was shown to suppress the production of TGF-b via Y box-binding protein YB-1(YB-1) and an orally administered compound that promotes nuclear translocation of YB-1 resulted in the improvement of murine liver fibrosis and TNBS-induced

murine chronic colitis [70-72]. Several other models have demonstrated the potent antifibrotic activity of IFNγ. In the case of schistosomiasis-induced fibrosis, treatment with IFNγ reduces collagen deposition associated with chronic granuloma formation [73]. Similar results were obtained in models of pulmonary and kidney fibrosis[74, 75]. These outcomes were not replicated in human studies, however. A randomized trial of subcutaneously injected recombinant IFNγ did not demonstrate improvement in survival of patients with idiopathic pulmonary fibrosis (Table 1)[76].

Th17 Cytokines

The family of IL-17 cytokines is comprised of IL-17A-F, which act through the IL-17 receptor. Early evidence suggested that a main function of IL-17 is the promotion of chemokine production for granulocyte activation and increasing inflammation[77]. With regards to pro-fibrotic effector functions, IL-17 stimulates activation pathways in human colonic myofibroblasts[78] and maintains fibrotic activity in various other cell types including stellate cells[79] and lung epithelial cells[80]. Treatment of mice with anti-IL-17A monoclonal antibody administered after the onset of myocarditis abrogates cardiac fibrosis and preserves ventricular function[81]. Similarly, IL-17A increases the synthesis and secretion of collagen and promotes the epithelial-mesenchymal transition in alveolar epithelial cells in a TGFβ1-dependent manner. Neutralization of IL-17A promotes the resolution of bleomycin-induced acute inflammation, attenuates pulmonary fibrosis, and increases survival in this model[80]. In IBD, however, IL-17's contribution to disease pathogenesis is complex as both human and animal data suggest a dual inflammatory and protective role. With regards to its role in intestinal fibrosis, *in vitro* intestinal samples from fibrosing CD patients express elevated levels of IL-17A and IL17-stimulated myofibroblasts

from CD strictures generate more collagen and TIMP-1[82]. The role of IL-17 concerning clinical disease development in animal models of IBD has yielded disparate results depending on the model used[83]. In a clinical trial of patients with inflammatory CD, blockade of IL-17A by administration of the anti-IL-17A antibody, secukinumab, failed to meet its primary endpoint (Table 1)[84]. Post hoc analysis, identified that a subgroup of patients who responded to IL-17 blockade carried a *TNFSF15* (rs4263839) SNP. The potential functional consequences of this allele include elevated production of TL1A protein. Under TL1A-upregulated conditions in adoptive transfer-induced colitis, IL-17A deficiency ameliorated colonic inflammation via reducing Th1 and Th9 effector responses while enhancing regulatory responses[85]. Thus, there exists a subset of patients (those that overexpress TL1A due to e.g. a *TNFSF15* variant) who could potentially benefit from IL-17 blockade. Given the potential profibrotic role of TL1A overexpression in this subset of patients who have a propensity towards fibrostenosis (as discussed below), IL-17 blockade may have a positive impact on fibrosis, as well as inflammation.

TL1A

TL1A (a protein encoded by *TNFSF15*) is a member of the TNF superfamily that binds to death domain receptor 3 (DR3, also known as TNFRSF25), expressed on a variety of cell types[86-88]. Modulating an array of immune responses, TL1A can be expressed by endothelial cells induced by IL-1β and TNFα, macrophages and dendritic cells in response to Toll-like receptor stimulation, as well as in some lymphoid lineage cells[89-92]. Role of TL1A in fibrosis is further described below and summarized in Table 1.

Table 1: Cytokine and drug targets in fibrosis

Cytokine	Effect on Fibrosis	Cellular/Molecular Mechanism	Drug (mechanism of action)
TNFα	↑/↓	Induction of TIMP, ↓ MMP ↓ fibroblast collagen, CTGF, TGFβ	Infliximab, Adalimumab (anti-TNF Ab)
IL-4 **IL-13**	↑ ↑	Fibroblast activation, ↑ collagen ↓ MMP , ↑ TGFβ	 Lebrikizumab, Tralokinumab (anti-IL-13 Ab)
IFNγ	↓	↓ Fibroblast proliferation, migration ↓ collagen production	Recombinant Human IFNγ, HSc025 (upregulates YB-1)
IL1β	↑/↓	↑TNF-α, IL-6; transcription of TGF-β ↑collagenase, ↓ collagen production	Canakinumab (anti-IL1β Ab)
IL-17	↑	Activation of fibroblasts, ↑ collagen Promotion of EMT, ↑ TGFβ	Secukinumab (anti-IL-17 Ab)
TL1A	↑	Activation of fibroblasts, ↑ collagen ↑TIMP	In development
TGFβ	↑	↑fibroblast activation, proliferation ↑ Collagen, fibronectin, TIMP ↓ MMP Promotion of EMT and EndoMT	Metelimumab, Fresolimumab (anti-TGFβ Ab) SD-208, EW-7197, IN-1130, SM16 (TGFβR inhibitor) Pirfenidone, ACEi/ARB, Statin (↓TGF syn/signaling) Cilenglitide (integrin inhibitor, ↓TGFβ activation)
IL-10	↓	T-reg associated suppression of cell activation	Recombinant Human IL-10

Much of the previous literature has focused on the TL1A-DR3 pathway with regards to immune function with recent evidence now suggesting its importance concerning fibrosis. As DR3 shares homology with TNFR1, consequently, like with other TNF receptors, developmental, immunoregulatory and pro-inflammatory effects have been described. DR3 activation of NF-KB in human cell lines upregulates c-IAP2, an NF-KB-dependent anti-apoptotic protein, which protects against apoptosis[93]. Conversely, however, DR3 in embryonic cells can induce FADD- and caspase-8-dependent apoptosis, and early work on DR3-deficient mice demonstrated that it is required for negative selection in the thymus[94, 95]. Despite its initially recognized role as a pro-apoptotic receptor, DR3 has been shown to be upregulated on Th17 cells, and TL1A-DR3 interaction promotes T cell expansion and cytokine production during immune responses[96-98]. Along these lines, the pro-inflammatory effects of TL1A-DR3 binding likely contribute to this

pathway's effect on fibrosis. More direct evidence has demonstrated that DR3 is an important receptor for fibroblast development, maturation and function. Indeed, DR3 is expressed on human and mouse primary intestinal fibroblasts, and DR3-deficient mice display reduced number of colonic fibroblasts.[99] DR3 deletion also results in reduced fibroblast activation (as evidenced by decreased expression of alpha smooth muscle actin) and expression of collagen induced by TL1A stimulation[99].

Specifically with regards to the TL1A-DR3 pathway in IBD, a TNFSF15 haplotype is associated with higher TL1A expression, increased risk of CD, intestinal fibrostenosis, and greater need for surgery[100-102]. In mice, constitutive TL1A overexpression causes spontaneous ileitis with increased collagen deposition[103, 104]. Under colitogenic conditions induced by chronic DSS treatment or adoptive T cell transfer, increased inflammation, fibrosis, and fibrostenotic lesions in the gut are seen[105]. These results support the role of TL1A in induction of intestinal inflammation and suggest its contribution to fibrogenesis in the gut. The potential for TL1A as a therapeutic target in intestinal fibrosis was demonstrated in a recent study evaluating the effect of anti-TL1A Ab in chronic DSS and adoptive T-cell transfer models of IBD. Treatment with neutralizing TL1A Ab attenuated disease and reversed colonic fibrosis. Additionally, TL1A blockade reduced the number of fibroblasts and myofibroblasts in colonic cell isolates and lowered expression of CTGF, TGFβ1 and IGF-1[99].

Thus, TL1A has emerged as a potential candidate target for therapy in fibrotic diseases. A crucial question that arises regarding the ability of TL1A to mediate fibrosis is whether its effects are due to direct signaling on profibrotic effector cells (e.g. fibroblasts); indirect due to effects on inflammation and immune cells, which then promote fibrosis via release of other cytokines

mentioned above; or work in concert with entities that are technically peripheral to the immune system, but undeniably and mutually involved in its functions, such as the microbiome, which has become increasingly relevant for many chronic diseases.

Studies of the role of TNFSF15/TL1A in fibrogenesis will help to define pathogenesis and identify potential therapeutic targets for those most severely affected by IBD. The precise mechanisms and specific contributors to TL1A-driven fibrosis have yet to be determined, however. As fibroblasts are a predominant cell type responsible for fibrosis, the mechanisms by which TL1A drives intestinal fibrosis possibly involve direct induction of fibroblast activation. Another (non-mutually exclusive) possibility is that TL1A has an indirect effect by regulating other cell types or the intestinal environment, including the microbiome, which may augment the fibrotic pathway. Studies addressing these issues will present novel direct and indirect effects of TNFSF15/TL1A on fibrosis and IBD severity. Moreover, their results have the potential to modify prevailing hypotheses concerning the role of TL1A (and the microbiome) in IBD, which have emphasized inflammation over fibrosis. The following chapters will examine these potential contributors to intestinal fibrosis in the TNFSF15/TL1A pathway.

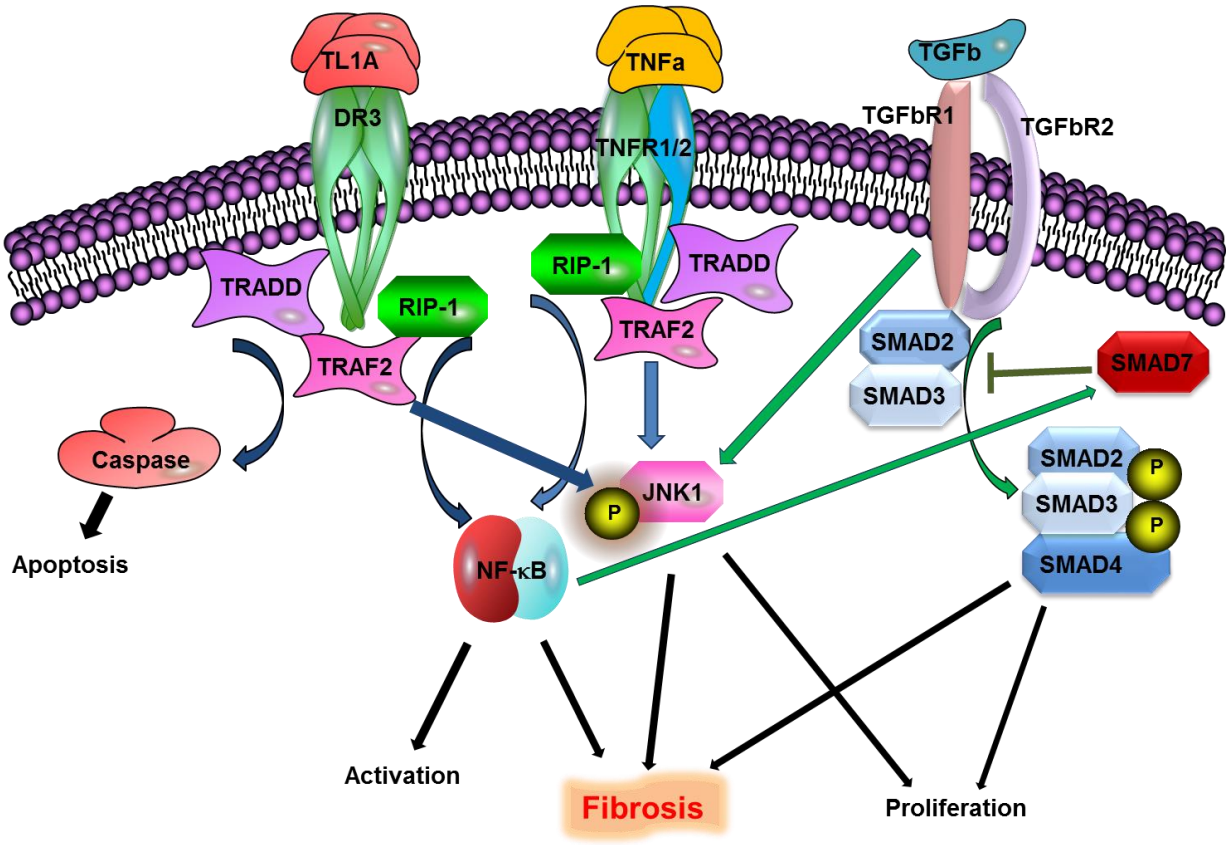

Figure 1. *Signaling of TL1A, TNFα, and TGFβ:* Both TL1A-DR3 and TNF-TNFR binding can lead to recruitment of TRADD, TRAF2 and RIP-1, which can activate pro-apoptotic caspase-mediated pathways (via TRADD) or activation and pro-fibrotic pathways via NFkB and JNK1. TGFb-TGFbR binding leads to phosphorylation of SMAD2/3, which complexes with SMAD4 and leads to activation of fibrotic and proliferation pathways. SMAD7 inhibits binding and phosphorylation of SMAD2/3, thereby attenuating TGFb signaling. TRADD (TNF receptor associated death domain); TRAF (TNF receptor associated factor); RIP (Receptor-interacting serine/threonine-protein kinase); NFkB (nuclear factor kappa-light-chain-enhancer of activated B cells); JNK (c-Jun N-terminal kinases); SMAD (Mothers against decapentaplegic homolog). Dark blue arrows represent TL1A pathway signaling; light blue arrows represent TNF pathway signaling; green arrows represent signaling in TGFb pathway; black arrows represent downstream end effects.

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Chapter 2:

Inflammation-independent TL1A-mediated Intestinal Fibrosis is Dependent on the Gut Microbiome

Introduction:

Tl1a (a protein encoded by *TNFSF15*) is a member of the tumor necrosis factor (TNF) superfamily that binds to death domain receptor 3 (DR3), expressed on a variety of cell types including immune cells, epithelial cells, and fibroblasts.[1-5] Modulating an array of immune responses, Tl1a can be produced by endothelial cells in response to IL-1β and TNFα, by macrophages and dendritic cells in response to Toll-like receptor stimulation, as well as in some lymphoid lineage cells.[2,3,6-9] A *TNFSF15* haplotype is associated with higher Tl1a production, increased risk of CD, intestinal fibrostenosis, and greater need for surgery.[10-13] In mice, constitutive Tl1a expression induced increased collagen deposition in the colon without detectable histologic colitis as well as increased collagen deposition in the ileum with spontaneous ileitis.[14-17] Under colitogenic conditions induced by chronic DSS treatment or adoptive T-cell transfer, there was increased collagen deposition with fibrostenotic lesions in the gut that caused intestinal obstruction in the Tl1a-Tg mice.[12] These results support the role of Tl1a in fibrogenesis that can lead to fibrostenosis in the setting of chronic inflammation, which is a common complication of CD leading to resection.

The intestinal microbiome has been linked with many inflammatory diseases including IBD.[18-21.] Although previous studies have found alterations in various bacterial taxa in IBD patients, and a recent study found an association with a fibrostenotic disease cohort, none have correlated specific microbes with degree of fibrosis and fibroblast phenotype.[22] Most of these studies have been largely associative without the ability (by design) to prove causality. The question remains, therefore, whether altered microbiota associated with IBD contribute to the disease phenotype or are its consequence. In rodent models, comparison of experimental IBD models under GF

conditions have yielded disparate results with development of colitis in most spontaneous genetically engineered models dependent on resident microbiota, or uniquely, potentiated DSS-induced colitis in GF mice.[23-25] No animal experiments have evaluated the contribution of the microbiome to fibrosis in the context of IBD. In this study, we show that the native murine fecal microbiota is required for optimal Tl1a-dependent fibroblast activation and transformation into myofibroblasts. Moreover, we provide evidence that the intestinal fibrotic phenotype requires specific microbial cues provided by mouse microbiota from an SPF facility but absent in human feces from a healthy donor. Our analysis further identified several candidate organisms that correlate directly with degree of fibrosis in reconstituted hosts and impact fibroblasts *in vitro*. To our knowledge, this is the first study to establish a potential causal role for the microbiome in intestinal fibrosis, fibroblast activation and function.

Results:

The intestinal microbiome is required for Tl1a-enhanced intestinal inflammation, collagen deposition and fibroblast migration

In agreement with our prior results, Tl1a-Tg mice raised under SPF microbial conditions display significant spontaneous ileitis, as evident by increased histopathological scoring under H&E, with over a 2-fold increase in average histopathology compared with wild type mice (Figure 1A).[16] No histologically apparent cecal inflammation was observed (Figure 1B). Despite this, Tl1a-Tg mice demonstrate increased cecal collagen deposition (Figure 1D), underscoring the importance of TL1A as a mediator of fibrosis that can act independently of its pro-inflammatory effects.

As the microbiome is relevant to inflammation in several diseases, we evaluated the effects of GF conditions on Tl1a-mediated intestinal inflammation. The absence of a microbiome abrogated the spontaneous ileitis induced by Tl1a-overexpression, as there were no significant differences in ileal histopathology between GF Tl1a-Tg and GF WT mice (Figure 1A).

We next evaluated if the fibrosis observed in Tl1a-Tg mice was dependent upon the microbiome. The absence of microbes significantly reduced ileal and cecal collagen deposition in GF Tl1a-Tg mice compared with microbiome intact Tl1a-Tg mice (Figure 1C-D). No difference was observed in WT mice.

Since we observed notable changes in fibrosis between Tl1a-Tg and WT mice under native SPF conditions that were abrogated under GF conditions, we sought to determine the impact of Tl1a-overexpression and resident microbiota on fibroblast phenotype. Colonic fibroblasts isolated from Tl1a-Tg mice raised under native microbial conditions displayed significantly increased

migratory capacity after simulated wounding compared with those from WT mice (Figure 2A-B). Tl1a-overexpression also increased fibroblast adhesion, which was unchanged in GF mice (Supplementary Figure 1). The enhanced rate of fibroblast gap-closure observed with Tl1a-overexpression under native conditions was eliminated under GF conditions, consistent with the observed reduction in histological fibrosis (Figure 2A-B). Interestingly, the absence of microbiome reduced fibroblast migratory capacity even in WT mice, but to a lesser extent than in Tl1a-Tg mice. These results indicate that the intestinal microbiome is required for Tl1a-mediated intestinal fibrosis and influences fibroblast migratory function.

An important question arising from these results is whether the observed findings are due to direct effects of SPF microbiota and Tl1a (alone or in concert) on fibroblasts themselves, or if the microbiome and Tl1a in Tl1a-Tg mice affect other non-fibroblast cell types, which then promote fibroblast activation and profibrotic phenotype. Bacterial components and products can induce fibroblast activation,[26] but it is unclear if they can promote fibroblast migration directly. Moreover, the direct effect of bacterial stimulus on fibroblasts in the context of host Tl1a-overexpression has not been evaluated. We therefore assessed if bacterial products isolated from the cecal luminal washings of native WT SPF mice could promote fibroblast migration directly, and if this effect was enhanced by host Tl1a-overexpression. WT fibroblasts exposed to native SPF cecal washings demonstrate significantly increased migration compared to those exposed to cecal washings from GF mice (Figure 2C). This direct effect of cecal bacterial components was enhanced by host Tl1a-overexpression in Tl1a-Tg mice. Thus, a significant part of the commensal microbiome's effect on fibroblast migration seen in Figure 2A-B may be due to direct effects mediated by bacterial components (or products) on fibroblasts themselves.

We next asked if this enhanced migratory phenotype in fibroblasts from Tl1a-Tg mice was partly due to direct Tl1a-mediated effects on fibroblasts; namely, can Tl1a promote fibroblast migration directly and do so in concert with the direct effect of SPF microbiota on fibroblasts seen in Figure 2C? We have previously demonstrated that fibroblasts express the Tl1a receptor DR3, and upon treatment with Tl1a *in vitro*, demonstrate expression of alpha-smooth-muscle actin (indicative of activation of myofibroblasts) and collagen.[27] Consequently, fibroblasts treated with Tl1a *in vitro* demonstrate expression of alph-smooth-muscle actin (activation to myofibroblasts) and collagen.[27] We therefore hypothesized that direct stimulation of fibroblasts with Tl1a would increase migration. WT fibroblasts treated with Tl1a (and without bacterial components) *in vitro* displayed significantly increased migration compared with untreated cells, suggesting a direct effect of Tl1a on fibroblast migration (Figure 2D). To determine if Tl1a can enhance the fibroblast migratory response to bacterial components, we conducted the same experiments as in Figure 2C in the context of exogenous Tl1a stimulation. WT fibroblasts treated with both Tl1a and SPF cecal bacterial products together demonstrated enhanced migration compared with those treated solely with the bacterial products (Figure 2D). These data demonstrate that resident bacteria and Tl1a can both stimulate fibroblasts, and the direct effect of resident bacteria on fibroblasts is enhanced directly by Tl1a.

Gavage with murine but not human fecal microbiota promotes intestinal inflammation and collagen deposition in Tl1a transgenic mice

As these data demonstrate that the microbiome is required for Tl1a-mediated fibrosis in intestinal regions with and without underlying inflammation, we sought to evaluate if this phenotype is due to the absence of a unique bacterial population, as opposed to any bacterial colonization *per se*.

We used two distinct microbiota to test the hypothesis that the pro-fibrotic phenotype observed in Tl1a-Tg mice under native microbial conditions was due to a specific bacterial population adapted to the mouse intestine rather than the presence of any gut bacteria. GF mice were gavaged with stool collected from wild-type mice housed in SPF or with stool from a healthy human (Hu) donor and evaluated 2 months later.

WT mice displayed no increase in intestinal inflammation or collagen deposition when colonized with either SPF or Hu flora, indicating that in the absence of Tl1a-overexpression the species-specific microbiome does not induce intestinal inflammation or fibrosis (Figure 3A-D). Tl1a-transgenic mice colonized with SPF microbiota demonstrated increased collagen deposition in both inflamed ileum and non-inflamed cecum, consistent with findings in mice under native conditions (Figure 3A-D, Figure 1). In contrast, Tl1a-transgenic recipients of Hu microbiota showed no increase in ileal or cecal collagen deposition or inflammation. Together, these data indicate that Tl1a-mediated intestinal fibrosis is modulated by the composition of the intestinal microbiome and suggest that this phenotype is induced by microbes selectively contained in the SPF mouse microbiota but missing from the human microbiota.

Murine microbiota potentiate Tl1a-mediated intestinal fibroblast differentiation to myofibroblasts

Fibroblast activation has been shown to occur after bacterial stimulation e.g. with lipopolysaccharide.[26] Previously, we showed that there is an increase in the proportion of intestinal myofibroblasts in Tl1a-Tg mice raised under conventional SPF conditions.[27] We investigated whether absence of microbial stimulation (i.e. under GF conditions) impairs fibroblast differentiation to myofibroblasts. GF Tl1a-Tg mice did not display an increased

number or proportion of activated fibroblasts in the cecum compared to GF WT mice (Figure 4A-B). Colonization with SPF microbiota induced intestinal myofibroblasts in both WT and Tl1a-Tg mice relative to GF conditions or colonization with Hu microbiota (Figure 4A-B). SPF microbiota, but not Hu microbiota also restored the increased proportion of myofibroblasts in Tl1a-Tg mice compared to WT controls (54.7% vs. 36.7%). Interestingly, GF Tl1a-Tg mice had reduced myofibroblast proportion compared to GF WT mice, which was not seen in the presence of Hu microbiota (Figure 4A-B). These results show that fibroblast activation in the cecum induced by Tl1a overexpression is microbiota-dependent and that microbial composition affects fibroblast differentiation into myofibroblasts.

To assess whether Tl1a mediated fibroblast activation in the ileum also requires the microbiome, we quantitated myofibroblasts in Tl1a-Tg mice under GF conditions. In contrast to the cecum, there was an increased proportion of activated myofibroblasts in the ileum of GF Tl1a-Tg mice compared with GF WT mice (Figure 4C-D). We next tested whether the degree of Tl1a-mediated fibroblast activation is affected by the specific microbiome. SPF gavage increased myofibroblast numbers and proportion in both WT and Tl1a-Tg mice while preserving the relative increase in myofibroblasts in Tl1a-Tg mice (Figure 4C-D). In contrast, mice colonized with Hu microbiota had reduced proportion of fibroblast activation compared with GF conditions, suggesting that members of the Hu microbiota may have inhibited fibroblast activation. This did not result in significant histopathological differences in collagen deposition, however. Taken together, these results suggest greater modulation of Tl1a-mediated fibroblast activation by the microbiome in the cecum (without concomitant changes in inflammation), for which Tl1a can potentially compensate in the ileum, (and in which there is significant increases in inflammation). This may reflect distinct microbial communities and mucosal immunity in the ileum, reflected in the

significant increase in ileal inflammation in Tl1a-Tg mice. Indeed, differing microbial composition between the ileum and the cecum has been well-documented in mice and humans, including patients with IBD who show distinct microbiome profiles between subsets with ileal vs. colonic disease.[48] Consistent with this, sequencing of mucosal and luminal microbial communities in the ileum and cecum demonstrated distinct microbial populations in the ileum vs. the cecum in both Tl1a-Tg and WT mice colonized with SPF microbiota (Supplemental Figure 2). These data illustrate biogeographic differences in the host-microbe interactions underlying intestinal collagen deposition. Additionally, our data demonstrated that microbial composition modulates the degree of Tl1a-mediated myofibroblast activation independent of the intestinal location.

Fibrosis severity is associated with the abundance of specific microbes found in the mouse SPF microbiome

Since SPF and Hu microbiota had differential effects on collagen deposition and fibroblast activation, we hypothesized that the abundance of specific bacteria would be associated with the degree of fibrosis seen in recipient mice. To evaluate this, we performed 16S rRNA sequencing to characterize the ileal and cecal microbiome of colonized mice and then employed multivariate models to identify microbes with a statistically significant positive or negative association with fibrosis score. These microbes were then used to construct co-occurrence/co-exclusion networks with fibrosis severity to identify the microbes that were directly associated with increased or decreased fibrosis rather than merely having a co-occurrence or co-exclusion relationship with fibrosis-associated microbes. Separate analyses were performed of the cecum and ileum of humanized and SPF-colonized ex- GF mice (Figure 5). In the cecum of SPF-colonized mice,

which demonstrated significant collagen deposition and fibroblast activation, we identified several microbes not present in Hu-gavaged mice that clustered tightly with fibrosis (Figure 5A). This included groups of mucolytic bacteria such as *Mucispirillum schaedleri* and *Ruminococcus*. Additionally, *Anaeroplasma* were also significantly associated with fibrosis in the cecum of SPF-colonized mice. Members of *Oscillospira* and *Coprococcus* were negatively correlated with fibrosis in the cecum (Figure 5A).

In the ileum of SPF-colonized mice, we observed that there were competing sets of microbes associated with enhanced or reduced fibrosis severity (Figure 5B). For example, members of the *Streptococcus* and *Lactobacillus* genera were found to be positively associated with fibrosis, whereas *Faecalibacterium prausnitzii* and members of *Bacteroides* were negatively associated with fibrosis. Consistent with the absence of histological fibrosis, we observed only negative correlations between microbial species and fibrosis in both the cecum and ileum of Hu-colonized mice (Figure 5C-D).

Differential effects of bacteria positively or negatively correlated with fibrosis on in vitro fibroblast function

Next, we determined whether bacterial strains that positively or negatively correlated with cecal fibrosis severity *in vivo* could alter fibroblast function directly in *in vitro*. Cell lysates of *Ruminoccocus* and *M. schaedleri*, two bacterial strains that were positively correlated with the degree of fibrosis, promoted fibroblast migration and collagen expression compared with negatively-correlated *Oscillospira*, which had comparatively less pronounced effects (Figure 6). These results show that microbes that positively or negatively correlate with fibrosis *in vivo* can

directly and disparately impact fibroblasts *in vitro*. Furthermore, one potential causal mechanism is now suggested by which specific organisms in the gut microbiome mediate fibrosis.

Discussion:

To our knowledge, this is the first study that causally implicates the intestinal microbiome in intestinal fibrosis, demonstrating that fibrosis requires the presence of resident microbiota and that Tl1a-mediated fibrosis is dependent upon specific bacteria or bacterial consortia. Furthermore, we show that microbes that positively or negatively correlate with intestinal fibrosis *in vivo* have direct (and opposing) effects on fibroblast function *in vitro*. These results also suggest that microbiome-TL1A interactions may influence the degree and location of intestinal fibrosis in IBD, which has up to now been attributed to the severity of inflammation. Accordingly, no histologically significant cecal inflammation was observed under SPF microbial conditions but despite this, Tl1a-Tg mice demonstrate increased cecal collagen deposition, underscoring the importance of TL1A as a modulator of the location and severity of mucosal inflammation, as well as a pro-fibrotic mediator that can act independently of its pro-inflammatory effects. Indeed, this disjunction between inflammation and fibrosis is clinically significant. While inflammatory disease may be associated with significant fibrotic change, as increased inflammation perpetuates the cascade of mucosal repair, the frequency of fibrostenosing complications remains significant despite immunosuppressive therapy in CD patients in the form of steroids or immunomodulators.[45] Findings that provide insight into unique pro-fibrotic mediators—whether cytokine- or microbiome-driven (or both)—are highly relevant for clinical disease.

This theme apparent in the histopathological results was mirrored in our results for fibroblast activation, which further underscore the relevance of a pro-fibrotic SPF consortia and Tl1a. Given their expression of Toll-like-receptors, fibroblasts have the capacity to become activated

by bacterial products.[26] Consistent with this, Tl1a-mediated fibroblast activation required the microbiome in the cecum (despite no concomitant changes in inflammation). However, in the ileum, (in which there is significant increase in inflammation), Tl1a could partially compensate for the lack of the microbiome and promote some fibroblast activation. This might point to unique effects of Tl1a on fibroblast activation that are tissue-specific, but may still ultimately require a specific consortium of organisms in SPF to yield histopathologically evident fibrosis, as Tl1a-Tg mice still had reduced ileal fibrosis under GF conditions and with Humanized microbiome compared with conventional SPF microbiota (Figure 1 and 3). The effect of SPF microbiota on ileal fibrosis was not as dramatic as in the cecum. One possible explanation for this effect is the differing microbial communities that colonize the ileum vs. the cecum. We show that fibrosis correlated much more closely with several organisms in the cecum compared with the ileum where there were equal positive and negative "pulls" at fibrosis, with a few positively correlated organisms and numerous negatively correlated organisms. The organisms that are tightly correlated with increased fibrosis in the cecum are less abundant in the ileum. Similarly, the ileum harbors numerous organisms that are negatively correlated with fibrosis compared with the cecum where there are fewer such organisms. A related potential explanation, is the quantity, structure, and type of mucous seen in the small intestine vs. the large intestine. The differing mucin composition may account for observed differences in bacterial composition in different regions of the gut.[49] The mucin-rich large intestine can harbor anaerobic organisms with a repertoire of glycosidic enzymes that disassemble complex mucus glycans to be used as a carbon source.[50] We identified mucin-degrading bacteria *(M. schaedleri*) to correlate with fibrosis in the cecum. The effect of differing microbial compositions in the cecum vs. ileum on fibrosis may be

mediated through bacterial modulation of fibroblast phenotype, since specific bacteria may have direct and opposing effects on fibroblast, as we demonstrated *in vitro*.

Cecal and ileal colonization with SPF microbiota induced intestinal myofibroblasts in *both* WT and Tl1a-Tg mice relative to GF conditions or colonization with Hu microbiota. Notably, however, colonization with SPF microbiota (but not Hu microbiota), *in addition to* Tl1a-overexpression, resulted in overall increased proportion of myofibroblasts. Interestingly, GF Tl1a-Tg mice had *reduced* myofibroblast proportion in the cecum compared to GF WT mice, which was not seen in the presence of Hu microbiota. This raises the question as to the specific contribution of Tl1a-overexpression vs. microbial changes in previously referenced colitigenic models conducted in native microbiome-intact mice.[12, 27] It would, therefore, be important to determine what effects Tl1a-overexpression, independent of any bacterial stimulation, but in the presence of other mucosal stimulation, such as inflammatory insults due to DSS for example, may have on intestinal fibrosis and inflammation; or the effects that differing microbial populations may impart on experimental colitis.

These results also have novel implications for a microbiome effect on fibroblast function in concert with Tl1a. Consistent with the above noted points regarding the disconnect between pro-inflammatory and pro-fibrotic stimuli, colonic fibroblasts do not migrate in response to classic pro-inflammatory cytokines such as TNFα or IL-1, but rather require traditionally "pro-fibrotic" cytokines such as TGFβ to induce migration.[46] It is notable that in our migration assay Tl1a significantly and directly increased fibroblast migration compared with controls, again suggesting a direct contribution of Tl1a to the pro-fibrotic pathway, which may act independently of inflammation. Importantly, our data demonstrate that the gut microbiota can

promote this effect directly in concert with Tl1a. These findings propose novel roles for both Tl1a and SPF microbiota in fibroblast function.

In this study, we utilized a novel correlation of direct changes in fibrosis with specific bacterial abundance in a region-specific manner. In the cecum of SPF-colonized mice, which demonstrated significant collagen deposition and fibroblast activation, we identified several microbes that clustered tightly with fibrosis. This included *Mucispirillum schaedleri*, a mucous degrading organism that has been reported to discriminate between colitis and remission[28] in a mouse model but has not been linked to fibrosis. *Ruminococcus* are another group of mucolytic bacteria that have been observed to be increased in CD in some studies, associated with the stricturing phenotype in a recent pediatric CD study, and contribute to experimental colitis.[22,29,30,44] *Ruminococcus* and *M. schaedleri* were capable of directly modulating fibroblast function *in vitro*. Therefore, further studies demonstrating potential causal efficacy of these correlated organisms *in vivo* are warranted. It would be interesting to assess in future studies whether *M. schaedleri*, or previously identified species of *Ruminococcus*, are present in Tl1a-Tg mice with fibrostenosis under colitic conditions and in CD patients with the high risk *TNFSF15* haplotype and stricturing disease. Furthermore, it would be informative to assess mucus structure, mucosal barrier function, and fibroblast activation in mice and humans with and without these mucous degrading organisms. Lastly, *Anaeroplasma*, a genus which has been previously associated with experimental colitis,[31] was also significantly associated with fibrosis in the cecum of SPF-colonized mice. In terms of organisms that were associated with reduced fibrosis in the cecum, *Coprococcus*, a genus that has been reported to be depleted in patients with CD was associated with reduced fibrosis in both, mice reconstituted with SPF microbiota and human microbiota. [34] *Oscillospira* have been associated with gut health, and their reduced

abundance has been implicated in a variety of diseases including CD.[47] Notably, compared with positively correlated organisms, *Oscillopsira* mitigated fibroblast function *in vitro*.

In the ileum of SPF-colonized mice, we observed that there were competing sets of microbes associated with either enhanced or reduced fibrosis severity. It is worth noting that ileal inflammation may have impacted the microbial variations and associations with fibrosis seen, compared with the cecum which had very tight microbial associations with fibrosis in the context of no significant inflammation. Members of the *Streptococcus* and *Lactobacillus* genera were found to be positively associated with fibrosis in the ileum. This is concordant with recent human data indicating that fecal abundance of these microbes is associated with another fibrotic complication of IBD, primary sclerosing cholangitis.[32] Many organisms that were associated with reduced fibrosis in SPF-colonized mice have previously been observed to be depleted in patients with CD, including *Faecalibacterium prausnitzii*, which has been well-described to have anti-inflammatory properties.[29] Additionally, members of the Lachnospiraceae family, which contains many butyrate producers that are decreased in CD patients,[33] were associated with reduced fibrosis in the ileum of SPF-colonized mice. To our knowledge, this is the first study to link these microbes not just to protection from inflammation, but also fibrotic disease. Thus, it would be important to determine mechanistically how these short-chain fatty acid producers affect intestinal fibrosis in addition to inflammation. One possibility is a direct effect of these microbes (or their products) on fibroblast function, as our data suggest.

Interestingly, our microbiome-fibrosis correlation studies also underscore the disjunction between inflammation and fibrosis noted above. Sulfite-reducing bacteria such as *Bilophila* have been associated with a pro-inflammatory T helper type 1 immune response and an ability to induce experimental colitis.[35] Despite the potential pro-inflammatory effects of such bacteria, we

found that *Bilophila* correlated with a *reduction* in fibrosis in the ileum of SPF-colonized mice. Thus, hydrogen sulfide, one of the metabolic products of these bacteria, may have opposing effects on intestinal inflammation compared with fibrosis.[36,37]

To our knowledge, this is the first study that causally implicates the intestinal microbiome in intestinal fibrosis, demonstrating that Tl1a-mediated fibrosis is dependent upon specific bacteria or bacterial consortia and that those bacteria can directly affect fibroblast function. Thus, a focus on TL1A pathways acting in concert with the microbiome may identify future therapeutic targets for fibrostenosing Crohn's disease.

Materials and Methods:

Gnotobiotic experiments: Tl1a-Tg (which have sustained TL1A expression) and WT mice, both on C57Bl/6 background were re-derived into germ-free status and bred under sterile conditions at the National Gnotobiotic Rodent Resource Center, Chapel Hill, NC. Tl1a-Tg mice and WT littermates at 2-4 months of age were orally gavaged with 200 uL of a 1:10 suspension of stool from either Cedars-Sinai specific pathogen free (SPF) mice or a healthy human donor diluted in pre-reduced phosphate-buffered saline. Mice were euthanized after 2 months of colonization for assessment of intestinal fibrosis and histopathology. Mucosal areas of collagen deposition identified by Picrosirius red-stained gut sections were quantitated for the relative degree of fibrosis using ImageJ software, as previously described.[27] Two animal pathologists scored H&E stained sections in a blinded manner using previously described histopathological scoring system used in GF experiments.[38]

Fibroblast gap-closure assays: Mouse primary colonic fibroblasts were isolated as previously described.[27] Equal numbers of fibroblasts per group ($1x10^5$ cells) were seeded in 8 chamber slides and cultured for 24-48h until a monolayer was formed. A scratch was created with a P200 pipette tip. Cell debris was removed by washing cells with PBS and then cell-culture medium was replaced with time-lapse images taken every 4 hours under an Olympus CK2 microscope at 100x magnification. The area of the gap between the two migrating fronts of the cells was quantified using ImageJ software and relative percent area of gap closed at the indicated time points was calculated as (area t_0 – area t_x)/area t_0. For assays involving supplementation with cecal washings, cecal contents from native SPF WT and GF mice were released by flushing with 1 ml of distilled deionized water, as previously described.[38] The washings were then

homogenized by vortexing, and pelleted by centrifugation. Supernatant were collected, filtered through a 0.22 um filter, and was added directly to the cells after simulated wound, at a 1:20 dilution (5% volume). In the indicated assays, mouse recombinant Tl1a (R&D Systems, Minneapolis, MN) was added at a concentration 100 ng/ml for 4 hours prior to simulated wound and then maintained during the indicated migration period. For assays involving the addition of bacterial lysates, *Oscillospira sp., Mucispirillum schaedleri* and *Ruminococcus gnavus* were cultured anaerobically on chocolate blood agar. Fresh bacterial colonies were resuspended in sterile PBS and lysed. After simulated wound, 25 µg/ml lysate was added to the culture chamber, as describe previously.[35] Cell migration was assessed after 16 h of incubation.

For fibroblast adhesion assays, an equal number of cells were seeded into 24-well plates and allowed to settle for either 20 or 80 minutes, after which the wells were washed twice with PBS to remove non-adherent cells. Adherent cells were counted for 5 visual fields/well (representing four quadrants and the center of the well) at 200x magnification, then averaged. The average number of adherent cells per visual field is then displayed for each well.

Histological myofibroblast quantification: Fibroblast and myofibroblasts were quantified by anti-vimentin and anti-α-Smooth Muscle Actin immunofluorescence-stained OCT tissue sections. 4 µm frozen sections were fixed with 10% formalin, blocked in 10% BSA, 0.1% Triton X-100 TBST, and stained overnight at 4 degree C with primary antibodies: rabbit polyclonal anti-αSMA Ab (Abcam, Cambridge, MA) at 1:100 dilution and chicken polyclonal anti-Vimentin Ab (Abcam, Cambridge, MA) at 1:2000 dilution. Secondary antibody at 1:500 dilution was added for 2 hours at room temperature with donkey anti-rabbit IgG-Alexa-fluor-647 and goat anti-chicken IgY- DyLight 488 (Abcam, Cambridge, MA). Images were captured with Leica TCS spectral microscope. Total numbers and percentage of myofibroblasts (that co-localize

fluorescence) over total vimentin-positive cells per HPF in ileum or cecum were quantitated by two independent investigators.

Quantitative real-time PCR analysis: Total RNA was isolated from cultured fibroblasts using Qiagen RNeasy Micro Kit according to the manufacturer's protocol. Two-hundred and fifty nanograms of total RNA was used in each RT reaction, with oligo(dT) as primer, using the Omniscript kit and protocol (Qiagen). Collagen 1a2 and β-actin transcripts were amplified by quantitative real-time RT-PCR with TaqMan probes and primers (ThermoFisher Scientific, Waltham MA, USA). PCR was done on 1/4 the RT reaction in duplicate as follows: 50°C for 2 min, 95°C for 2 min, then 45 cycles at 95°C for 15 s, and 60°C for 1 min. Assays were performed following the predeveloped TaqMan assay reagents protocol for Platinum qPCR mix (Invitrogen Life Technologies) in a Mastercycler Ep realplex^2 (Eppendorf). The Mastercycler System Interface was used to analyze samples. Duplicates differing by less than one cycle were averaged and amount of transcript was analyzed. Replicate Ct values were normalized to replicate reference gene (β-actin) Ct values (ΔCt), and relative expression was calculated with respect to the indicated reference sample (ΔΔCt), expressed as percentage of β-actin.

Microbial correlation with fibrosis: Cecal and ileal luminal content was released by flushing with distilled deionized water then the mucosa-associated bacteria were released by DTT treatment according to our published protocol.[38] DNA extraction and sequencing of the 16S ribosomal RNA gene was then performed for luminal and mucosal samples as previously described.[39] In brief, bacterial DNA was extracted using the MO BIO Powersoil kit with bead beating. The V4 region of the 16S gene was amplified and barcoded using 515f/806r primers then 150x2 bp sequencing was performed on an Illumina HiSeq 2500. Raw data was processed in QIIME 1.9.1 and 97% operational taxonomic units (OTUs) were identified by closed

reference OTU picking against the Greengenes database.[40] OTUs associated with fibrosis were identified using DESeq2, an algorithm that employs multivariate negative binomial models to identify differentially abundant features.[41] Separate analyses were performed of the cecum and ileum of humanized and SPF colonized ex-GF mice. The models included fibrosis score by Sirius Red staining and sample type, sex, and TL1a genotype as covariates. Results were adjusted for multiple hypothesis testing.[42] OTUs with adjusted p-values <0.05 were inputted into CoNet along with fibrosis scores to generate co-occurrence networks.[43] This analysis involved four metrics (Spearman correlation, Bray Curtis dissimilarity, Jensen Shannon dissimilarity, scaled variance of log ratios) from which a merged p-value was obtained by Fisher's method and corrected with the Benjamini-Hochberg method. The resulting network was visualized in Cytoscape 3.2.1 (http://cytoscape.org) with an edge-weighted, spring-embedded layout.

Statistics: Data are presented as dot plots and means with group differences tested using standard methods depending on variables measured: Student's t-test for comparisons between two groups or Mann Whitney test for comparisons between two groups requiring non-parametric testing. When indicated, 1-way Analysis of Variance (ANOVA) with Tukey's honestly significant difference (HSD) test for multiple comparisons was used. In all settings, a P value of less than 0.05 indicated a statistically significant difference in the parameter being compared. Additional statistical methods were used for microbial correlations with fibrosis, as described.

Study approval: This study was carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Animal studies were approved by the CSMC Animal Care and Use Committee, under IACUC protocol 4942.

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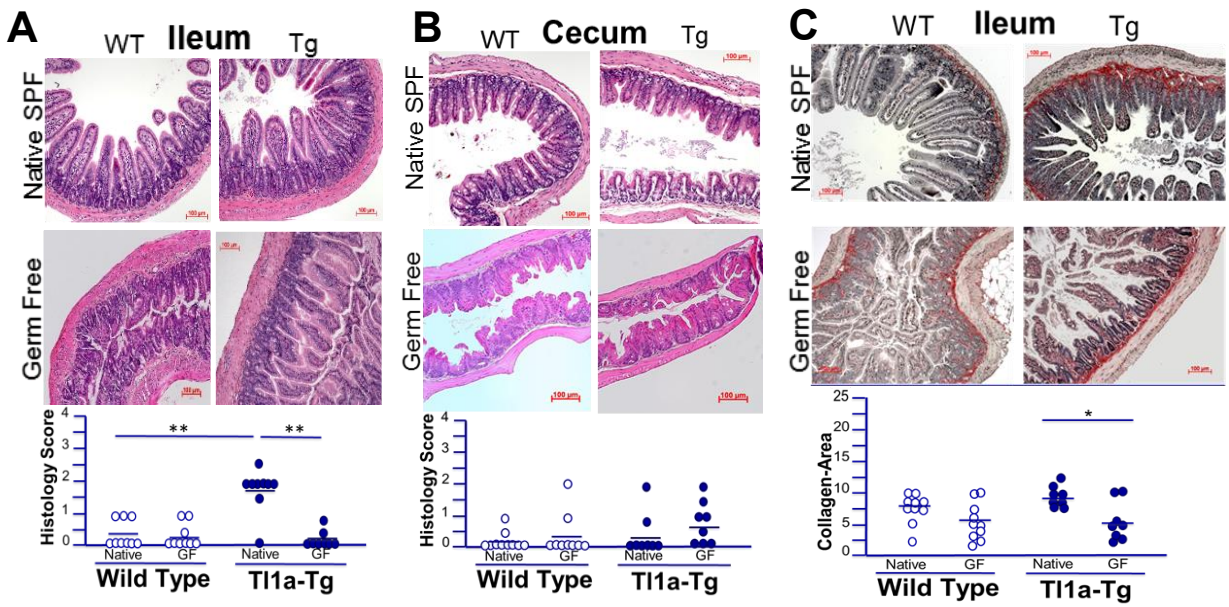

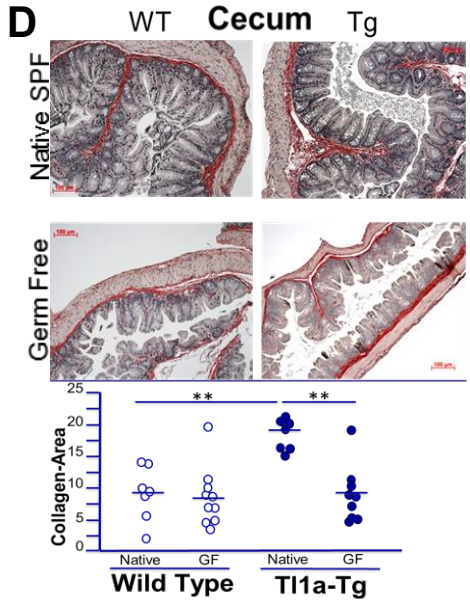

Figure 1. *The intestinal microbiome is required for Tl1a-mediated location and degree of intestinal inflammation and collagen deposition*: A-B) Representative H&E stains of ileal and cecal sections of 5-month-old WT and Tl1a-Tg mice, raised in germ free and native SPF conditions. C-D) Representative Sirius red staining of collagen deposition in ileal and cecal sections of 5-month-old WT and Tl1a-Tg mice, raised in GF and native SPF conditions. Dot plots with means displayed; n = 7-10 mice per group. * indicates $p<0.05$; ** $p<0.01$.

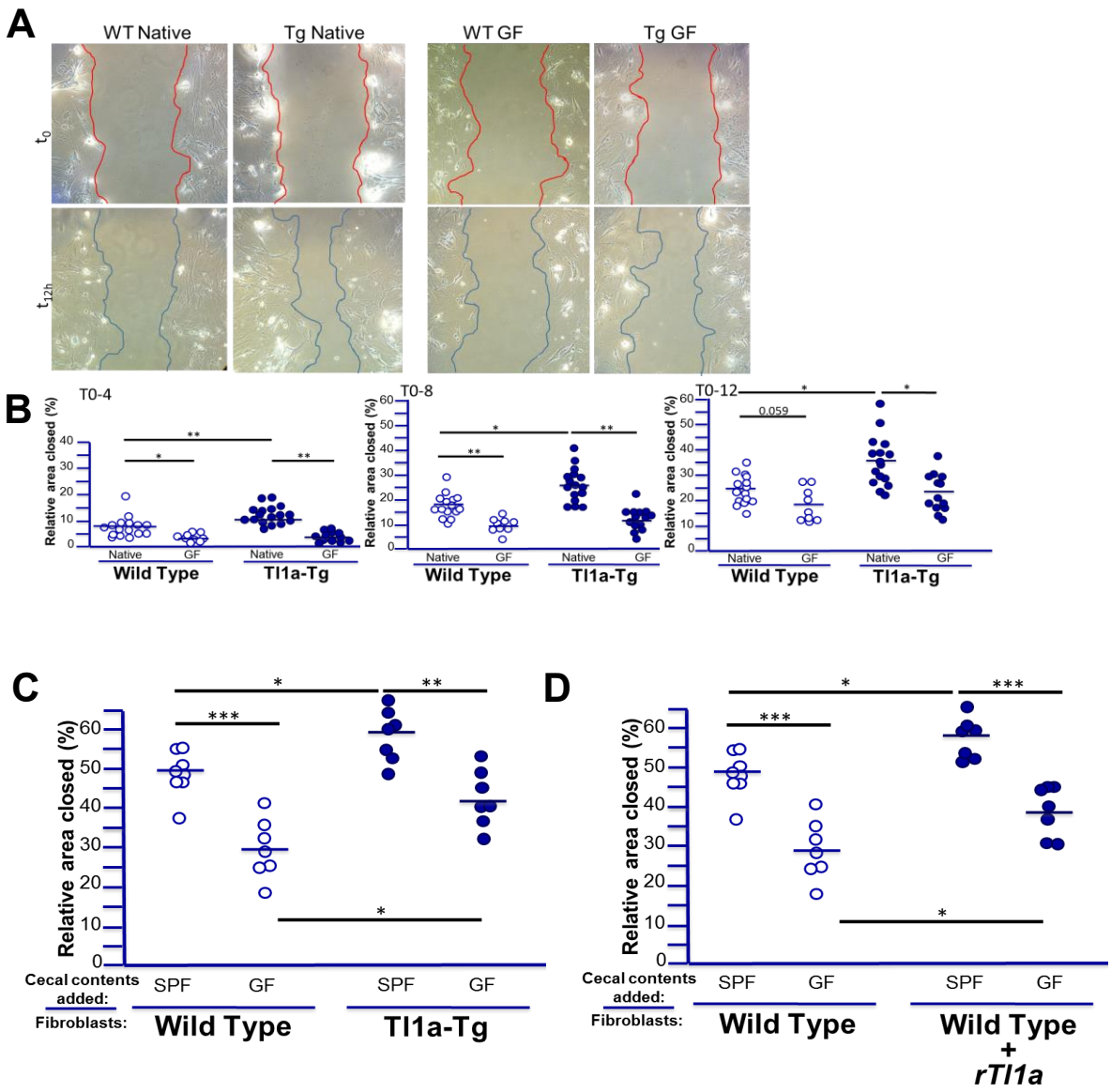

Figure 2. *The intestinal microbiome is required for Tl1a-enhanced fibroblast migration*: A) Representative images of gap-closure assay after simulated wound shown for WT and Tl1a-Tg mice under native (left) and GF conditions (right) at initial wound (upper panels) and after migration (lower panel). B) Relative % area of gap closed indicated for fibroblasts isolated from WT and Tl1a-Tg mice raised under native SPF or GF conditions with 4, 8, and 12 hours of migration after initial wound. C) Relative % area of gap closed indicated for fibroblasts isolated from WT and Tl1a-Tg mice raised under native SPF conditions and supplemented with cecal washings from either native SPF or GF WT mice during 16 hours of migration after initial wound. D) Relative % area of gap closed indicated for fibroblasts isolated from native SPF WT mice and supplemented with cecal washings as in (C), or with either of the cecal washings and recombinant mouse Tl1a (100ng/ml). Data points represent fibroblast isolation from individual mice and bars represent means. * indicates $p<0.05$, ** $p<0.01$, *** $p<0.005$.

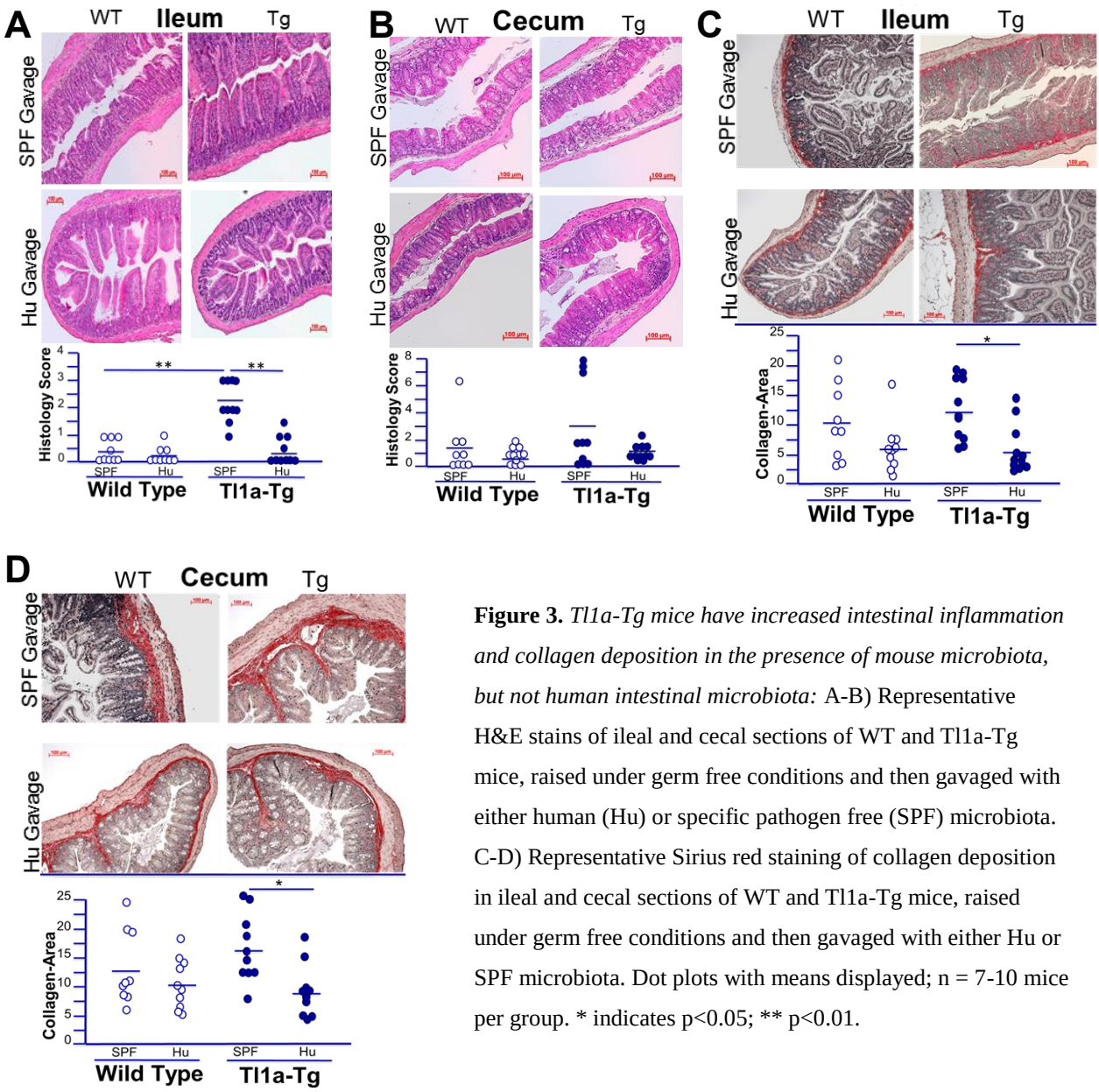

Figure 3. *Tl1a-Tg mice have increased intestinal inflammation and collagen deposition in the presence of mouse microbiota, but not human intestinal microbiota:* A-B) Representative H&E stains of ileal and cecal sections of WT and Tl1a-Tg mice, raised under germ free conditions and then gavaged with either human (Hu) or specific pathogen free (SPF) microbiota. C-D) Representative Sirius red staining of collagen deposition in ileal and cecal sections of WT and Tl1a-Tg mice, raised under germ free conditions and then gavaged with either Hu or SPF microbiota. Dot plots with means displayed; n = 7-10 mice per group. * indicates $p<0.05$; ** $p<0.01$.

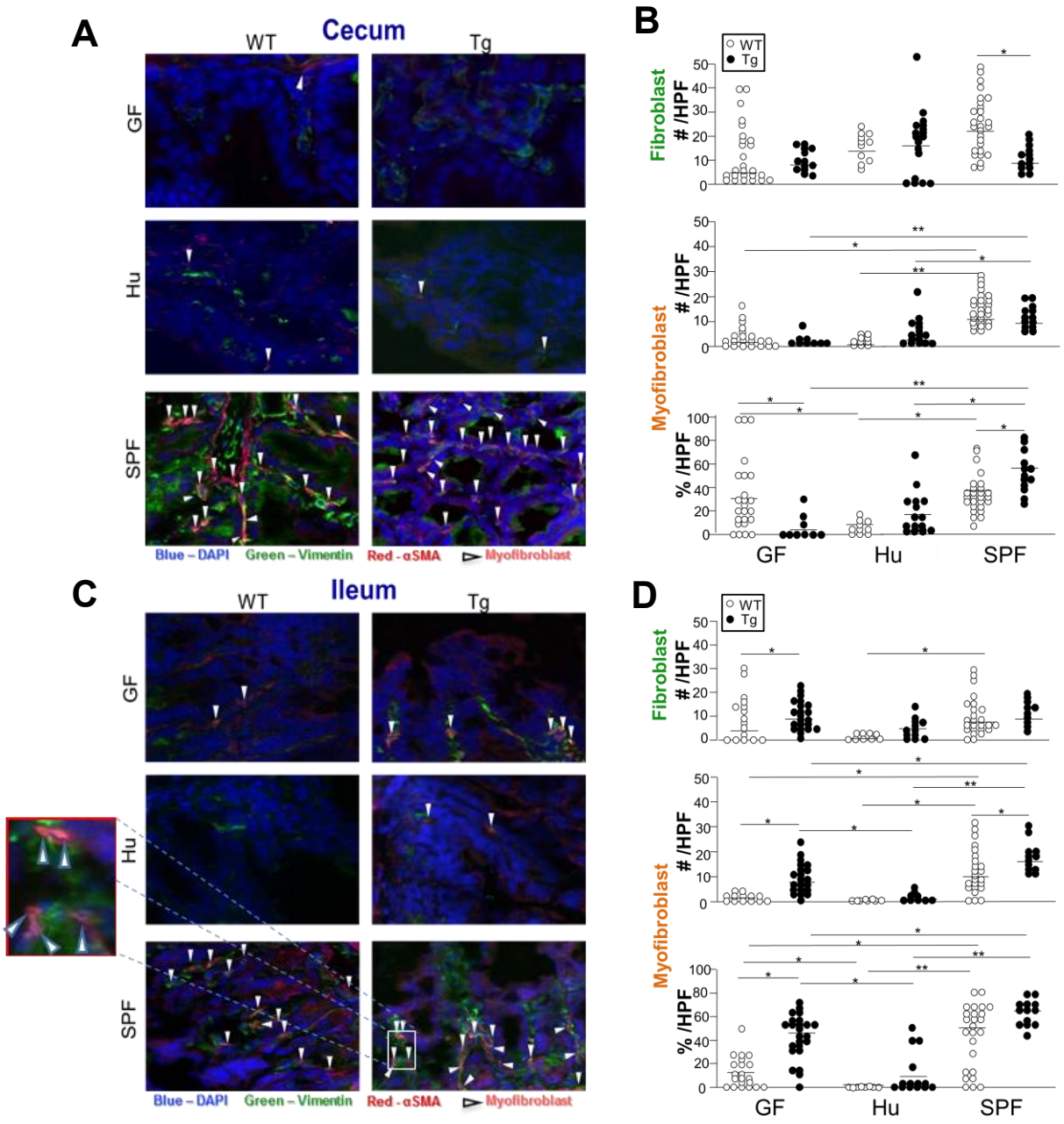

Figure 4. *Tl1a-Tg mice have increased fibroblast activation in the presence of mouse microbiota, but not human intestinal microbiota*: Immunofluorescent staining of vimentin (green) and αSMA (red) from cecal (A) and ileal (C) sections of 5-month-old WT and Tl1a-Tg mice raised in germ free conditions and then gavaged with either Hu or SPF microbiota. White arrows denote myofibroblasts that co-localize vimentin and αSMA at 400x magnification. Total numbers and percentage of myofibroblasts over total vimentin-positive cells per individual HPF in cecum (B) or ileum (D) were quantitated and displayed as dot plots with means. Pooled data of total individual HPFs, representative of 4-6 mice per group. * indicates $p<0.05$; ** $p<0.01$.

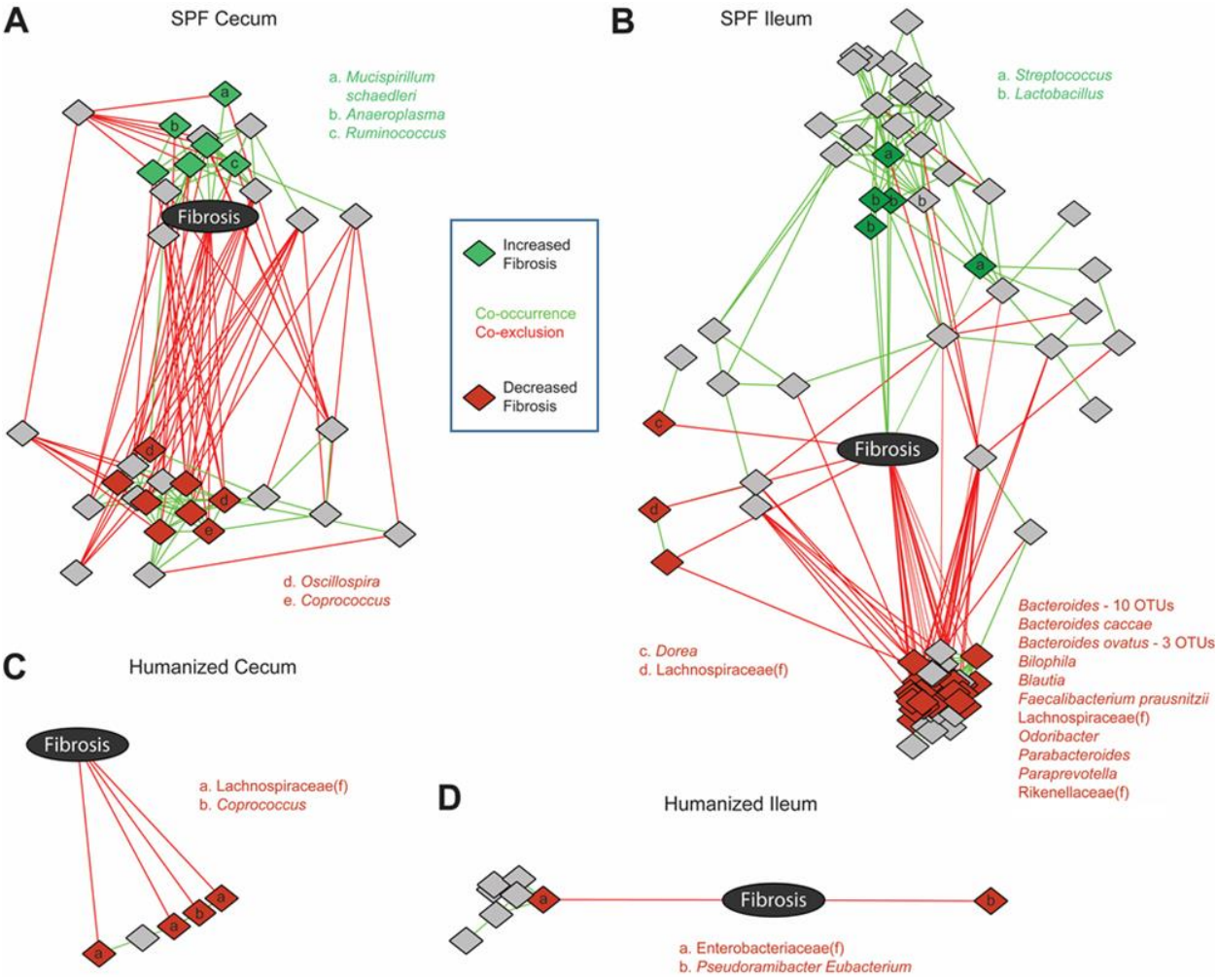

Figure 5. *Microbial networks associated with cecal and ileal fibrosis in germ-free mice colonized with SPF or Hu microbiota.* A) Microbes associated with fibrosis in DESeq2 models were used to construct co-occurrence/co-exclusion networks with fibrosis score. Networks were constructed in Cytoscape with an edge-weighted, spring-embedded layout, which groups nodes with highly interconnected nodes. Diamonds represent OTUs, colored by association with fibrosis: green (associated with increased fibrosis), red (associated with reduced fibrosis), gray (indirect association only). Letters indicate the genus of fibrosis-associated OTUs; unmarked OTUs are unclassified members of the *Clostridiales* order. Similar network plots were constructed for fibrosis in the ileum of SPF colonized mice (B), cecum of Hu mice (C), and ileum of Hu mice (D). In (B), the names and numbers of OTUs associated with reduced fibrosis are shown next to a dense cluster in which it is difficult to discern individual OTUs. OTUs that are unclassified members of microbial families are indicated with (f).

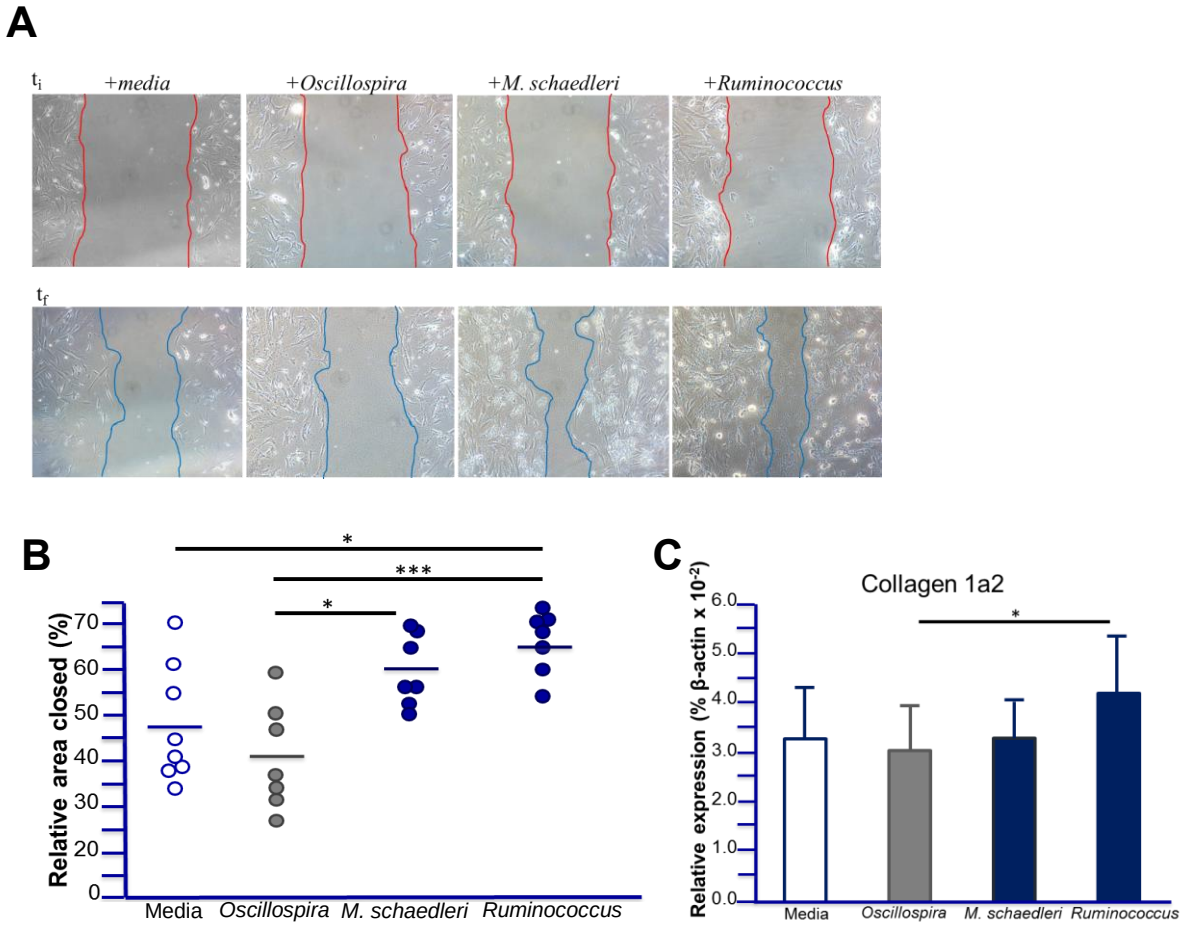

Figure 6. *Differential effects of bacteria positively or negatively correlated with fibrosis on fibroblast migration and collagen expression.* A) Representative images of gap-closure assay after simulated wound on fibroblasts isolated from native WT mice at initial wound (upper panels) and after migration (lower panel) in the presence of bacterial lysates. B) Relative % area of gap closed for fibroblasts isolated from native WT mice and supplemented with indicated bacterial lysates (25 μg/ml) during migration. Data points represent fibroblast isolation from individual mice and bars represent means. C) Relative expression of collagen from fibroblasts in (B) after migration in the presence of the specified bacterial lysates. Data are represented as means ± SD; * indicates $p<0.05$, ** $p<0.01$, *** $p<0.005$.

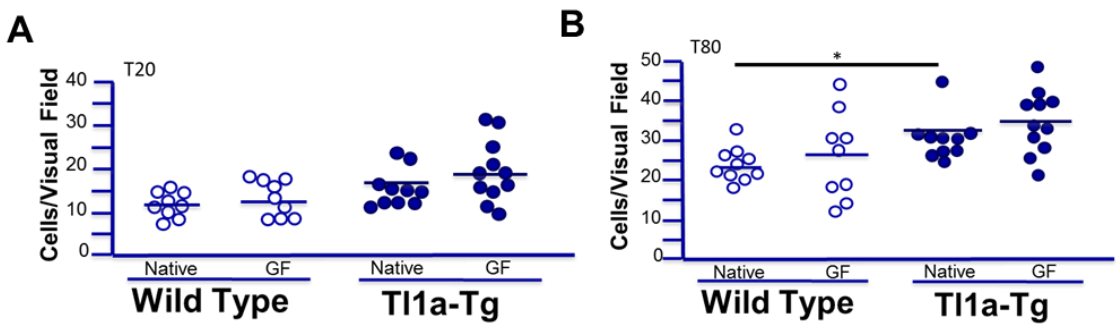

Figure 7 [Supplemental Figure 1]. *Fibroblast adhesion under native and germ-free conditions.* Representative assays at 20 minutes (A) and 80 minutes (B) for fibroblasts isolated from 5-month-old WT and Tl1a-Tg mice, raised in GF and native SPF conditions. Dot plots with means displayed; representative of 3 independent experiments of 3-4 assays each; * indicates $p<0.05$.

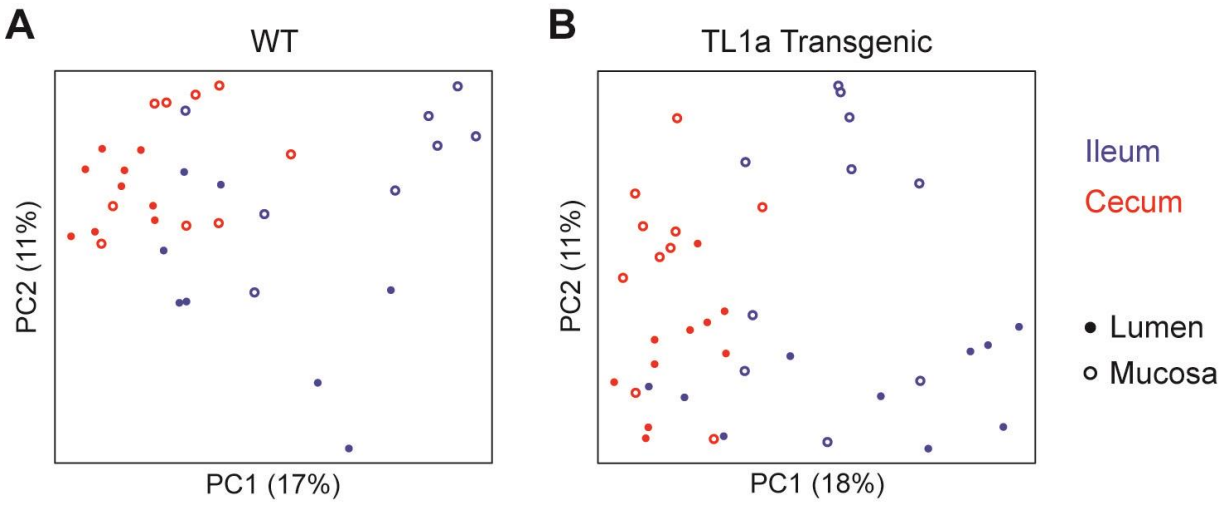

Figure 8 [Supplemental Figure 2]. *Distinct microbiota in the ileum and cecum of WT and Tl1a-Tg mice colonized with SPF microbiota.* Differences in microbial composition across samples are represented on principal coordinates plots using unweighted UniFrac distances. Separate analyses were performed for WT (A) and Tl1aTg (B) recipients. Samples are colored by region sampled (ileum vs. cecum), with fill representing sample type (lumen or mucosa). Microbial differences by region were highly statistically significant ($p < 10^{-5}$ for both WT and Tl1a-Tg mice) by multivariate Adonis after adjusting for sample type.

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Chapter 3:

Direct Signaling of TL1A-DR3 on Fibroblasts Induces Intestinal Fibrosis *In Vivo*

Introduction:

TL1A (a protein encoded by *TNFSF15*), a member of the TNF superfamily, modulates numerous cellular functions by binding to death domain receptor 3 (DR3, also known as TNFRSF25), which is expressed on a broad array of cells[1-3]. TL1A is produced by endothelial cells induced by IL-1β and TNFα, macrophages and dendritic cells in response to Toll-like receptor stimulation, as well as in lymphoid lineage cells[4-7].

Developmental, immunoregulatory and pro-inflammatory effects have been described for DR3, which shares homology to TNFR1. Early work on DR3-deficient mice demonstrated that it is required for negative selection in the thymus, and in embryonic cells it can induce FADD- and caspase-8-dependent apoptosis[8,9]. Conversely, however, DR3 activation of NF-KB in human cell lines upregulates c-IAP2, an NF-KB-dependent anti-apoptotic protein, which protects against apoptosis[10]. DR3 can also be upregulated on Th17 cells, promote T cell expansion and cytokine production during immune responses[11-13].

Pertaining to IBD, genetic studies found that TNFSF15 haplotypes are associated with higher TL1A expression, increased risk of CD, intestinal fibrostenosis, and greater need for surgery[14-16]. Consistent with these findings, TL1A overexpression in mice causes spontaneous ileitis with increased collagen deposition.[17, 18] Under colitogenic conditions induced by chronic DSS treatment or adoptive T cell transfer, increased inflammation, fibrosis, and fibrostenotic lesions in the gut are seen in TL1A-overexpressing mice[19]. Administration of anti-TL1A monoclonal antibodies inhibits DSS-induced colonic inflammation in mice, as TL1A synergizes with IL-12 or IL-23 to induce both IFN-γ and IL-17 secretion in CD4+ T cells derived from gut-associated lymphoid tissue[20]. These results support the role of TL1A in induction of intestinal inflammation

and suggest its contribution to fibrogenesis in the gut. The potential for TL1A as a therapeutic target in intestinal fibrosis was demonstrated in a study evaluating the effect of anti-TL1A Ab in chronic DSS and adoptive T-cell transfer models of IBD. Treatment with neutralizing TL1A Ab attenuated disease and reversed colonic fibrosis. Notably, TL1A blockade reduced the number of fibroblasts and myofibroblasts in colonic cell isolates and lowered expression of CTGF, TGFβ1 and IGF-1[21]. The pro-inflammatory effects of TL1A-DR3 may partly contribute to this pathway's effect on fibrosis, but more direct evidence has shown that DR3 is an important receptor for fibroblast development, maturation and function. DR3-deficient mice display reduced number of colonic fibroblasts and reduced fibroblast activation (as evidenced by decreased expression of alpha smooth muscle actin) [21]. *In vitro*, TL1A-DR3 signaling was shown to have direct effects on fibroblasts by inducing expression of alpha smooth muscle actin and collagen,[21] as well as fibroblast migration.

An important mechanistic question arising from the results of pan-DR3-deficient mice is the contribution of direct TL1A-DR3 signaling on fibroblasts *in vivo*. Specifically, it has not been determined if the reductions in fibroblast activation due to pan-DR3-dificiency are due to absence of direct TL1A-DR3 signaling on fibroblasts, rather than due to that on other cells such as regulatory T cells (Tregs), which can support fibroblast activation via production of TGFβ. Indeed, it has been previously shown that DR3-deficient mice harbor reduced numbers of Tregs and DR3 stimulation *in vivo* can induce selective expansion of Tregs.[22,23] Accordingly, abrogation of DR3 signaling on T cells (in pan-DR3-deficient mice) may partly account for the reduction in myofibroblasts noted previously.[21] Thus, the extent of *in vivo* fibroblast activation and fibrosis due to direct TL1A-DR3 activation of fibroblasts had yet be determined. We hypothesized that direct TL1A-DR3 signaling on fibroblasts plays a significant role in a TL1A

specific fibrogenic pathway responsible for fibroblast activation and subsequent intestinal fibrosis *in vivo.* Using the adoptive T cell transfer model, and to obviate the effects of DR3-deficiency on T cells noted above, transfers of DR3-intact, TL1A over-expressing naïve T cells (Tl1a-Tg) were used to induce severe intestinal inflammation and fibrosis in *Rag*$^{-/-}$ mice (as previously reported); *Rag*$^{-/-}$ mice lacking DR3 in all cell types (*Rag*$^{-/-}$*Dr3*$^{-/-}$); or *Rag*$^{-/-}$ mice lacking DR3 only on fibroblasts (*Rag*$^{-/-}$*Dr3*$^{\Delta Col1a2}$). We show that *Rag*$^{-/-}$*Dr3*$^{-/-}$ recipients demonstrated reduced disease activity, inflammation, and an accompanying reduction in fibrosis and fibroblast activation compared with DR3-sufficient *Rag*$^{-/-}$ recipients. In contrast to pan-DR3-deficient recipients, *Rag*$^{-/-}$*Dr3*$^{\Delta Col1a2}$ recipients exhibited a similar degree of clinical disease and inflammation as *Rag*$^{-/-}$ recipients. Despite the presence of abundant inflammation, however, fibroblast-selective DR3-deficiency significantly reduced intestinal fibrosis and decreased activation of fibroblasts. *Ex vivo*, DR3-deficient fibroblasts isolated from these colitic mice exhibited a significant reduction in gap-closure compared to those from DR3-intact *Rag*$^{-/-}$ mice. These data demonstrate that direct TL1A-DR3 signaling on fibroblasts *in vivo* significantly contributes to TL1A-mediated intestinal fibrosis, independent of inflammation. Moreover, in the context of TL1A-mediated fibrosis, loss of this pathway in fibroblasts results in reduced activation and migration. These results may have profound ramifications for clinical fibrosis and underscore the importance of TL1A as a fibrotic mediator that can act independently of its pro-inflammatory effects.

Results:

Host DR3-deficiency results in reduced disease activity, colonic gross pathology, and histological inflammation

As TL1A overexpression promotes inflammation, both spontaneously and exaggerated under colitogenic conditions via adoptive T cell transfer, we sought to determine the effects of abrogating TL1A-DR3 signaling in recipient hosts. Consistent with previous studies, adoptive transfer of Tl1a-Tg naïve T cells into *Rag*$^{-/-}$ recipients resulted in significant weight-loss beginning at week 3 after transfer. This weight-loss was accompanied by development of loose and bloody stools, and reflected in a high disease activity index (DAI) (Figure 1). Compared with *Rag*$^{-/-}$ recipients, *Rag*$^{-/-}$*Dr3*$^{-/-}$ recipients displayed decreased severity in these disease parameters, with a reduction in initial weight-loss and significantly less loose stools, stool blood and DAI by week 6 after transfer (Figure 1).

The reduction in clinical disease activity imparted by pan-DR3-deficient hosts was mirrored in their gross- and histopathology scores. *Rag*$^{-/-}$ recipients developed notable colonic edema, hyperemia, and colonic shortening in the setting of TL1A-mediated colitis. This gross pathology was most pronounced in the cecum. In contrast, *Rag*$^{-/-}$*Dr3*$^{-/-}$ recipients had notably reduced gross pathology and a trend towards less colonic shortening (Figure 2A).

As the cecum was most involved on gross pathology, we next evaluated microscopic histopathological inflammation by H&E. *Rag*$^{-/-}$ recipients developed severe mononuclear cell infiltrates extending into the submucosa and transmurally, along with widespread crypt architectural destruction. *Rag*$^{-/-}$*Dr3*$^{-/-}$ recipients developed a more moderate degree of cellular

infiltrates and, although crypt architectural distortion was noted, it was not as extensive as in DR3-intact *Rag*$^{-/-}$ recipients (Figure 2B). These data demonstrate that in the context of TL1A-mediated exacerbated colitis, host DR3-deficiency mitigates clinical and histopathological disease elements.

Fibroblast selective DR3-deficiency results in severe disease activity, colonic edema and hyperemia, and cecal histopathologic inflammation similar to DR3-intact recipients.

To evaluate the direct effects of TL1A-DR3 signaling on fibroblasts *in vivo* in the context of experimental IBD, we generated *Rag*$^{-/-}$ mice with selective DR3-deficiency on fibroblasts (*Rag*$^{-/-}$ *Dr3*$^{\Delta Col1a2}$) and induced TL1A-exacerbated colitis, as above. We tested the hypothesis that in virtue of selective DR3 deficiency on fibroblasts, but otherwise intact on the other cell types, colitis would progress as in DR3-intact *Rag*$^{-/-}$ recipients. Indeed, *Rag*$^{-/-}$*Dr3*$^{\Delta Col1a2}$ recipients of TL1A-Tg T cells developed as severe clinical disease activity as *Rag*$^{-/-}$ recipients; and like *Rag*$^{-/-}$ recipients, demonstrated relative heightened severity of disease compared with pan-DR3-deficient recipients (Figure 1). In contrast to host pan-DR3-deficiency, selective DR3-deficiency did not modulate colonic gross pathology, as *Rag*$^{-/-}$*Dr3*$^{\Delta Col1a2}$ recipients displayed marked colonic edema and hyperemia, also most notably in the cecum (Figure 2A). As in *Rag*$^{-/-}$ recipients, the gross pathology observed in *Rag*$^{-/-}$*Dr3*$^{\Delta Col1a2}$ recipients was significantly more severe than in *Rag*$^{-/-}$*Dr3*$^{-/-}$ recipients, and the intensity of cecal gross pathology reflected an underlying histopathology replete with inflammatory cell infiltration and crypt destruction (Figure 2B).

Reduction in cecal collagen deposition accompanies the reduction in histopathological inflammation in pan-DR3-deficient recipients

While host DR3-deficiency improved elements of disease activity and inflammation, a reduction in inflammation does not necessarily equate with a reduction in fibrosis, as has been observed in many fibrostenosing CD patients treated with steroids or immunomodulators.[24,25] However, since TL1A can potentially mediate fibrosis independently of its pro-inflammatory effects, and TL1A antagonism reduces fibrosis experimentally, we hypothesized that abrogation of TL1A-DR3 signaling in host cells would result in a reduction in mucosal collagen deposition along with the attendant improvement in inflammation noted above. We confirmed that host pan-DR3-deficiency significantly reduces mucosal collagen deposition as evidenced by an average total mucosal area of 4.9%±1.3 Sirius Red (SR) in the cecums of *Rag*$^{-/-}$*Dr3*$^{-/-}$ recipients compared with 8.3%±2.5 in DR3-sufficient *Rag*$^{-/-}$ recipients; $p<0.01$ (Figure 3).

Fibroblast selective DR3-deficiency attenuates cecal mucosal collagen deposition despite significant histopathological inflammation

While pan-DR3-deficient hosts developed less severe inflammation and fibrosis, we asked if the effects of TL1A-mediated inflammation and fibrosis could segregate at the level of DR3 signaling on fibroblasts. We thus evaluated mucosal collagen deposition in *Rag*$^{-/-}$*Dr3*$^{\Delta Col1a2}$ recipients, which developed severe inflammatory colitis. Compared with *Rag*$^{-/-}$ recipients, *Rag*$^{-/-}$ *Dr3*$^{\Delta Col1a2}$ recipients displayed significantly less collagen deposition in the cecum, with 5.9%±1.7 total mucosal area of SR compared with 8.3%±2.5 in *Rag*$^{-/-}$ recipients (Figure 3). This was not significantly different from pan-DR3-deficient recipients. These data underscore the importance

of TL1A as a fibrotic mediator that can act independently of its pro-inflammatory effects and demonstrate that TL1A may have divergent effects depending on cell-type. Thus, abrogation of direct TL1A signaling on fibroblasts significantly reduces fibrosis despite pronounced inflammation resulting from TL1A overexpression and signaling on other cell-types. These results have the potential to modify prevailing hypotheses concerning the role of TL1A in IBD, which have emphasized inflammation over fibrosis.

Fibroblast-selective DR3-deficiency reduces mucosal fibroblast activation

While TL1A induces, and pan-DR3-deficiency reduces, fibroblast activation *in vitro* and *in vivo*, the effects of TL1A-overexpression or DR3-deficiency on fibroblast activation in the context of experimental IBD has not be established. Moreover, it has not been determined if the reductions in fibroblast activation *in vivo* due to pan-DR3-dificiency are due to absence of direct TL1A-DR3 signaling specifically on fibroblasts, rather than due to that on other cell types that promote fibroblast activation, as mentioned earlier. To address these issues, we examined fibroblast activation in the context of fibroblast-selective DR3-deficiency under colitogenic conditions in the adoptive T cell transfer model. Consistent with previous results, pan-DR3-deficient hosts demonstrated reduced total numbers of myofibroblasts and percentage of fibroblast activation in cecal sections from colitic mice (Figure 4). There was also a trend towards reduction in overall fibroblast numbers. Notably, abrogation of TL1A-DR3 signaling selectively on fibroblasts also resulted in a significant reduction in myofibroblast number and relative fibroblast activation, while maintaining total fibroblast numbers. This suggests that direct TL1A-DR3 signaling on fibroblasts during colitis significantly contributes to fibroblast activation into myofibroblasts.

Fibroblast-selective DR3-deficiency attenuates fibroblast migration ex vivo

As selective DR3-deficiency reduced fibroblast activation *in vivo* despite the presence of significant inflammation, we sought to evaluate additional functional phenotypes of colonic fibroblasts isolated from these colitic mice *ex vivo.* The ability of effector cells to migrate to and remain in sites of disease activity has become increasingly relevant for therapeutic targeting. Adhesion and migration of fibroblasts have been used as markers of functional responses in these cells and may be relevant to their production and maintenance of fibrotic processes. We observed no differences in fibroblast adhesion after 20 minutes or 80 minutes for cells obtained from either *Rag*$^{-/-}$*Dr3*$^{-/-}$ or *Rag*$^{-/-}$*Dr3*$^{\Delta Col1a2}$ adoptive transfer recipients compared with *Rag*$^{-/-}$ recipients (Figure 5A).

While TL1A has been shown to enhance WT fibroblast wound closure *in vitro,* other "inflammatory" cytokines released during colitis may partly diminish fibroblast migration.[26] Thus, it was important to evaluate the migratory capacity of fibroblasts in response to TL1A-exacerbated colitis both in the context of severe inflammation seen in *Rag*$^{-/-}$*Dr3*$^{\Delta Col1a2}$ recipients, as well as during relatively reduced inflammation seen in *Rag*$^{-/-}$*Dr3*$^{-/-}$ recipients. Fibroblasts isolated *ex vivo* from colitic *Rag*$^{-/-}$*Dr3*$^{-/-}$ or *Rag*$^{-/-}$*Dr3*$^{\Delta Col1a2}$ recipients demonstrated significantly reduced migration 8 hours after simulated wound compared with DR3-intact fibroblasts isolated from colitic *Rag*$^{-/-}$ recipients (Figure 5B). These data suggest that TL1A-DR3 activation of fibroblasts *in vivo* promotes functional responses during varying states of inflammation.

Discussion:

Fibrosis often accompanies many chronic inflammatory diseases, but mechanisms responsible for this process and potentially distinct pathways independent of inflammation are warranted. The frequency of fibrosing Crohn's disease is significant, with approximately 40% of patients developing clinically apparent strictures throughout their lifetime.[27] Despite anti-inflammatory therapy in the form of steroids or immunomodulators, the frequency of fibrostenosing complications has remained significant.[24,25] Studies evaluating targetable pathways that broach both inflammation and fibrosis are therefore acutely relevant. We reveal a new role for TL1A-DR3 signaling contributing to intestinal fibrosis distinct from its previously reported role in enhancing inflammatory responses. We provide novel insight into effects of TL1A on intestinal fibroblasts, demonstrating that TL1A functions directly at the level of DR3 signaling on fibroblasts by promoting activation, migration, and collagen production (summarized in Table 1).

Table 1: Summary of in vivo (adoptive transfer colitis) and ex vivo (gap closure) experiments

Recipients	In vivo					Ex vivo
	DAI	Gross Pathology	Intestinal Inflammation	Intestinal Fibrosis	Fibroblast Activation	Fibroblast Migration
***Rag*$^{-/-}$**	++++	++++	++++	++++	++++	++++
***Rag*$^{-/-}$*Dr3*$^{-/-}$**	++	++	++	+	+	++
***Rag*$^{-/-}$*Dr3*$^{\Delta Col1a2}$**	++++	++++	++++	++	++	++

+ indicates activity of parameter measured, ranging from +: mild activity, to ++++: severe activity

These data are consistent with recent reports of DR3-deficiency reducing intestinal inflammation in experimental IBD.[28] We further demonstrate that reduction in inflammation in pan-DR3 deficient recipients was accompanied by a reduction in intestinal fibrosis, an important feature of experimental IBD that has not yet been established due to host-DR3-deficiency. In this context, TL1A-DR3 signaling could potentially contribute to intestinal inflammation and fibrosis in both direct and indirect manners. DR3 was found to be expressed by Th2 cells and innate lymphoid

cells (ILC), and was shown to enhance expansion of these cells or their ability to secrete Th2 and other pro-inflammatory cytokines. In these reports, intestinal pathology was shown to result from IL-13 produced by ILC2s upon TL1A stimulation, as well as IL-22 from ILC3s.[29,30] As the T cells transferred into pan-DR3-deficient mice were DR3-intact, this raises the possibility that part of the reduction in inflammation in *Rag*$^{-/-}$*Dr3*$^{-/-}$ recipients may be due to deletion of DR3 signaling on non-T cells such as ILCs, which have further been implicated recently in inflammatory models, including experimental IBD.[28,31] Moreover, the relative amount of TL1A expression in these Tl1a-Tg cells has been shown to affect their pathogenicity *in vivo*. As these transferred T cells overexpress significant amounts of TL1A, they have a more pronounced inflammatory, as opposed to regulatory, phenotype.[32] Adoptive transfer of DR3-deficient T cells would be useful to evaluate the contribution of DR3 signaling on T cells to intestinal inflammation and fibrosis *in vivo*, as TL1A-DR3 signaling on T cells can promote both inflammatory and regulatory functions. Definitive evidence of the role of DR3 expression on Tregs in mitigating intestinal inflammation or promoting intestinal fibrosis and its mechanism of action awaits the generation and study of mice with selective deficiency of DR3 in Tregs.

While DR3 was intact on T cells in transferred mice, we also observed significant reductions in fibrosis in pan-DR3-deficient recipients, suggesting that a significant contribution to *fibrosis* in this model may be due to DR3 signaling in non-T cells. To evaluate one such potential-cell type, we turned to fibroblast-selective DR3-deficient recipients. We provide novel findings that direct TL1A-DR3 signaling on fibroblasts promotes intestinal fibrosis *in vivo*, impacting fibroblast activation and functional phenotypes. It is notable that the effects on fibrosis opposed those of inflammation, as *Rag*$^{-/-}$*Dr3*$^{\Delta Col1a2}$ recipients generated severe inflammatory responses due to

TL1A overexpression, but still exhibited attenuation in mucosal collagen deposition and harbored reduced myofibroblasts that were limited in their migratory function.

The source of TL1A overexpression in this context was due to transfer of TL1A-Tg T cells, but as shown previously, TL1A can be produced by various cell types such as macrophages, dendritic cells, and endothelial cells.[6,33-34] An interesting possibility is the notion that structural cells central to tissue remodeling, such as fibroblasts, may produce TL1A. This could promote an autocrine feedback loop whereby fibroblasts can drive their own fibrotic processes independent of adaptive immune functions, which may help explain disjunctions seen between inflammation and fibrosis in the TL1A-DR3 pathway. Thus, disruption of DR3-signaling on fibroblasts may also abrogate this potential effect with regards to fibrotic function in fibroblasts, but still preserve inflammatory effects with DR3-inatct on other cell types. Indeed, synovial fibroblasts and intestinal myofibroblasts were found to be capable of producing TL1A after stimulation with inflammatory cytokines, TNF or IL-1.[35,36] Further studies are underway with selective deletion of TL1A in fibroblasts to evaluate the contribution of this potential mechanism.

The most severe inflammation and fibrosis noted was in the cecum, which we have previously associated with fibrosis in states of TL1A overexpression. In previous studies, the cecum was also the site of significant microbial dysbiosis where pro-fibrotic bacteria capable of affecting fibroblast phenotype were abundant. As we have shown, the microbiome and TL1A both affect fibroblast functions. Studies evaluating the effect of the microbiome on fibrosis in the context of selective DR3-deficiency would therefore be highly informative to expand our understanding of these contributors to intestinal fibrosis.

Our studies suggest that TL1A-DR3 antagonism is a potential source for therapeutics aimed at mitigating inflammation and fibrosis in IBD, as well as potentially other inflammatory/fibrotic diseases. The heterogeneity of patients with IBD in terms of the extent and type of inflammatory responses that underlie their clinical symptoms is becoming increasingly relevant. Recognizing differential expression of TL1A, as well as DR3, in different patient populations and various cell types/tissues in these patients will become paramount in determining who may benefit most from treatments targeting this pathway.

In summary, we reveal novel functions of TL1A in promoting intestinal inflammation and fibrosis *in vivo* and extend the knowledge regarding its mechanistic biology to highlight its direct action on fibroblasts. These findings further emphasize the importance of TL1A as mediator of fibrosis that can act independently of its pro-inflammatory effects and suggest its continued importance as a candidate target for therapy in fibrotic diseases.

Materials and Methods:

Generation of Transgenic Mice: LCK-CD2-Tl1a-GFP (L-Tg) mice were generated and genotyped as described.[17] *Dr3*$^{-/-}$ were generated as previously described[21]: Cloning of *Dr3* targeting vector and generation of *Dr3*$^{+/-}$ founder mice were performed in collaboration with genOway (genOway, Lyon, France). Briefly, *Dr3* endogenous locus containing 1.5 kb upstream of exon 1 and 3 kb downstream of exon 8 were generated by PCR amplification using genomic DNA from C57BL/6J mice and cloned into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA). Subsequently, two *lox*P sites were inserted flanking *Dr3* exons 2 to 5. Positive selection neomycin gene flanked by FRT sites was inserted to the intron between exon 1 and 2 to generate the targeting vector. Every step of the cloning process was validated through restriction enzyme analysis and sequencing. The *Dr3* gene targeting construct was linearized and electroporated into genOway proprietary embryonic stem (ES) cells with C57BL/6J background. Homologous recombinants were selected by G418 and confirmed by Southern blot analysis. ES clones with correct 5' and 3' recombination were microinjected into C57BL/6J blastocysts and introduced into pseudopregnant C57BL/6J mice. Male chimeric offspring were bred to obtain germ line mutant mice which were then bred to *Flpe* delete mouse strain to remove the neomycin cassette, then bred to Cre delete mice to excise the loxP flanked sequences, and maintained on the C57BL/6J genetic background. To generate *Dr3*$^{\Delta Col1a2}$ mice, *DR3*$^{f/f}$ mice above were bred to *Col1alpha2-Cre*$^{+}$ mice.[37] These mice were then bred to *Rag1*$^{-/-}$ (The Jackson Laboratory, Sacramento, CA) mice to generate *Rag*$^{-/-}$*Dr3*$^{-/-}$or *Rag*$^{-/-}$*Dr3*$^{\Delta Col1a2}$ mice for adoptive transfer experiments.

Induction of Chronic Colitis, Disease Activity Index, Macroscopic and Histopathological Analyses: The adoptive-transfer model of colitis was induced by intraperitoneal injection of 500,000 $CD4^{+}CD45RB^{hi}$ naive T cells isolated from L-Tg mice to *Rag1*$^{-/-}$ mice; *Rag*$^{-/-}$*Dr3*$^{-/-}$or *Rag*$^{-/-}$ *Dr3*$^{\Delta Col1a2}$.[38] All mice were maintained under specific pathogen-free conditions in the Animal Facility at Cedars-Sinai Medical Center (CSMC). Littermate control mice were used.

DAI was calculated by scoring from 0 to 4 abnormalities regarding changes in body weight (0, no weight loss; 1, 1–5% weight loss; 2, 5–10% weight loss; 3, 10–15% weight loss; 4, more than 15% weight loss), stool consistency (0, firm dry stool; 1, moist stool; 2, soft adherent stool; 3, large soft pliable stool; 4, liquid stool), stool blood performed on Hemoccult Sensa (Beckman Coulter, Brea, CA; 0, no color; 1, flecks of blue; 2, up to 50% blue; 3, more than 50% blue; 4, gross red blood) and summing the results.[19] DAI score was determined twice a week for the adoptive-transfer model. Macroscopic evidence of inflammation was scored blinded to the mice genotype using the established classification.[20,38] Briefly, normal gut morphology was assigned a score of 0; mild bowel wall thickening without hyperemia was assigned a score of 1; moderate bowel wall thickening with hyperemia was assigned a score of 2; severe bowel wall thickening with rigidity and hyperemia was assigned a score of 3; and severe bowel wall thickening with rigidity, hyperemia, and adhesions was assigned a score of 4. Tissue samples were processed and stained with hematoxylin and eosin (H&E) by the CSMC Histology-Core. Two animal pathologists scored H&E stained sections in a blinded manner using previously published histopathological scoring system.[38] Mucosal area of collagen deposition was identified by Sirius red staining using the NovaUltra Sirius Red Stain Kit according to manufacturer's protocol (IHC World, Woodstock, MD). Stained gut sections were quantitated for the relative degree of fibrosis using ImageJ software, as previously described.[21]

Fibroblast assays: Mouse primary colonic fibroblasts were isolated as previously described.[21] Equal numbers of fibroblasts per group ($1x10^5$ cells) were seeded in 8 chamber slides and cultured for 24-48h until a monolayer was formed. A scratch was created with a P200 pipette tip. Cell debris was removed by washing cells with PBS and then cell-culture medium was replaced with time-lapse images taken every 4 hours under an Olympus CK2 microscope at 100x magnification. The area of the gap between the two migrating fronts of the cells was quantified using ImageJ software and relative percent area of gap closed at the indicated time points was calculated as (area t_0 – area t_x)/area t_0.

For fibroblast adhesion assays, an equal number of cells were seeded into 24-well plates and allowed to settle for either 20 or 80 minutes, after which the wells were washed twice with PBS to remove non-adherent cells. Adherent cells were counted for 5 visual fields/well (representing four quadrants and the center of the well) at 200x magnification, then averaged. The average number of adherent cells per visual field is then displayed for each well.

Histological myofibroblast quantification: Fibroblast and myofibroblasts were quantified by anti-vimentin and anti-α-Smooth Muscle Actin immunofluorescence-stained OCT tissue sections. 4 μm frozen sections were fixed with 10% formalin, blocked in 10% BSA, 0.1% Triton X-100 TBST, and stained overnight at 4 degree C with primary antibodies: rabbit polyclonal anti-αSMA Ab (Abcam, Cambridge, MA) at 1:100 dilution and chicken polyclonal anti-Vimentin Ab (Abcam, Cambridge, MA) at 1:2000 dilution. Secondary antibody at 1:500 dilution was added for 2 hours at room temperature with donkey anti-rabbit IgG-Alexa-fluor-647 and goat anti-chicken IgY- DyLight 488 (Abcam, Cambridge, MA). Fluorescent images were captured by the CSMC Imaging Core and analyzed using TissueGnostics TissueFAXS 200 system (Tissue

Gnostics GmbH, Vienna, Austria) for cell-based counting of automatically recognized positive cells in a FACS-like manner of scattergram analysis, as described previously.[39,40]

Statistics: Data are presented as dot plots and means with group differences tested using standard methods depending on variables measured: Student's t-test for comparisons between two groups or Mann Whitney test for comparisons between two groups requiring non-parametric testing. When indicated, 1-way Analysis of Variance (ANOVA) with Tukey's honestly significant difference (HSD) test for multiple comparisons was used. In all settings, a P value of less than 0.05 indicated a statistically significant difference in the parameter being compared.

Study approval: This study was carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Animal studies were approved by the CSMC Animal Care and Use Committee, under IACUC protocol 4942.

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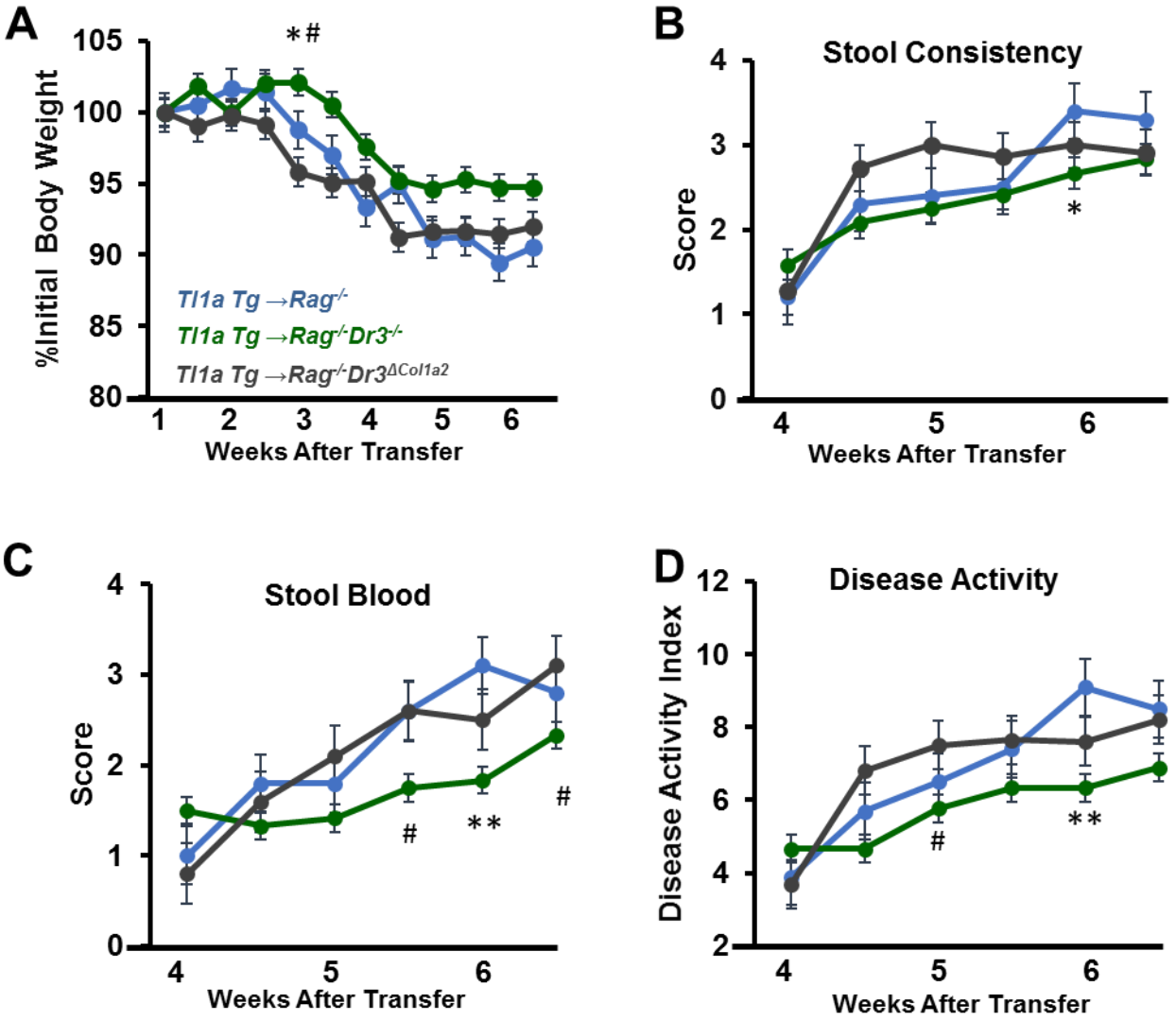

Figure 1. *Fibroblast-selective DR3 deficiency results in severe disease activity*: Percent body weight loss (A), stool consistency (B), stool blood (C), and composite disease activity index (D) are shown for the adoptive transfer model of colitis, at indicated time points for transfers of naïve Tl1a-Tg T cells into *Rag*$^{-/-}$, *Rag*$^{-/-}$*Dr3*$^{-/-}$, or *Rag*$^{-/-}$*Dr3*$^{\Delta Col1a2}$ mice. Data are represented as means ± SEM; * indicates p<0.05, ** p<0.01 Rag$^{-/-}$Dr3$^{-/-}$ vs Rag$^{-/-}$; # indicates p<0.05 Rag$^{-/-}$Dr3$^{-/-}$ vs Rag$^{-/-}$Dr3$^{\Delta Col1a2}$; n=9-10 mice per group.

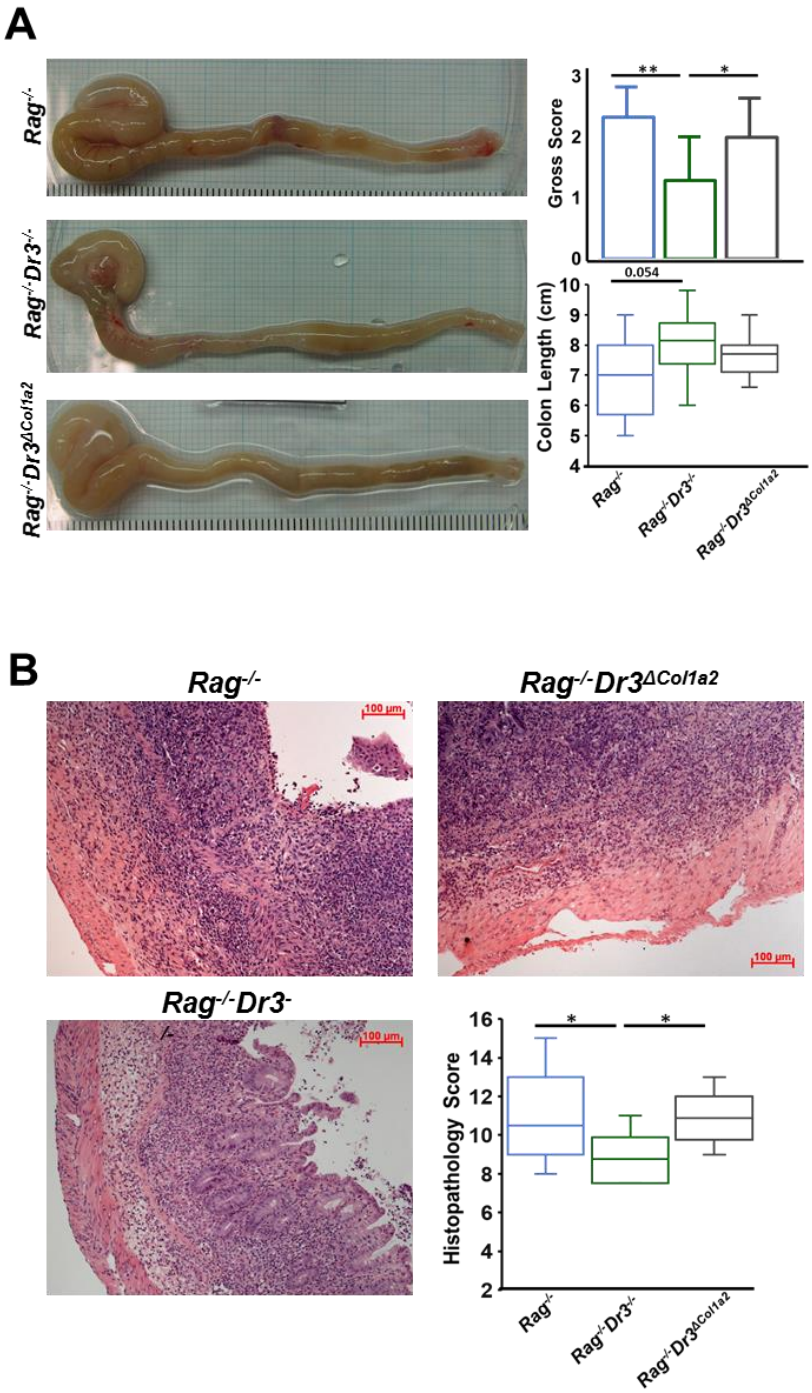

Figure 2. *Fibroblast-selective DR3 deficiency results in macroscopic and histopathological inflammation similar to DR3-intact mice*: A) Representative gross colonic specimens, quantitated macroscopic pathology scores, and colon lengths; B) Representative H&E cecal sections and quantitated histopathological scores are shown for the adoptive transfer model of colitis of *Rag*$^{-/-}$, *Rag*$^{-/-}$*Dr3*$^{-/-}$, or *Rag*$^{-/-}$*Dr3*$^{\Delta Col1a2}$ recipient mice. Data are represented as means ± SD; * indicates $p<0.05$, ** $p<0.01$; n=6-9 mice per group.

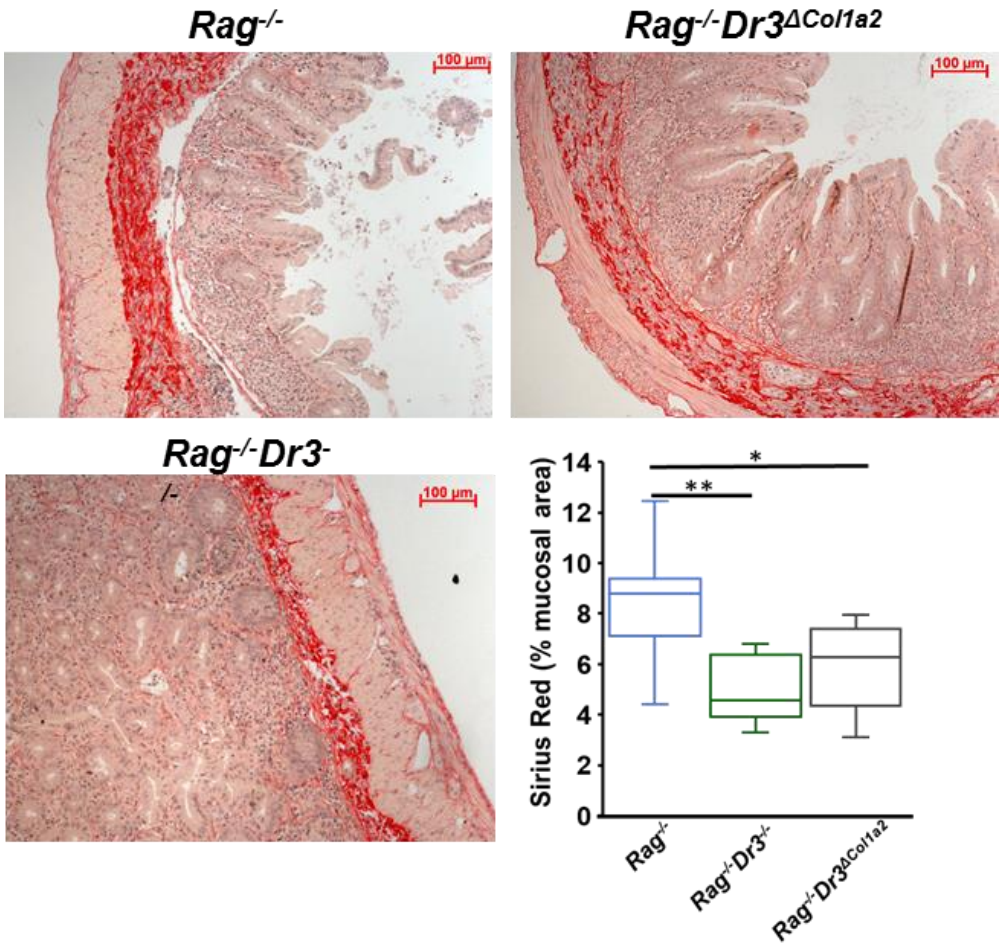

Figure 3. *Fibroblast-selective DR3-deficiency attenuates mucosal collagen deposition:* Representative Sirius red staining of collagen deposition in cecal sections and quantitated percent mucosal area shown for the adoptive transfer model of colitis of *Rag*$^{-/-}$, *Rag*$^{-/-}$*Dr3*$^{-/-}$, or *Rag*$^{-/-}$*Dr3*$^{\Delta Col1a2}$ recipient mice. Data are represented as means ± SD; * indicates $p<0.05$, ** $p<0.01$; n=6-9 mice per group.

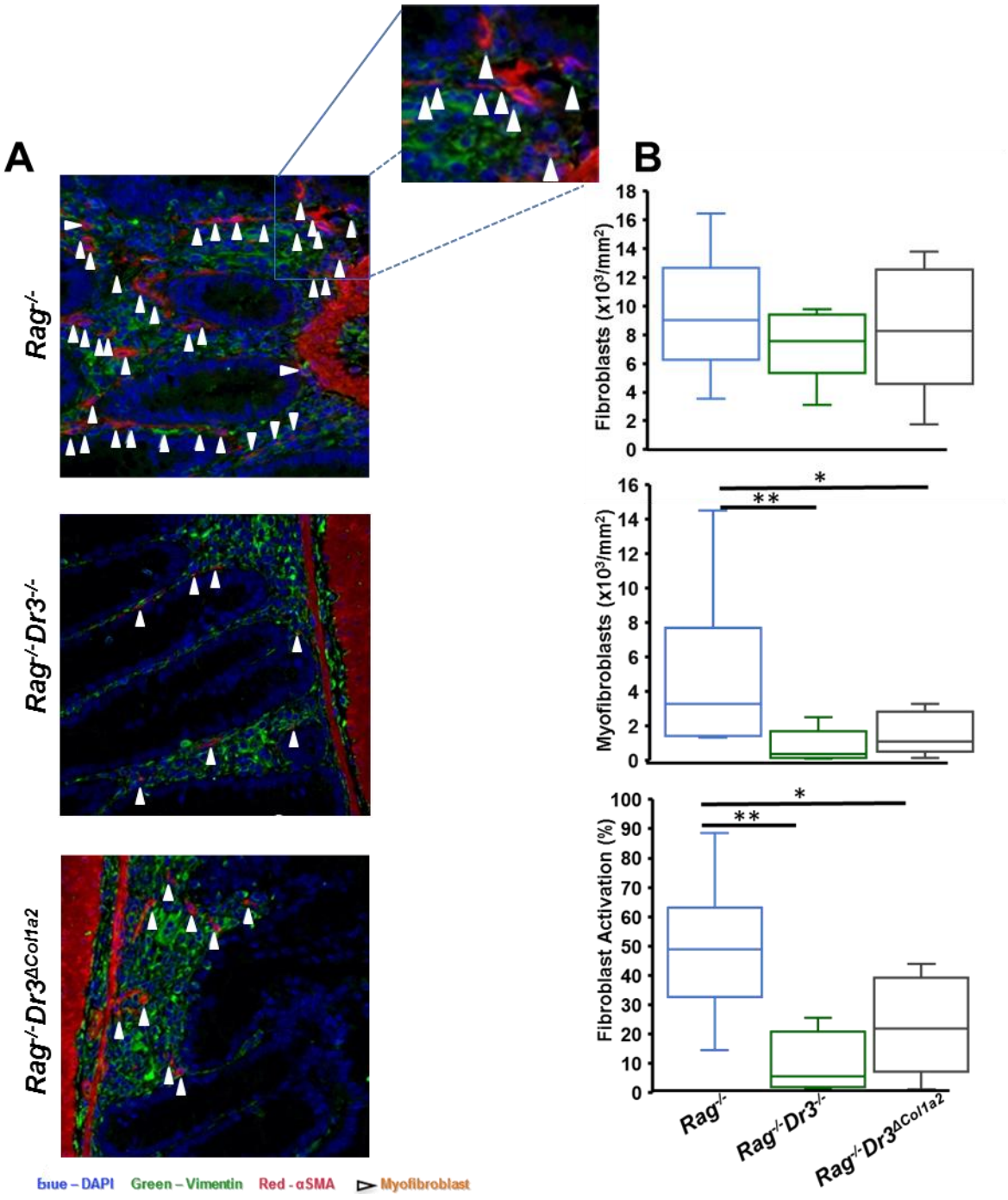

Figure 4. *Fibroblast-selective DR3-deficiency reduces fibroblast activation:* A) Immunofluorescent staining of vimentin (green) and αSMA (red) from cecal sections of adoptive transfer *Rag*$^{-/-}$, *Rag*$^{-/-}$*Dr3*$^{-/-}$, or *Rag*$^{-/-}$*Dr3*$^{\Delta Col1a2}$ recipient mice. White arrows denote myofibroblasts that co-localize vimentin and αSMA at 400x magnification. B) Total numbers and percentage of myofibroblasts over total vimentin-positive cells per mm^2 of tissue area were quantitated and displayed; * indicates $p<0.05$, ** $p<0.01$; n=8 mice per group.

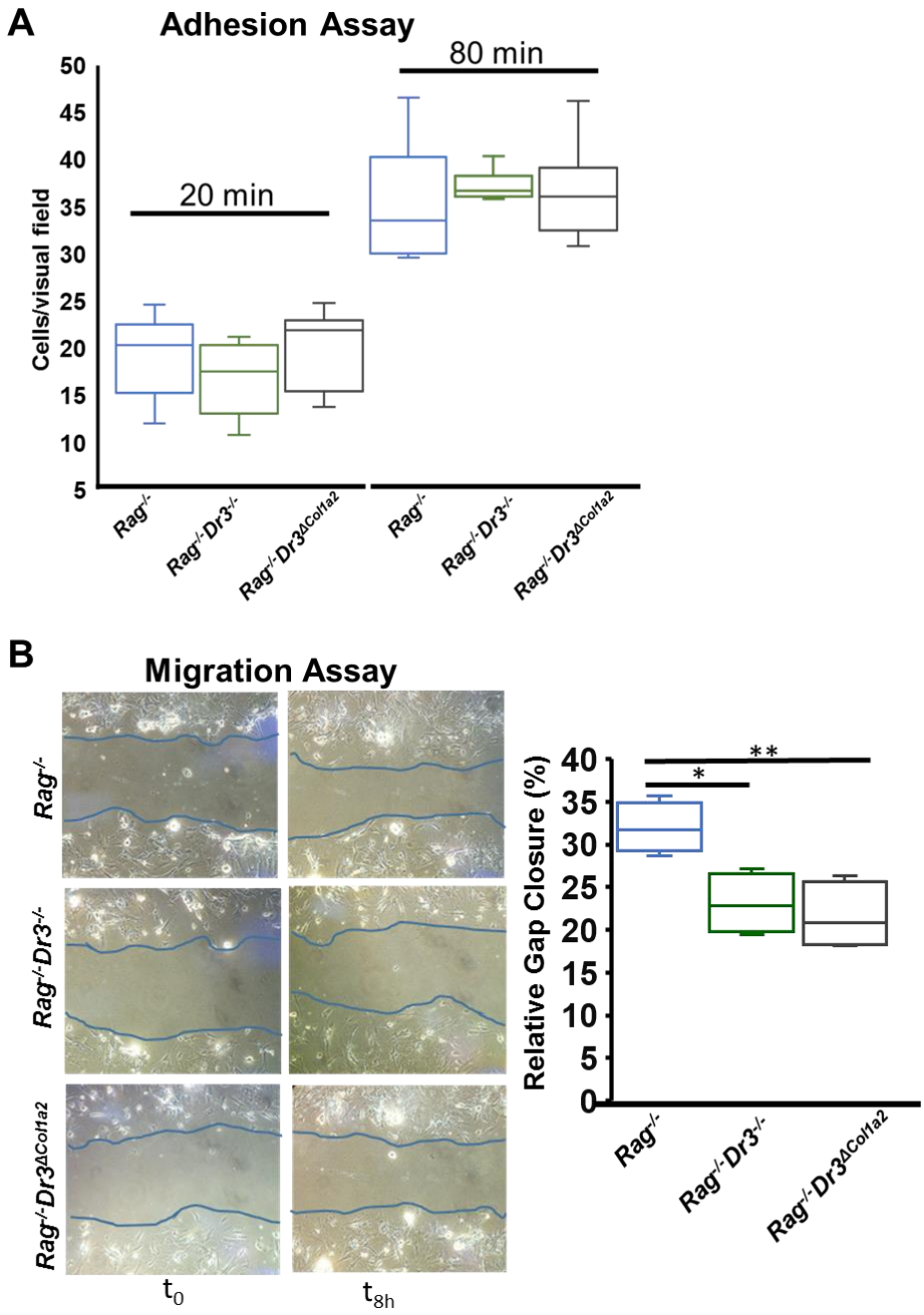

Figure 5. *Fibroblast-selective DR3-deficiency attenuates fibroblast migration ex vivo:* A) Number of adherent cells/visual field indicated during adhesion assays at time points of 20 minutes and 80 minutes for fibroblasts isolated *ex vivo* from adoptive transfer *Rag*$^{-/-}$, *Rag*$^{-/-}$*Dr3*$^{-/-}$, or *Rag*$^{-/-}$*Dr3*$^{\Delta Col1a2}$ recipient mice. B) Representative images of gap-closure assay after simulated wound on fibroblasts isolated *ex vivo* from adoptive transfer *Rag*$^{-/-}$, *Rag*$^{-/-}$*Dr3*$^{-/-}$, or *Rag*$^{-/-}$*Dr3*$^{\Delta Col1a2}$ recipient mice at initial wound (t_0) and 8 hours after migration (t_8) with relative % area of gap closed quantitated; representative of 3 independent experiments; * indicates $p<0.05$, ** $p<0.01$; n=4-6 mice per group.

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Chapter 4:

Concluding Remarks and Future Directions

We have shown that both TL1A-DR3 signaling on fibroblasts and the microbiome (with specific organisms in particular) contribute to intestinal fibrosis. Indeed, while we have determined the direct effect of fibrotic disease *in vivo* due to TL1A signaling on intestinal fibroblasts, the specific molecular mechanisms and pathways of direct TL1A-DR3 signaling of fibroblasts are not known. The known downstream mediators of TL1A-DR3 signaling (but not evaluated in fibroblasts), include TRADD, RIP1, TRAF2, NFkB, and JNK.[1-5] We may, therefore, hypothesize that TL1A-DR3 signaling transduces TRADD, RIP1, TRAF2, NFkB, and JNK to activate intestinal fibroblasts and initiate a TL1A specific fibrogenic program that leads to intestinal fibrosis. Future studies are planned to determine if the known signaling components will work collectively, and if fibroblasts stimulated directly with TL1A will express increases in proliferative and fibrogenic signaling responses, with accompanying decreases in apoptotic markers. One or more of the signaling components may mediate independent functional consequences, however: TRADD may induce fibroblast apoptosis whereas JNK1 may induce fibroblast proliferation. We hope to dissect downstream signaling activity of TRADD, RIP1, TRAF2, NFkB, and/or JNK1 to either fibroblast proliferation, apoptosis, activation or downstream function such as expression of fibrogenic proteins. Whole transcriptome sequencing may also be used to identify additional TL1A-DR3 directed molecular pathways and fibrogenic programs, which may point to functions involved in cytoskeletal extension, contraction, and cellular migration given the likely relevance of these parameters in TL1A-mediated fibroblast phenotypes presented in this work.

The direct contribution of specific bacteria raises the topic of bacterial ligands vs. bacterial products, i.e. metabolites, on stimulating fibroblast function. Indeed, metabolomic profiling of

patients is becoming a burgeoning area of research for both diagnostic/predictive and therapeutic potential.[6] Metabolomic screening of intestinal luminal content derived from TL1A-Tg mice presented in Chapter 2 is in process. Mechanistically, some host-microbiome effects may be mediated by short-chain fatty acids (SCFAs)[7], which are produced in in the colonic lumen during the anaerobic fermentation of dietary fiber by certain gut bacteria.[8] Many negatively-correlated bacteria that we identified can produce SCFAs.[6] The three most abundant SCFAs, namely acetate, butyrate and propionate, can exert their physiological effects directly on effector cells by activating the mammalian G protein-coupled receptors, FFAR2 (also known as GPR43 and FFA2) and FFAR3 (also known as GPR41 and FFA3).[9-11] Interestingly, the effects of SCFA-signaling can be both pro-inflammatory and immunoregulatory depending on the length of the fatty acid chain (i.e. 2, 3, 4, or 5 carbon atoms). Mouse monocytes express FFAR2 and FFAR3, and when exposed to acetate upregulate TIMP-1 (a profibrotic molecule), but also down-regulate CXCL-10, which has been associated with migration and invasiveness of synovial fibroblasts in rheumatoid arthritis.[12,13] Thus, determining the expression of such receptors on intestinal fibroblasts and the direct effects of these metabolites on their function will potentially illuminate downstream mechanisms responsible for the results presented thus far. Alternatively, direct stimulation with bacterial ligands may promote fibroblast function. Just recently presented, selective deletion of Toll-like receptor adaptor protein, MyD88, in α-SMA positive cells (myofibroblasts) decreased amount of colonic fibrosis in experimental IBD. Among all bacterial ligands, human intestinal myofibroblasts selectively responded to flagellin with production of fibronectin and collagen 1 via MyD88 and post-transcriptional regulation. Preliminary results suggested that all bacterial ligands induced phosphorylation of AKT and mTOR. Only flagellin, but not activation of TLR4 or NOD1, dephosphorylated eIF2alpha and phosphorylated 4EBP,

suggesting that the selectivity of flagellin-dependent ECM secretion is translationally regulated.[14] Notably, both candidate pro-fibrotic bacteria we identified and found to affect fibroblasts directly, *M. schaedleri* and *Ruminococcus*, can express flagella. Future studies down these avenues will shed light on additional mechanisms by which the microbiome can modulate fibrosis.

Cytokine targeting has proven to be robustly effective in targeting inflammation in IBD. Given the pleiotropy of many cytokines, cytokine targeting with regards to intestinal fibrosis has been challenging. Moreover, the genetic variability present across patient populations may result in different pathogenesis of disease with regards to cytokine pathways, and thus broad cytokine targeting may result in disparate rates of response. Indeed, this has been observed with regards to anti-TNF agents in terms of inflammation and may be one source of failure of some clinical trials with newer anti-cytokine agents. A potential future approach to overcome this difficult aspect of cytokine-targeting may require careful selection of patients based on genetic or biochemical characteristics. As noted in Chapter 1, post hoc analysis of the trail with anti-IL17, identified that a subgroup of patients who responded to IL-17 blockade carried a *TNFSF15* (rs4263839) SNP. Subsequent work in animal models suggested that under elevated levels of TL1A conditions, IL-17 blockade could be beneficial. Thus, patient selection for such genetic or biochemical traits as candidates for a specific anti-cytokine treatment may result in greater success with these agents. An additional attractive possibility from our findings may be the combined utilization of cytokine *and* microbial profiling to identify such patients that are pre-disposed to fibrotic disease and may benefit most from early application of these treatments. Additionally, there are promising lines of targets used for other fibrotic conditions that may be of benefit in CD and warrant investigation. Given the variables that contribute to fibrostenosis in CD, targeting of

multiple points in the fibrotic pathway, including the cytokines themselves and their related microbiomal dysbiosis, may be an option. Future investigations into novel fibrogenic molecules and pathways may lead to additional and more selective therapeutic targets as well as the identification of specific patient groups that could best benefit from such individualized treatment.

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