Inflammation-independent TL1A-mediated intestinal fibrosis is dependent on the gut microbiome

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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 lead to spontaneous ileitis, and exacerbated induced proximal colitis and fibrosis. 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 investigated. We demonstrate that the pro-fibrotic and inflammatory phenotype resulting from TL1a-overexpression is abrogated in the absence of resident microbiota. To evaluate if this is due to the absence of a unique bacterial population, as opposed to any bacteria per se, we gavaged germ-free (GF) wild-type and TL1a-transgenic (TL1a-Tg) mice with stool from specific pathogen free (SPF) mice and a healthy human donor (Hu). Reconstitution with SPF, but not Hu microbiota, resulted in increased intestinal collagen deposition and fibroblast activation in TL1a-Tg mice. Notably, there was reduced fibroblast migration and activation under GF conditions compared to native conditions. We then identified several candidate organisms that correlated directly with increased fibrosis in reconstituted mice and showed that these organisms directly impact fibroblast function in vitro. Thus, TL1a-mediated intestinal fibrosis and fibroblast activation are dependent on specific microbial populations.

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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.1–3 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.2,3,4–9 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.15 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.16–21 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 T1α-enhanced intestinal inflammation, collagen deposition and fibroblast migration. In agreement with our prior results, T1α-Tg mice raised under SPF microbial conditions display significant spontaneous ileitis, as evident by increased histopathological scoring under H&E, with over a twofold increase in average histopathology compared with wild-type mice (Fig. 1a). No histologically apparent cecal inflammation was observed (Fig. 1b). Despite this, T1α-Tg mice demonstrate increased cecal collagen deposition (Fig. 1d), underscoring the importance of T1α 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 T1α-mediated intestinal inflammation. The absence of a microbiome abrogated the spontaneous ileitis induced by T1α-overexpression, as there were no significant differences in ileal histopathology between GF T1α-Tg and GF WT mice (Fig. 1a).

We next evaluated if the fibrosis observed in T1α-Tg mice were dependent upon the microbiome. The absence of microbes significantly reduced ileal and cecal collagen deposition in GF T1α-Tg mice compared with microbiome intact T1α-Tg mice (Fig. 1c, d). No difference was observed in WT mice.

As we observed notable changes in fibrosis between T1α-Tg and WT mice under native SPF conditions that were abrogated under GF conditions, we sought to determine the impact of T1α-overexpression and resident microbiota on fibroblast phenotype. Colonic fibroblasts isolated from T1α-Tg mice raised under native microbial conditions displayed significantly increased migratory capacity after simulated wounding compared with those from WT mice (Fig. 2a, b). T1α-overexpression also increased fibroblast adhesion, which was unchanged in GF mice (Supplementary Figure 1). The enhanced rate of fibroblast gap-closure observed with T1α-overexpression under native conditions was eliminated under GF conditions, consistent with the observed reduction in histological fibrosis (Fig. 2a, b). Interestingly, the absence of microbiome reduced fibroblast migratory capacity even in WT mice, but to a lesser extent than in T1α-Tg mice. These results indicate that the intestinal microbiome is required for T1α-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 T1α (alone or in concert) on fibroblasts themselves, or if the microbiome and T1α in T1α-Tg mice affect other non-fibroblast cell types, which then promote fibroblast activation and profibrotic phenotype. Bacterial components and products can induce fibroblast activation, 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 T1α-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 T1α-overexpression. WT fibroblasts exposed to native SPF cecal washings demonstrate significantly increased migration compared to those exposed to cecal washings from GF mice (Fig. 2c). This direct effect of cecal bacterial components was enhanced by host T1α-overexpression in T1α-Tg mice. Thus, a significant part of the commensal microbiome’s effect on fibroblast migration seen in Fig. 2a, b may be due to direct effects mediated by bacterial components (or products) on fibroblasts themselves.

We next asked whether this enhanced migratory phenotype in fibroblasts from T1α-Tg mice were partly due to direct T1α-mediated effects on fibroblasts; namely, can T1α promote fibroblast migration directly and do so in concert with the direct effect of SPF microbiota on fibroblasts seen in Fig. 2c? We have previously demonstrated that fibroblasts express the T1α receptor DR3, and upon treatment with T1α in vitro, demonstrate expression of alpha-smooth-muscle actin (indicative of activation of myofibroblasts) and collagen. Consequently, fibroblasts treated with T1α in vitro demonstrate expression of alpha-smooth-muscle actin (activation of myofibroblasts) and collagen.

We therefore hypothesized that direct stimulation of fibroblasts with T1α would increase migration. WT fibroblasts treated with T1α (and without bacterial components) in vitro displayed significantly increased migration compared with untreated cells, suggesting a direct effect of T1α on fibroblast migration (Fig. 2d).

To determine whether T1α can enhance the fibroblast migratory response to bacterial components, we conducted the same experiments as in Fig. 2c in the context of exogenous T1α stimulation. WT fibroblasts treated with both T1α and SPF cecal bacterial products together demonstrated enhanced migration compared with those treated solely with the bacterial products (Fig. 2d). These data demonstrate that resident bacteria and T1α can both stimulate fibroblasts, and the direct effect of resident bacteria on fibroblasts is enhanced directly by T1α.

Gavage with murine but not human fecal microbiota promotes intestinal inflammation and collagen deposition in T1α transgenic mice. As these data demonstrate that the microbiome is required for T1α-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 T1α-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 T1α-overexpression the species-specific microbiome does not induce intestinal inflammation or fibrosis (Fig. 3a–d). T1α-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 (Fig. 3a–d, Fig. 1). In contrast, T1α-transgenic recipients of Hu microbiota showed no increase in ileal or cecal collagen deposition or inflammation. Together, these data indicate that T1α-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 potentiates T1α-mediated intestinal fibroblast differentiation to myofibroblasts. Fibroblast activation has been shown to occur after bacterial stimulation e.g., with lipopolysaccharide. Previously, we showed that there is an increase in the proportion of intestinal myofibroblasts in T1α-Tg mice raised under conventional SPF conditions. We investigated whether absence of microbial stimulation (i.e., under GF conditions) impairs fibroblast differentiation to myofibroblasts. GF T1α-Tg mice did not display an increased number or proportion of activated fibroblasts in the
cecum compared to GF WT mice (Fig. 4a, b). Colonization with SPF microbiota-induced intestinal myofibroblasts in both WT and T11a-Tg mice relative to GF conditions or colonization with Hu microbiota (Fig. 4a, b). SPF microbiota, but not Hu microbiota also restored the increased proportion of myofibroblasts in T11a-Tg mice compared to WT controls (54.7% vs. 36.7%). Interestingly, GF T11a-Tg mice had reduced myofibroblast proportion compared to GF WT mice, which was not seen in the presence of Hu microbiota (Fig. 4a, b). These results show that fibroblast activation in the cecum induced by T11a overexpression is microbiota-dependent and that microbial composition affects fibroblast differentiation into myofibroblasts.

To assess whether T11a mediated fibroblast activation in the ileum also requires the microbiome, we quantitated myofibroblasts in T11a-Tg mice under GF conditions. In contrast to the cecum, there was an increased proportion of activated myofibroblasts in the ileum of GF T11a-Tg mice compared with GF WT mice (Fig. 4c, d). We next tested whether the degree of T11a-mediated fibroblast activation is affected by the specific microbiome. SPF gavage increased myofibroblast numbers and proportion in both WT and T11a-Tg mice while preserving the relative increase in myofibroblasts in T11a-Tg mice (Fig. 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 T11a-mediated fibroblast activation by the microbiome in the cecum (without concomitant changes in inflammation), for which T11a can potentially

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Fig. 1 The intestinal microbiome is required for T11a-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 T11a-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 T11a-Tg mice, raised in GF and native SPF conditions. Dot plots with means displayed; n = 7–10 mice per group. *p < 0.05; **p < 0.01
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 T11a-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. 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 T11a-Tg and WT mice colonized with SPF microbiota (Supplemental Fig. 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 T11a-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 (Fig. 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 (Fig. 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 (Fig. 5a).

In the ileum of SPF-colonized mice, we observed that there were competing sets of microbes associated with enhanced or reduced fibrosis severity (Fig. 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 (Fig. 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 vitro. Cell lysates of *Ruminococcus* 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 (Fig. 6). These results show that microbes that positively or negatively correlate with intestinal 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 T11a-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-T11A 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, T11a-Tg mice demonstrate increased cecal collagen deposition, underscoring the importance of T11A 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.29 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 T11a. Given their expression of Toll-like receptors, fibroblasts have the capacity to become activated by bacterial products.26 Consistent with this, T11a-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), T11a could partially compensate for the lack of the microbiome and promote some fibroblast activation. This might point to unique effects of T11a 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 T11a-Tg mice still had reduced ileal fibrosis under GF conditions and with Humanized microbiome compared with conventional SPF microbiota (Figs. 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 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.30 The mucin-rich large intestine can harbor anaerobic organisms with a repertoire of glycosidic enzymes that...
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 colitogenic 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 impact 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.35 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 remission35 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.22,34–36 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 stricture 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. Finally, *Anaeroplasma*, a genus which has been previously associated with experimental colitis, 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. *Oscillospira* have been associated with gut health, and their reduced abundance has been implicated in a variety of diseases including CD. Notably, compared with positively correlated organisms, *Oscillospira* 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. 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. Additionally, members of the Lachnospiraceae family, which contains many butyrate producers that are reduced in CD patients, 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. 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.

To our knowledge, this is the first study that causally implicates the intestinal microbiome in intestinal fibrosis, demonstrating that T11a-mediated fibrosis is dependent upon specific bacteria or bacterial consortia and that those bacteria can directly affect fibroblast function. Thus, a focus on T11a pathways acting in concert with the microbiome may identify future therapeutic targets for fibrostenosing Crohn’s disease.

### MATERIALS AND METHODS

**Gnotobiotic experiments**

T11a-Tg (which have sustained T11A 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. T11a-Tg mice and WT littermates at 2–4 months of age were orally gavaged with 200 µL 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 killed after 2 months of colonization for assessment of intestinal fibrosis and histopathology. Mucosal areas of collagen deposition identified by Picrosirisid red-stained gut sections were quantitated for the relative degree of fibrosis using ImageJ software, as previously described. Two animal pathologists scored H&E stained sections in a blinded manner using previously described histopathological scoring system used in GF experiments.

**Fibroblast gap-closure assays**

Mouse primary colonic fibroblasts were isolated as previously described. Equal numbers of fibroblasts per group (1 × 10⁵ cells) were seeded in 8 chamber slides and cultured for 24–48 h 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 h under an Olympus CK2 microscope at ×100 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 t₀ – area tₜ)/area t₀. 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. The washings were then homogenized by vortexing, and pelleted by centrifugation. Supernatant were collected, filtered through a 0.22 µm filter, and was added directly to the cells after simulated wound, at a 1:20 dilution (5% volume). In the indicated assays, mouse recombinant T11a (R&D Systems, Minneapolis, MN) was added at a concentration 100 ng/ml for 4 h prior to simulated wound and maintained during the indicated migration period. For assays involving the addition of bacterial lysates, *Oscillospira sp.*, *Mucispirillum schaeideri* 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. 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 min, 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 ×200 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-a-Smooth Muscle Actin immunofluorescence-stained OCT tissue sections. A total of 4 µm frozen sections were fixed with 10% formalin, blocked in 10% BSA, 0.1% Triton X-100 TBST, and stained overnight at 4 °C with primary antibodies: rabbit polyclonal anti-aSMA 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 h 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. A total of 250 ng 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 quantita-
tive real-time RT-PCR with TaqMan probes and primers (Thermo-
Fisher 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
Masterecycler Ep realplex2 (Eppendorf). The Masterecycler 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 expres-
sion 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.45 DNA extraction and sequencing of the 16S ribosomal
RNA gene was ampli-
fied using the MO BIO Powersoil kit with bead beating. The V4 region
reference gene (16S) was analyzed. Replicate Ct values were normalized to replicate
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 expres-
sion was calculated with respect to the indicated reference sample
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AUTHOR CONTRIBUTIONS
N.J., J.J., C.W.Y.H., S.D., J.B., K.S.M., R.B.S., S.R.T., and D.Q.S. designed experiments; N.J.,
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analyzed data; and N.J., J.J., R.B.S., K.S.M., S.R.T., and D.Q.S wrote the manuscript.

ADDITIONAL INFORMATION
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