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Flagellin-mediated activation of IL-33-ST2 signaling by a pathobiont promotes intestinal fibrosis

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Abstract

Intestinal fibrosis is a severe complication in patients with Crohn's disease (CD). Unfortunately, the trigger leading to the development of intestinal fibrosis in the context of CD remains elusive. Here, we show that colonization by a CD-associated pathobiont adherent-invasive *Escherichia coli* (AIEC) promotes the development of intestinal fibrosis. Exogenously inoculated AIEC strain LF82 and commensal *E. coli* HS were gradually eradicated from the intestine in healthy mice. In *Salmonella*- or dextran sodium sulfate-induced colitis models, AIEC exploited inflammation and stably colonize the gut. Consequently, persistent colonization by AIEC LF82 led to substantial fibrosis. In contrast, commensal *E. coli* HS was unable to derive a growth advantage from inflammation, thereby failing to colonize the inflamed intestine or promote intestinal fibrosis. AIEC colonization potentiated the expression of the IL-33 receptor ST2 in the intestinal epithelium, which is crucial for the development of intestinal fibrosis. The induction of ST2 by AIEC LF82 was mediated by flagellin, as the FliC mutant failed to induce ST2. These observations provide novel insights into pathobiont-driven intestinal fibrosis and can lead to the development of novel therapeutic approaches for the treatment of intestinal fibrosis in the context of CD that target AIEC and/or its downstream IL-33-ST2 signaling.

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J.I. and N.K. conceived and designed experiments. J.I. conducted most of the experiments with help from S.K., K.S., H.N.-K., A.H., T.L.M., and P.K.. P.D.R.H. and N.B. provided advice and discussion. N.B. provided critical materials. J.I., S.K., K.S., and N.K. analyzed the data. J.I. and N.K. wrote the manuscript with contributions from all authors.

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Keywords

Crohn's disease; fibrosis; gut microbiota; interleukin-33; flagellin

Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder of the gastrointestinal tract, which comprises two major disease types, Crohn's disease (CD) and ulcerative colitis (UC) ¹. Although the precise etiology of IBD remains unknown, genetic predispositions and environmental factors, including the gut microbiota, diet, stress, smoking, cooperatively promote abnormal immune responses as well as the impairment of intestinal barrier function, thus leading to disease ². Chronic inflammation often results in tissue fibrosis, especially in CD, frequently causing extensive local intestinal fibrosis and mechanical obstruction ³. Approximately 40% of CD patients with the ileal disease develop clinically apparent strictures ³. The strictures are clinically classified as fibrotic, inflammatory, or mixed types. Standard anti-inflammatory therapies, including anti-tumor necrotic factor-alpha (TNF- α) antibody treatment, can improve inflammatory strictures through the reduction of inflammation-mediated edema. On the other hand, conventional medical treatment is largely ineffective for fibrotic strictures. Hence, surgical resection and endoscopic dilation are utilized as treatment options for fibrotic strictures. Surgical resection is an option for more than 80% of stenosis patients ³. However, the post-surgery recurrence rate is close to 50% at 10 to 15 years following ileocecal resection. Likewise, the cumulative redilation-free rate after initial balloon dilation was 64% at 2 years and 47% at 3 years ⁴. Thus, existing strategies for stricture management in the context of CD are insufficient and new treatment options are desperately needed.

Accumulating evidence indicates that the gut microbiota plays a pivotal role in the development and progression of IBD. The gut microbial communities in IBD patients are known to be perturbed - a condition called gut dysbiosis ⁵. Gut dysbiosis results in the enrichment of potentially pathogenic members of commensal bacteria (pathobionts) and/or the reduction of protective bacteria, thereby rendering the microbiota pathogenic ⁶. Indeed, colonization by the dysbiotic gut microbiota isolated from IBD patients is sufficient to elicit abnormal immune activation in the intestinal mucosa ⁷. A pathotype of *Escherichia coli*, namely adherent-invasive *E. coli* (AIEC), has been identified as a potential pathobiont associated with IBD, particularly CD ⁸. Although colonization by AIEC only results in mild inflammation in normal mice ⁹, AIEC causes massive intestinal inflammation in genetically susceptible hosts. Moreover, AIEC aggravates dextran sulfate sodium (DSS)-induced or enteric pathogen-induced colitis ^{10, 11}. Thus, AIEC is involved in the augmentation of inflammation in the context of IBD. However, the potential role of AIEC in the development of intestinal fibrosis remains incompletely understood. In CD patients, AIEC is isolated most frequently from the terminal ileum ⁸. Given that terminal ileum is the most common site for the formation of fibrotic stricture in CD ³, it is conceivable that AIEC strains target this section of the gut and contribute to the promotion of fibrosis.

Although an experimental model that would fully replicate the pathogenesis of intestinal fibrosis seen in IBD patients currently does not exist, various animal models have been used to study the mechanism of intestinal fibrosis associated with IBD¹². Chemically-induced colitis models (e.g. dextran sodium sulfate (DSS), trinitrobenzene sulfonic acid) are widely used to model fibrogenesis in IBD. However, these substances used to induce colitis and fibrosis are toxic to the intestinal epithelium and, therefore, these models might not mimic the natural course of fibrogenesis in IBD. In addition to the chemically-induced colitis models, infection with pathogenic bacteria can be used to induce intestinal fibrosis. It has been reported that *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) infection results in the development of severe fibrosis¹³. *S. Typhimurium* infection recapitulates various pathogenic features of intestinal fibrosis seen in CD patients, such as Th1/Th17-skewed immune responses and induction of pro-fibrotic and extracellular matrix proteins. *S. Typhimurium* infection is therefore the optimal model for the investigation of immune pathways associated with fibrogenesis in CD¹³. However, infection with this pathogen has not been implicated in the pathogenesis of CD. Hence, in this study, we have modified the *Salmonella*-induced intestinal fibrosis model and established an intestinal fibrosis model in which fibrosis is induced by the colonization of a CD-associated pathobiont AIEC. An attenuated strain of *S. Typhimurium* can cause Th1/Th17-skewed CD-like colitis, however it is insufficient for the induction of fibrosis¹³. Here, we show that co-colonization with a CD-associated pathobiont AIEC promotes the development of intestinal fibrosis in mice infected with the attenuated strain of *S. Typhimurium*. AIEC colonization likewise promotes intestinal fibrosis in the DSS model of colitis, implying that the impact of AIEC colonization on the development of fibrosis is not model specific. AIEC colonization potentiates IL-33-ST2 signaling in the intestinal epithelium via production of flagellin. The AIEC-induced up-regulation of ST2 is critical for the induction of fibrosis. These observations are novel and identify new avenues for the treatment of intestinal fibrosis in IBD that focus on AIEC and AIEC-mediated IL-33-ST2 signaling.

Results

AIEC colonization persists in the inflamed intestine.

Infection with a murine non-typhoidal *Salmonella* is known to model various pathogenic features of intestinal fibrosis seen in CD patients and is hence widely used to study the immune pathways that are associated with fibrogenesis in CD¹³. In this study, in order to address the extent to which colonization by CD-associated pathobionts contributes to intestinal fibrosis, we utilized a modified *Salmonella*-induced colitis and fibrosis model. A *S. Typhimurium* mutant strain that is deficient in type 3 secretion system encoded by the *Salmonella* pathogenicity island (SPI) 2 (*spiA* mutant) was used. This mutant strain replicates normally in the intestine but fails to disseminate systemically, thereby causing chronic colitis without killing the infected hosts¹³. Importantly, SPI-2 mutant *Salmonella* is unable to induce the development of intestinal fibrosis¹³. SPF C57BL/6 mice were pre-treated with 20mg of streptomycin (Strep) (Fig. 1a). After 8 days, mice were challenged with the *S. Typhimurium spiA* mutant strain (ST) and *Salmonella* colonization was monitored until day 21 post infection. To address the effect of AIEC colonization, mice were colonized with either a CD patient-derived AIEC strain LF82⁸ or a human commensal *E.*

coli strain HS¹⁴ 7 days prior to *Salmonella* or mock infection (Fig. 1a). *Salmonella* colonization levels reached $>10^7$ CFU/g feces on day 2 post-infection and then gradually diminished in the gut (Fig. 1b). Colonization levels of *Salmonella* were not affected by the presence of *E. coli* strains (Fig. 1b). Next, we assessed the colonization potential of *E. coli* strains. In the absence of *Salmonella* (mock infection), both HS and LF82 did not stably colonize the intestine and were gradually eradicated. In contrast, in *Salmonella*-infected mice, only LF82 persisted on days 7, 14 and 21 post *Salmonella* infection (Fig. 1c). These results suggest that AIEC, unlike commensal *E. coli*, is able to exploit *Salmonella*-caused inflammation and stably colonize the inflamed gut.

AIEC colonization promotes colonic fibrosis.

Next, we assessed the effects of persistent AIEC colonization on the development of intestinal inflammation and fibrosis. In the acute phase (day 2), *Salmonella*-induced colitis (the expression of *Tnf*, *Il6*, *Ifng*) was not affected by the presence of *E. coli* strains (HS or LF82) (Fig. 2a). However, in the chronic phase (day 21), the presence of LF82 amplified the expression of pro-inflammatory cytokines induced by *Salmonella* infection in the colonic mucosa (Fig. 2b). In contrast, *E. coli* HS did not influence the severity of colitis caused by *Salmonella*, even in the chronic phase (Fig. 2b). Next, we examined colonic fibrosis to further assess the impact of AIEC colonization on *Salmonella*-induced chronic inflammation. Fibrosis was evaluated by assessing the expression of fibrosis-related factors *Tgfb1* and *Col1a2* in the colonic mucosa, Picro-sirius red staining and quantifying the amount of collagen deposited in the colonic tissues. Consistent with a previous report¹³, infection with the mutant *S. Typhimurium* deficient in SPI-2 (*spiA*) did not upregulate fibrosis-related genes or increase collagen deposition (Fig. 2c-e). Notably, upregulation of fibrosis genes and a massive deposition of collagen in the extracellular matrix (ECM), especially in the submucosal layer, were observed in the colonic mucosa of *Salmonella*/AIEC co-infected group (ST/LF82) (Fig. 2c-e). In contrast, HS colonization or *Salmonella*/HS co-colonization did not promote colonic fibrosis (Fig. 2c-e). These results indicate that persistent AIEC colonization results in chronic inflammation and fibrosis in the colon.

AIEC colonization promotes intestinal fibrosis in DSS-induced colitis.

Next, we examined the impact of AIEC colonization on the development of intestinal fibrosis in another IBD model. SPF C57BL/6 mice were pre-treated with 20mg of streptomycin 1 day prior to AIEC LF82 or commensal *E. coli* HS colonization. *E. coli*-colonized mice were then treated with 3.0% DSS for 5 days followed by regular water until day 21 (Fig. 3a). Commensal *E. coli* HS colonization did not affect body weight loss in DSS-treated mice (Fig. 3a). In contrast, mice colonized by AIEC LF82 lost significantly more body weight on day 14 compared to control mice not colonized by exogenous *E. coli* (none) (Fig. 3a). Consistent with the *Salmonella*-induced colitis model, LF82 colonized more efficiently than HS in DSS-treated mice during inflammation (Fig. 3b). Moreover, the colon tissue isolated from DSS-treated mice colonized by AIEC was significantly thicker than that isolated from DSS-treated mice colonized by HS or control DSS-treated mice not colonized by exogenous *E. coli* (Fig. 3c). However, colonic inflammation was not significantly enhanced by AIEC colonization (Fig. 3d). This evidence suggests that colon

thickening in AIEC-colonized mice may not result from increased levels of inflammation. Rather, it is conceivable that AIEC colonization promoted colonic fibrosis in this model. Consistent with this notion, massive deposits of collagen were observed in the colonic tissue of DSS-treated mice colonized by LF82 compared to those colonized by HS or without *E. coli* colonization (Fig. 3e and 3f). These results suggest that, in addition to the colitis induced by a microbial pathogen, persistent AIEC colonization leads to the development of fibrosis in the colon in a chemically-induced colitis model.

AIEC does not directly promote collagen deposition in fibroblasts.

To unravel how AIEC colonization promotes the development of fibrosis, we first tested whether AIEC colonization directly initiates collagen deposition in fibroblasts, which play a central role in organ fibrogenesis. We stimulated human primary colonic fibroblasts with LF82 or HS, and then measured the expression of fibrosis-related factors *TGFBI* and *COL1A2*. As shown in Fig. 4, stimulation with neither LF82 nor HS elicited the expression of *TGFBI* and *COL1A2* in human fibroblasts. In contrast, stimulation with *E. coli* significantly upregulated the expression of pro-inflammatory cytokine *TNF* (Fig. 4). These results suggest that AIEC does not directly induce fibrotic programs in fibroblasts, although these cells can sense the bacterium and elicit inflammatory responses.

AIEC colonization promotes intestinal fibrosis via the activation of IL-33-ST2 signaling.

IL-33, an IL-1 family cytokine, is implicated in the pathogenesis of fibrotic diseases in various organs¹⁵. IL-33 binds to its receptor ST2, leading to the activation of nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinases (MAPKs) that cooperatively drive the production of pro-inflammatory and type 2 helper T cell (Th2)-associated cytokines¹⁵. Th2 cytokines, especially IL-13, promote TGF- β production and collagen deposition in the ECM¹⁶. In our model, mono-colonization with *Salmonella* or *E. coli* strains (commensal HS or AIEC LF82) did not induce the expression of *Il33* and *Il1rl1* (gene encodes ST2) (Fig. 5a). In contrast, co-colonization with *Salmonella* and AIEC (group ST/LF82) resulted in a marked upregulation of *Il33* and *Il1rl1* in the colon, while HS colonization did not augment the expression of these genes in *Salmonella*-infected animals (HS/ST group) (Fig. 5a). Moreover, AIEC LF82 colonization, unlike commensal *E. coli* HS, induced the expression of *Il1rl1* in the DSS model of colitis (Fig. 5b). Next, we validated these results in human CD patients. Using a publically available dataset (GSE75214)¹⁷, we discovered that the expression of IL-33 (*IL33*) and ST2 (*IL1RL1*) are significantly upregulated in the ileal mucosa of active CD patients compared to that of normal controls or inactive CD patients (Fig. 5c). To assess the role of IL-33-ST2 signaling in AIEC-elicited intestinal fibrosis, we attempted to block this pathway during AIEC and *Salmonella* co-infection. Streptomycin-treated mice were colonized with LF82 for 7 days, followed by *Salmonella* infection for 21 days. The anti-ST2 blocking antibody or an isotype control IgG was injected intraperitoneally 3 times a week starting from day 7 post *Salmonella* infection (Fig. 5d). *Salmonella* and *E. coli* (LF82) colonization was not influenced by blockade of IL-33-ST2 signaling (Fig. 5e). Likewise, blockade of IL-33-ST2 signaling did not affect the expression of pro-inflammatory cytokines, such as TNF- α , IL-6, IFN- γ and IL-17A (Fig. 5f). In contrast, IL-33-ST2 signaling blockade suppressed AIEC colonization-induced up-regulation of fibrosis-related genes in the colonic mucosa (Fig. 5g). Consistently, diminished

IL-33-ST2 signaling significantly attenuated the accumulation of collagen in the colon (Fig. 5h. i). These data suggest that AIEC colonization promotes intestinal fibrosis via the activation of IL-33-ST2 signaling.

CD-associated *E. coli* strains induce ST2 expression in IECs.

The IL-33 receptor ST2 is known to be expressed in various immune and non-immune cells. In the gastrointestinal tract, it is reported that intestinal epithelial cells (IECs) express high levels of ST2¹⁸. Likewise, IECs are major producers of its ligand IL-33¹⁹. Hence, we asked whether AIEC colonization can elicit the expression of ST2 and/or IL-33 in IECs. To this end, T84 cells, a human colonocyte cell line, were stimulated with *E. coli* HS or LF82 and the expression of IL-33 and ST2 was assessed by qPCR. As a control, human primary colonic fibroblasts were also stimulated with *E. coli* strains. As expected, only stimulation with AIEC LF82 elicited a marked upregulation of *IL33* (IL-33) and *IL1RL1* (ST2) expression (Fig. 6a). Neither *E. coli* strains induced the expression of these genes in fibroblasts (Fig. 6a). Next, we confirmed this result *in vivo*. To this end, we examined the expression of ST2 in the colonic mucosa of *Salmonella*-infected and *Salmonella/E. coli* co-infected mice (Fig. 6b). Consistent with the *in vitro* result, co-infection with *Salmonella* and AIEC (LF82/ST) resulted in the expression of ST2 protein in the colonic epithelium (Fig. 6b), whereas mono-infection with *Salmonella* (ST) or co-infection with *Salmonella* and *E. coli* HS (HS/ST) did not cause ST2 expression in the colonic mucosa (Fig. 6b). These results suggest that the CD-associated *E. coli* strain induces ST2 expression in the colonic epithelium.

Flagellin mediates the induction of ST2

Since one of AIEC's pathogenic features is its ability to adhere to and invade IECs⁸, we next assessed whether epithelial adhesion/invasion trigger the expression of these genes. To this end, we utilized a panel of AIEC and non-AIEC strains isolated from CD patients (except HS) and used them to stimulate IECs. Consistent with the results obtained from LF82, all tested AIEC strains (strain LF16, LF31, LF73, and LF82) elicited the expression of *IL1RL1* in IECs (Fig. 7a). The non-AIEC strains, with the exception of LF1, were not able to induce *IL1RL1* (Fig. 7a). Although two AIEC strains (LF82 and LF73) were able to induce the expression of *IL33*, all the other AIEC and non-AIEC strains failed to up-regulate *IL33* expression (Fig. 7a). These results suggest that the ability of *E. coli* to adhere/invade to IECs is not required for the induction of ST2. Notably, IL-33 induction was not induced by most of CD-associated *E. coli* strains, indicating that the induction of ST2, rather than its ligand IL-33, might be a key fibrosis-initiating event resulting from pathobiont colonization in the context of CD. Next, we sought to identify the microbial factor(s), present in the CD-associated pathobiont LF82, involved in the induction of ST2. We employed the isogenic mutant strains of LF82 that lack FimH (adhesin)²⁰, FliC (flagellin synthesis)²¹, OmpA or OmpC (outer membrane proteins)²². T84 cells were stimulated with WT LF82 or the mutant strains, and the expression of *IL1RL1* and *IL33* was assessed by qPCR. The LF82 *fimH* mutant induced *IL1RL1* at levels similar to WT LF82, indicating that epithelial adhesion is not required for this process (Fig. 7b). Similarly, the *ompA* and *ompC* mutants were able to induce the expression of *IL1RL1*. Notably, the LF82 *fliC* mutant was significantly impaired in its ability to induce *IL1RL1*, suggesting that flagellin is a key

bacterial factor driving the expression of ST2 in IECs. Interestingly, all tested mutant strains, including the LF82 *fliC* mutant, induced IL-33 with equal efficiency as their parental strain WT LF82.

To identify the receptor involved in AIEC-mediated ST2 expression in IECs, we next knocked-down the known flagellin receptors TLR5 and NLRC4 in IECs. The introduction of siRNA significantly reduced the expression of these receptors in T84 cells (Fig. 7c). Our results showed that both TLR5 and NLRC4 are involved in the induction of ST2 by AIEC (Fig. 7d). To validate the role of flagellin in the induction of intestinal fibrosis in the attenuated *Salmonella*/AIEC co-infection model, we next co-infected streptomycin-treated mice with the SPI-2 mutant of *Salmonella* and WT or the *fliC* mutant of LF82 (Fig. 8a). Flagellin production by AIEC did not affect the ability of *Salmonella* to colonize the intestine and *Salmonella* was eliminated from the mice in both WT LF82/ST and *fliC* LF82/ST groups with the same efficiency (Fig. 8b). Likewise, the WT and *fliC* LF82 strains colonized equally well in the inflamed gut, indicating that flagellin is not required for adaptation to the inflammatory microenvironment (Fig. 8b). The expression of pro-inflammatory cytokines in the colonic mucosa was not attenuated in the *fliC* LF82/ST group compared to the WT LF82/ST group (Fig. 8c), suggesting that LF82 is able to augment *Salmonella*-induced inflammation in the absence of flagellin secretion. However, the *fliC* mutant LF82 did not induce the expression of ST2 in the colonic mucosa (Fig. 8d, e). As a result of impaired ST2 induction, the expression of fibrosis-related genes was significantly reduced in the *fliC* LF82/ST group compared to the WT LF82/ST group (Fig. 8f), thereby leading to the attenuated accumulation of collagen in the colon (Fig. 8g, h). These data suggest that AIEC's flagellin promotes intestinal fibrosis.

Discussion

It has become evident that the gut microbiota in IBD patients is significantly perturbed compared to non-IBD subjects⁵. However, the impact of gut dysbiosis on disease outcomes remains poorly understood. A recent report has demonstrated that colonization of mice with dysbiotic microbiotas obtained from CD patients sufficiently recapitulates the pro-inflammatory responses seen in CD patients⁷. Thus, dysbiosis in IBD might be accompanied by an abnormal accumulation of pathobionts whose colonization likely elicits intestinal inflammation. However, the identities of IBD-associated pathobionts remain largely unknown. AIEC is an *E. coli* pathotype found to be more prevalent in the gastrointestinal tract of patients with CD²³. As of now, the consequences of chronic colonization by AIEC in CD patients are incompletely understood.

In the present study, we have demonstrated that persistent gut colonization by the AIEC strain LF82 potentiated the development of intestinal fibrosis in *Salmonella*-induced colitis model in mice. Interestingly, colonization by a human commensal *E. coli* strain HS (a symbiont strain) did not affect intestinal fibrosis, indicating that induction of fibrosis is a specific feature of AIEC. Notably, neither pathobiont nor symbiont *E. coli* strains influenced intestinal inflammation caused by *Salmonella* infection, suggesting that promotion of fibrosis, resulting from AIEC colonization, cannot be explained by considering AIEC-triggered inflammation alone. One possible explanation is that AIEC adapts better to

intestinal inflammation compared to the commensal *E. coli* strain and an increased abundance of *E. coli* might lead to intestinal fibrosis regardless of AIEC signatures. In this context, we have found that *Salmonella*-induced intestinal inflammation facilitated LF82 colonization, while not affecting HS. Neither *E. coli* strain was able to stably colonize the intestine in the absence of *Salmonella*. In IBD patients, it is well-documented that *Enterobacteriaceae* (phylum Proteobacteria) are over-represented in the gut microbiota⁵. In general, a healthy gut microbiota consists of obligate anaerobes, such as *Clostridiales* and *Bacteroidales*, while bacteria belonging to the family *Enterobacteriaceae*, including *E. coli*, are only minor constituents. On the other hand, intestinal inflammation tends to foster blooms of *Enterobacteriaceae* at the expense of obligate anaerobes. Intestinal inflammation elicits the infiltration of inflammatory immune cells in the mucosa, such as neutrophils. These immune cells express oxidative enzymes (e.g., iNOS, NOX1, DUOX2, PHOX, SOD, and MPO) capable of generating antimicrobial radicals, such as superoxide, peroxide, hypochloride, nitric oxide, and peroxyxynitrite. Further oxidation of these molecules yields nitrates, N-oxides, S-oxides and other compounds that serve as electron acceptors. Facultative anaerobes, including *Enterobacteriaceae*, rely on these products to utilize anaerobic respiration in the intestine^{24,25}. Since both AIEC and commensal *E. coli* can utilize anaerobic respiration, it is conceivable that AIEC employs specific, as yet unknown, mechanisms that allow it to adapt to the inflammatory environment with greater efficiency. Further study is needed to unravel the unique metabolic pathways utilized by AIEC to adapt to the inflammatory environment.

In this study, we have demonstrated that AIEC colonization elicits expression of the IL-33 receptor ST2 in IECs, which in turn augments IL-33 signaling and promotes the development of intestinal fibrosis. IL-33 is a member of the IL-1 cytokine family that has been discovered relatively recently¹⁵. IL-33 engages its receptor ST2 expressed on various immune and non-immune cells, and activates its downstream signaling¹⁵. The IL-33-ST2 signaling pathway is considered to be crucial for type 2 immune responses since it induces the production of IL-5 and IL-13 in Th2 cells and/or granulocytes. IL-33 is a protective cytokine important for promotion of wound healing¹⁵. However, excessive tissue regeneration mediated by IL-33 can lead to the development of fibrosis in various organs, such as liver cirrhosis, systemic sclerosis and pulmonary fibrosis^{26–28}. Thus, the role of IL-33-ST2 in IBD remains controversial. Experimental animal IBD models have been used to demonstrate that IL-33-ST2 signaling promotes intestinal inflammation via enhancing IL-4²⁹. On the other hand, mice deficient in ST2 develop more severe colitis than control mice in DSS-induced and TNBS-induced IBD models, indicating that IL-33-ST2 signaling has a protective role¹⁸. Likewise, a recent study has also reported that IL-33 aids tissue healing in the gut by enhancing the differentiation of goblet cells and M2 type macrophages, thus protecting mice from DSS and TNBS-induced colitis¹⁸. This discrepancy might in part be due to variability in the genetic backgrounds of experimental animals. The former study utilized Balb/c (Th2 skewed) animals whereas C57BL/6 (Th1 skewed) animals were used in the latter two studies. This may indicate that the IL-33-ST2 pathway plays different roles in UC and CD, as these two disease forms are associated with differently skewed adaptive immunity¹. In human IBD, IL-33 expression is reported to be elevated in the inflamed mucosa, mainly in UC and, to a lesser extent, in CD^{30,31}. In contrast, blood levels of IL-33

are found to decrease only in UC patients as compared to healthy control subjects³². Additionally, blood levels of soluble ST2 (sST2), which antagonizes the activity of IL-33, are elevated in both UC and CD³². In this study, we analyzed the publically available GEO dataset to discover that the expression of both IL-33 and ST2 is up-regulated in the ileal mucosa of patients with active CD, although this analysis cannot discriminate between soluble and membrane-bound ST2, which play opposite roles with respect to IL-33 signaling.

Various cell types are known to express membrane-bound ST2. ST2 is involved in IL-33 signaling and is primarily expressed, under normal conditions, in IECs in both mice and humans¹⁸. During intestinal inflammation, ST2-positive cells can also be found in subepithelial infiltrates in both murine colitis models as well as human IBD patients¹⁸. Therefore, it is possible that other ST2-expressing cells also contribute to AIEC-induced intestinal fibrosis. Recent accumulating evidence suggests that group 2 innate lymphoid cells (ILC2), known to express high levels of ST2, play a crucial role in the development of fibrosis via activating Th2 immune responses, as demonstrated in various models of fibrosis, such as liver and lung^{26,33}. However, a recent report has shown ILC2 to be dispensable for the development of intestinal fibrosis³⁴. Thus, ILC2 might not be involved in AIEC-induced intestinal fibrosis.

In this study, we have been able to demonstrate that AIEC colonization enhances ST2 expression in IECs, which in turn augments the sensing of IL-33 in the intestine. However, the identity of pathways downstream of IL-33-ST2 that leads to fibrosis remains elusive. Since the blocking of ST2 resulted in a decreased expression of TGF- β and collagen in the intestine, AIEC-mediated activation of the IL-33-ST2 pathway might eventually lead to the up-regulation of TGF- β , thereby promoting collagen deposition in fibroblasts. In the lung, pulmonary epithelial cells express TGF- β ³⁵. Thus, it is possible that IECs produce TGF- β in response to IL-33 and promote collagen expression in fibroblasts through mutual interaction. Another possibility is that IL-33 signaling in IECs alters the function of IECs, which in turn induce the activation and/or differentiation of other TGF- β producing cells, such as macrophage/dendritic cells (DCs). For example, thymic stromal lymphoprotein (TSLP), which is induced by IL-33, is known to condition DCs and skew them toward the type 2 phenotype, including up-regulation of TGF- β expression³⁶. It follows, then, that TSLP conditioned-DCs might mediate pro-fibrotic responses. Further research is required to identify the downstream mediators that link IL-33 signaling and intestinal fibrosis.

Our present results indicate that flagellin, a principal component of bacterial flagella, is a key molecule produced by AIEC that activates the expression of ST2 in IECs and subsequently causes the development of fibrosis. The role of bacterial flagella in locomotion is well documented³⁷. A decrease in the expression of type 1 pili, required for cell adhesion, has recently been observed in an isogenic mutant of LF82 that lacks *fliC*, the gene that encodes flagellin³⁷. As a result, the fitness and virulence of the LF82 *fliC* mutant were significantly impaired both *in vitro* and *in vivo*³⁷. In our study, the ST2 inducing capacity of AIEC LF82 remained intact when the *fimH* gene, which encodes adhesin and thus facilitates AIEC adhesion to IECs, was mutated. Consistently, a non-AIEC strain LF1, which cannot adhere/invade IECs, was also capable of inducing the expression of ST2 in IECs. It has been

reported that TLR5 plays an important role in the sensing of AIEC³⁸. Consistently, we found that AIEC colonization induces ST2 expression in IECs through TLR5. In addition to TLR5, our result indicates that the activation of NLRC4 signaling by flagellin may also be used for the induction of ST2.

Our study has demonstrated that flagellin, produced by AIEC, is critical for the induction of ST2 expression in IECs. However, it remains unclear why only AIEC induces ST2 and promotes intestinal fibrosis; after all, both AIEC and commensal *E. coli* HS have flagellin. In this regard, it has been recently shown that AIEC LF82, unlike commensal *E. coli* HS, produces flagellin when exposed to intestinal mucus³⁷. Thus, it is conceivable that AIEC produces more flagellin than commensal *E. coli* and this hypothesis would explain why only AIEC elicits flagellin-mediated pro-fibrotic signaling and subsequent development of intestinal fibrosis. Related to this notion, it also remains unclear why infection with the SPI-2 *Salmonella* mutant *per se* fails to induce fibrosis, since it also harbors flagellin. A simple explanation is that colonization of the mutant *Salmonella* peaked on day 2 post infection and was dramatically eradicated in the later phase of infection. On the other hand, AIEC colonization persisted longer and its abundance in the later phase of infection was higher than that of *Salmonella*. Thus, persistent colonization by a flagellated pathobiont resulted in intestinal fibrosis. Another possibility may be related to the capacity of the bacterium to survive intracellularly. It has been reported that SPI-2 is essential for the survival of the pathogen in phagocytic cells, such as macrophages³⁹. Since AIEC can survive within infected macrophages⁸, it is possible that intracellular survival of pathobionts within intestinal macrophages contributes to pro-fibrotic events induced by IL-33-ST2 signaling. However, it is largely unclear how macrophage activation by AIEC affects ST2 induction in the colonic epithelium. Thus, further study is required to fully understand the mechanisms by which CD-associated pathobionts induce intestinal fibrosis.

Similarly, the extent to which other bacteria, in addition to AIEC, contribute to susceptibility to intestinal fibrosis in CD patients remains uncertain. In this regard, it has been repeatedly reported that flagellated bacteria are enriched and the luminal flagellin load is elevated in CD patients compared to normal controls^{7,40}. Thus, dysbiotic microbiotas rich in flagellated bacteria are able to elicit intestinal fibrosis regardless of the abundance of AIEC. Consistently, colonization of germ-free mice with the gut microbiotas isolated from CD patients leads to ST2 expression in the colon⁷. Thus, CD-associated pathobionts, including AIEC, contribute to the development of intestinal fibrosis via production of flagellin.

Taken together, we have unveiled a novel link between gut dysbiosis and the risk for intestinal fibrosis. Treatment of gut dysbiosis by antibiotics, probiotics, or fecal microbiota transplantation to eradicate AIEC and/or other flagellated pathobionts could be a novel therapeutic approach used to prevent intestinal fibrosis in CD. Alternatively, blockade of IL-33-ST2 signaling has the potential to be a treatment option for fibrosis in the context of CD patients that does not perturb the gut microbiota.

Materials and Methods

Animals

Eight to ten week-old specific pathogen-free (SPF) wild-type C57BL/6 mice were originally purchased from Jackson Laboratories. Mice were then bred and maintained in a specific pathogen-free (SPF) animal facility at the University of Michigan. All animal studies were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Michigan.

Bacterial strains

Kanamycin- and streptomycin (Strep)-resistant *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) SL1344 *spiA*⁴¹ was used in mouse infection experiments. *Escherichia coli* strains used in this study were commensal *E. coli* strain HS¹⁴, AIEC strains LF82, LF16, LF31 and LF73 isolated from CD patients⁸, and non-AIEC-type strains LF1, LF6 and LF19 isolated from CD patients. Mutant LF82 strains *fimH*, *fliC*, *ompA*, and *ompC* were also used. All bacteria were grown in Luria-Bertani (LB) broth at 37°C with shaking (180 rpm).

Salmonella infection and E. coli colonization

Mice were pre-treated with 20 mg/100 β l/mouse of streptomycin (Strep) 1 day prior to *E. coli* inoculation. Strep-treated mice were then inoculated with human *E. coli* strain HS or LF82 (1×10^9 CFU). Seven days after *E. coli* colonization, mice were infected with 2×10^7 CFU of *S. Typhimurium* SL1344 *spiA*. To determine bacterial numbers in the feces, fecal pellets were collected from individual mice, homogenized in cold PBS, and plated at serial dilutions on MacConkey agar plates containing 50 μ g/ml Strep or ampicillin (Amp). CFU counts were determined after overnight incubation at 37°C. IL-33-ST2 signaling *in vivo* was blocked by the intraperitoneal administration of 50 μ g of an anti-mouse ST2 antibody or control rat IgG (Genentech, Inc) 3 times per week. 21 days following *Salmonella* infection, colonic tissues were harvested and fixed in buffered 4% paraformaldehyde. Fixed tissues were then embedded in paraffin, sectioned, and stained with hematoxylin and eosin or picro-sirius red (Picosciences, Inc.).

Quantitative real-time PCR

RNA was extracted from colonic tissues using the E.Z.N.A.® Total RNA Kit I (Omega Bio-tek). Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Quantitative PCR was performed with SYBR Green qPCR Kits (Radiant molecular tolls). The primers used are listed in supplementary table 1. The cycling conditions were 95°C for 3 mins and then 39 cycles of 95°C for 15 sec, 60°C for 1 min. The relative mRNA expression of target gene was normalized against that of the β -actin gene in the same RNA preparation. The following primer sets were used for amplifications: Human *hIL1RL1*-Fw; 5'- CTG TCT GGC CCT GAA TTT GC -3', Human *IL1RL1*-Rv; 5'- AGC AGA GTG GCC TCA ATC CA -3', Human *IL33*-Fw; 5'- ATC CCA ACA GAA GGC CAA AG -3', Human *IL33*-Rv; 5'- CCA AAG GCA AAG CAC TCC AC -3', Human *TGFBI*-Fw; 5'- CTA ATG GTG GAA ACC CAC AAC G -3', Human *TGFBI*-

Rv; 5'- TAT CGC CAG GAA TTG TTG CTG -3', Human *COL1A2*-Fw; 5'- TCT GGA TGG ATT GAA GGG ACA -3', Human *COL1A2*-Rv; 5'- CCA ACA CGT CCT CTC TCA CC -3', Human *TNF*-Fw; 5'- GCC TCT TCT CAT TCC TGC TT -3', Human *TNF*-Rv; 5'- TGG GAA CTT CTC ATC CCT TTG -3', Human *Actb*-Fw; 5'- CTC TTC CAG CCT TCC TTC CTG -3', Human *Actb*-Rv; 5'- GAA GCA TTT GCG GTG GAC GAT -3'. Mouse *Il1rl1*-Fw; 5'- AGA CCT GTT ACC TGG GCA AG -3', Mouse *Il1rl1*-Rv; 5'- CAC CTG TCT TCT GCT ATT CTG G -3', Mouse *Il33*-Fw; 5'- TCC TTG CTT GGC AGT ATC CA -3', Mouse *Il33*-Rv; 5'- TGC TCA ATG TGT CAA CAG ACG -3', Mouse *Tgfb1*-Fw; 5'- CTC CCG TGG CTT CTA GTG C -3', Mouse *Tgfb1*-Rv; 5'- GCC TTA GTT TGG ACA GGA TCT G -3', Mouse *Col1a2*-Fw; 5'- TGG ATA CGC GGA CTC TGT TG -3', Mouse *Col1a2*-Rv; 5'- CCC TTT CGT ACT GAT CCC GAT T -3', Mouse *Tnf*-Fw; 5'- GCC TCC CTC TCA TCA GTT CT -3', Mouse *Tnf*-Rv; 5'- CAC TTG GTG GTT TGC TAC GA -3', Mouse *Il6*-Fw; 5'- GAG GAT ACC ACT CCC AAC AGA CC -3', Mouse *Il6*-Rv; 5'- AAG TGC ATC ATC GTT GTT CAT ACA -3', Mouse *Ifng*-Fw; 5'- ATC TGG AGG AAC TGG CAA AA -3', Mouse *Ifng*-Rv; 5'- TTC AAG ACT TCA AAG AGT CTG AGG TA -3', Mouse *Il17a*-Fw; 5'- CAG GGA GAG CTT CAT CTG TGT -3', Mouse *Il17a*-Rv; 5'- GCT GAG CTT TGA GGG ATG AT -3', Mouse *Actb*-Fw; 5'- AAG TGT GAC GTT GAC ATC CG -3', Mouse *Actb*-Rv; 5'- GAT CCA CAT CTG CTG GAA GG -3'.

Collagen assay

Colonic specimens were homogenized in 2.5M acetic acid containing 0.1 mg/ml pepsin and stored at -80°C until sufficient quantity was obtained. The samples were then dried overnight at 37°C. Total soluble collagen was quantified by Soluble Collagen Assay (Cell Biolabs, Inc).

Cell culture

Human primary colonic fibroblasts were isolated from disease-free surgical margins found in surgically resected colonic specimens obtained from a diverticulitis patient. Human fibroblasts were then cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin/streptomycin). Human colon carcinoma cells (T84) were grown in Ham's F12 medium + DMEM (1:1) supplemented with 10% FBS and antibiotics. Cells (1×10^5 cells/500 μ l /well; 24 well plate) were stimulated with *E. coli* at a MOI=10 for 3 hr followed by 15 hrs of additional culture in the presence of gentamycin (100 μ g/ml) to prevent bacterial overgrowth.

Knock-down of flagellin receptors

SMARTpool siRNA for human *TLR5* and *NLRC4* were purchased from Dharmacon. The siRNAs or Non-targeting control siRNA (Dharmacon) were transfected to T84 cells using Lipofectamine RNAiMAX (Life Technologies) according to the manufacturers' instructions. Two days post-transfection, the cells were stimulated with *E. coli* (MOI=10) for 3 hr followed by 15 hrs of additional culture in the presence of gentamycin (100 μ g/ml).

Immunohistochemistry

The colonic tissues were harvested, fixed with 4% paraformaldehyde and embedded in paraffin. The tissue samples were then cut into 4 μm thick sections. Rehydrated paraffin sections were boiled in a microwave oven for epitope retrieval using a sodium citrate buffer (pH 6, 10 min). Slides were stained with an anti-mouse ST2 antibody (Abcam, ab25877, 1:500 dilution) overnight at 4°C and then incubated with a rabbit HRP polyclonal antibody for 1 h at room temperature. HRP was then visualized with DAB (VECTOR).

Statistical Analyses

Statistical analyses were performed using GraphPad Prism software version 5.0 (GraphPad Software, Inc, San Diego, CA). Differences between 2 groups were evaluated using Student's *t* test (parametric) or the Mann–Whitney *U* test (nonparametric). For comparisons of more than 3 groups, statistical analysis was performed using 1-way analysis of variance (parametric) or the Kruskal–Wallis test (nonparametric), followed by the Bonferroni correction for parametric samples, or the Dunn test for nonparametric samples as a post hoc test. *P* values less than 0.05 were considered significant.

Acknowledgments

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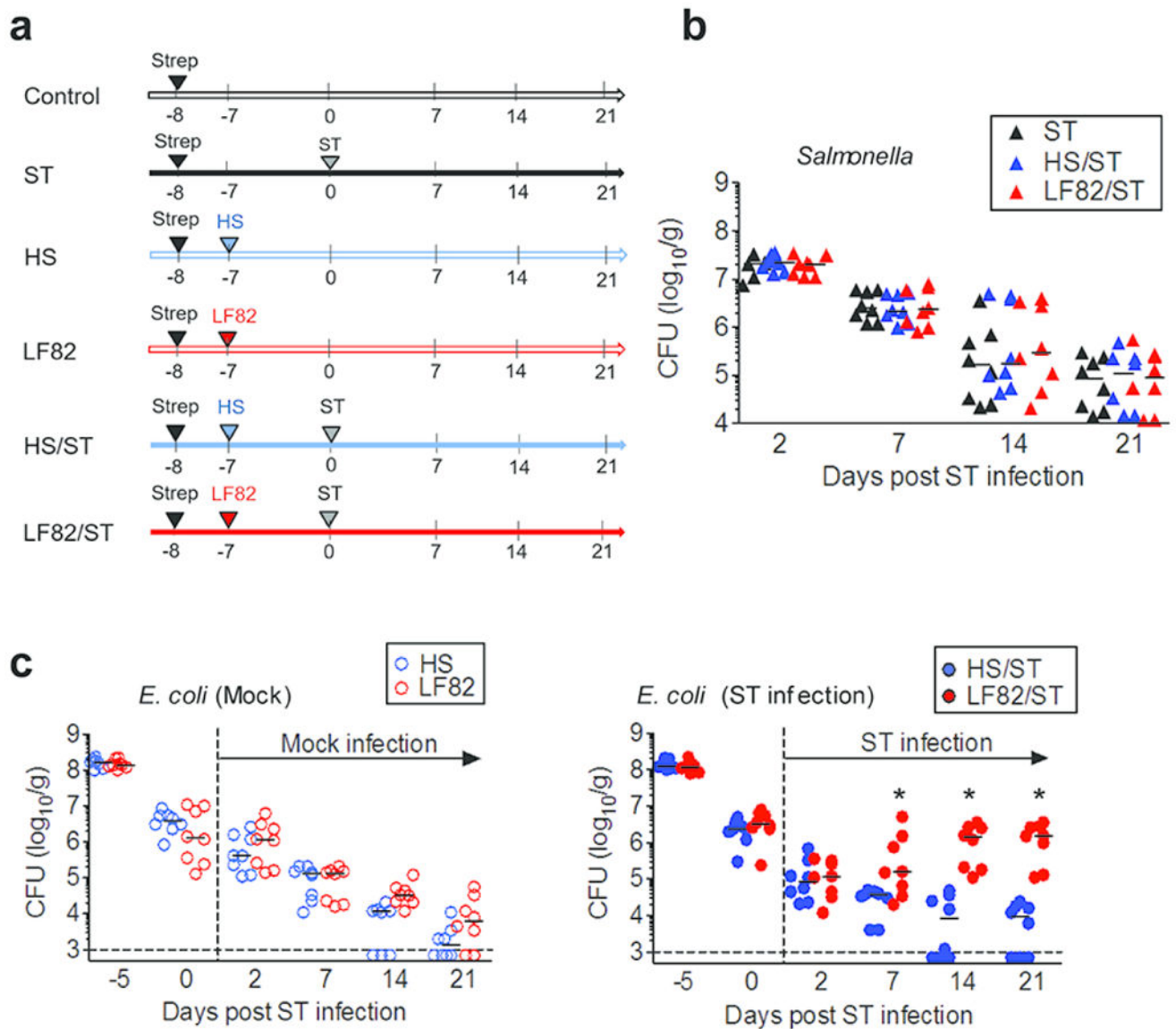


Figure 1. AIEC colonization persists in the inflamed intestine.

(a) SPF C57BL/6 mice were pre-treated with 20mg of streptomycin (Strep) 1 day prior to *E. coli* inoculation. Strep-treated mice were then inoculated with human commensal *E. coli* strain HS or human AIEC strain LF82 (1×10^9 CFU). The control and ST groups only received PBS. Seven days following *E. coli* colonization, mice were infected with 2×10^7 CFU of *S. Typhimurium spiA* (ST). (b, c) Intestinal colonization (feces) of *S. Typhimurium spiA* (b) and pre-colonized *E. coli* strains on indicated days post ST infection. Dots represent individual mice. Bars represent median. *: $P < 0.05$ by 2-Way ANOVA with Bonferroni post-hoc test.

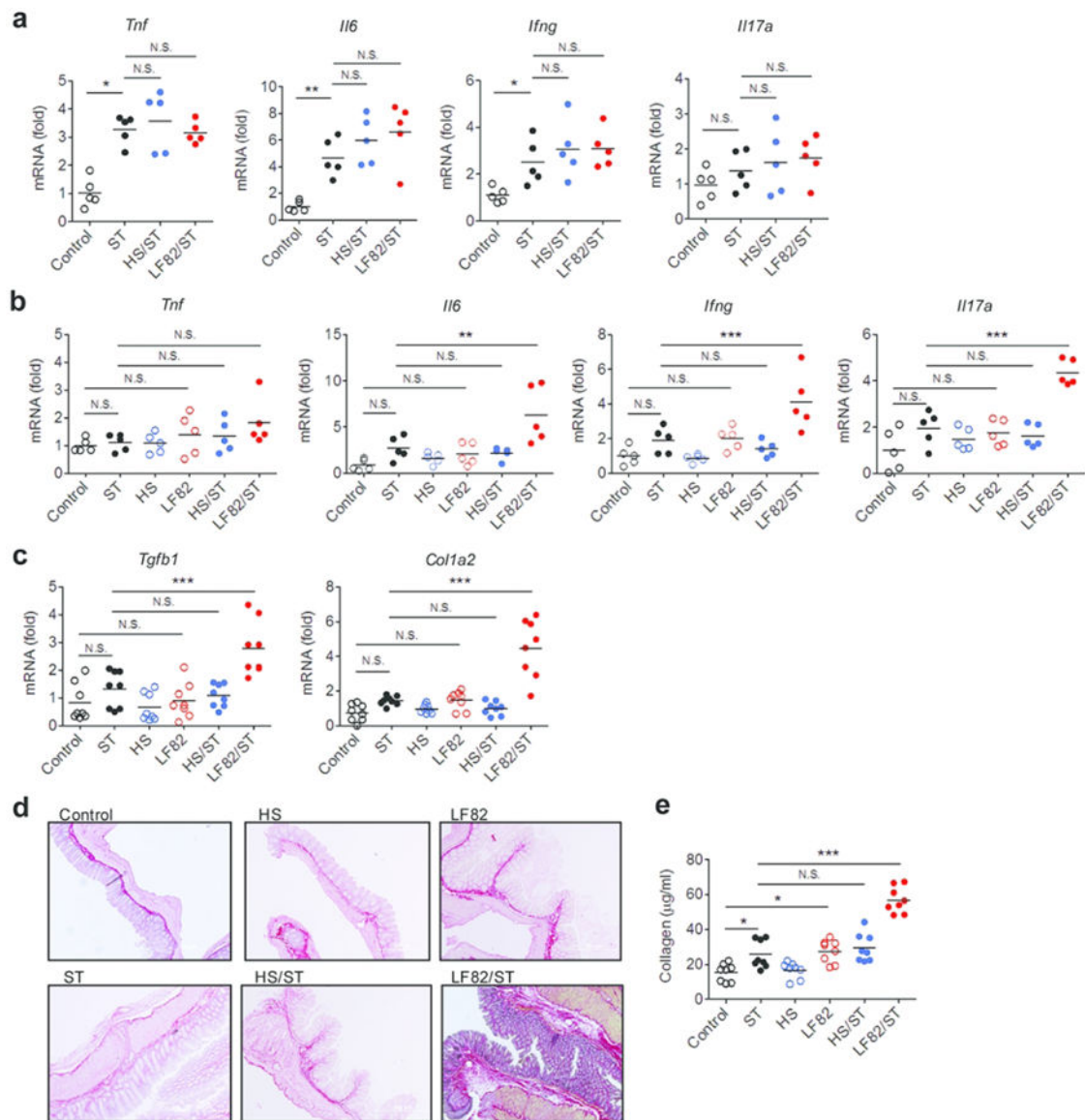


Figure 2. AIEC colonization promotes intestinal fibrosis in *Salmonella*-infected mice. *S. Typhimurium spiA* (ST) and *E. coli* (HS or LF82) infected mice were sacrificed on day 2 (a) and on day 21 post ST infection (b–e). Colonic tissues were harvested on day 2 (a) and on day 21 (b, c) post ST infection and mRNA expression of indicated cytokines was quantified by qPCR. The accumulation of collagen was examined by Picrosirius red staining on day 21 post ST infection (d) and collagen assay (e). Dots represent individual mice. Bars represent mean. N.S.; not significant, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ by 1-Way ANOVA with Bonferroni post-hoc test.

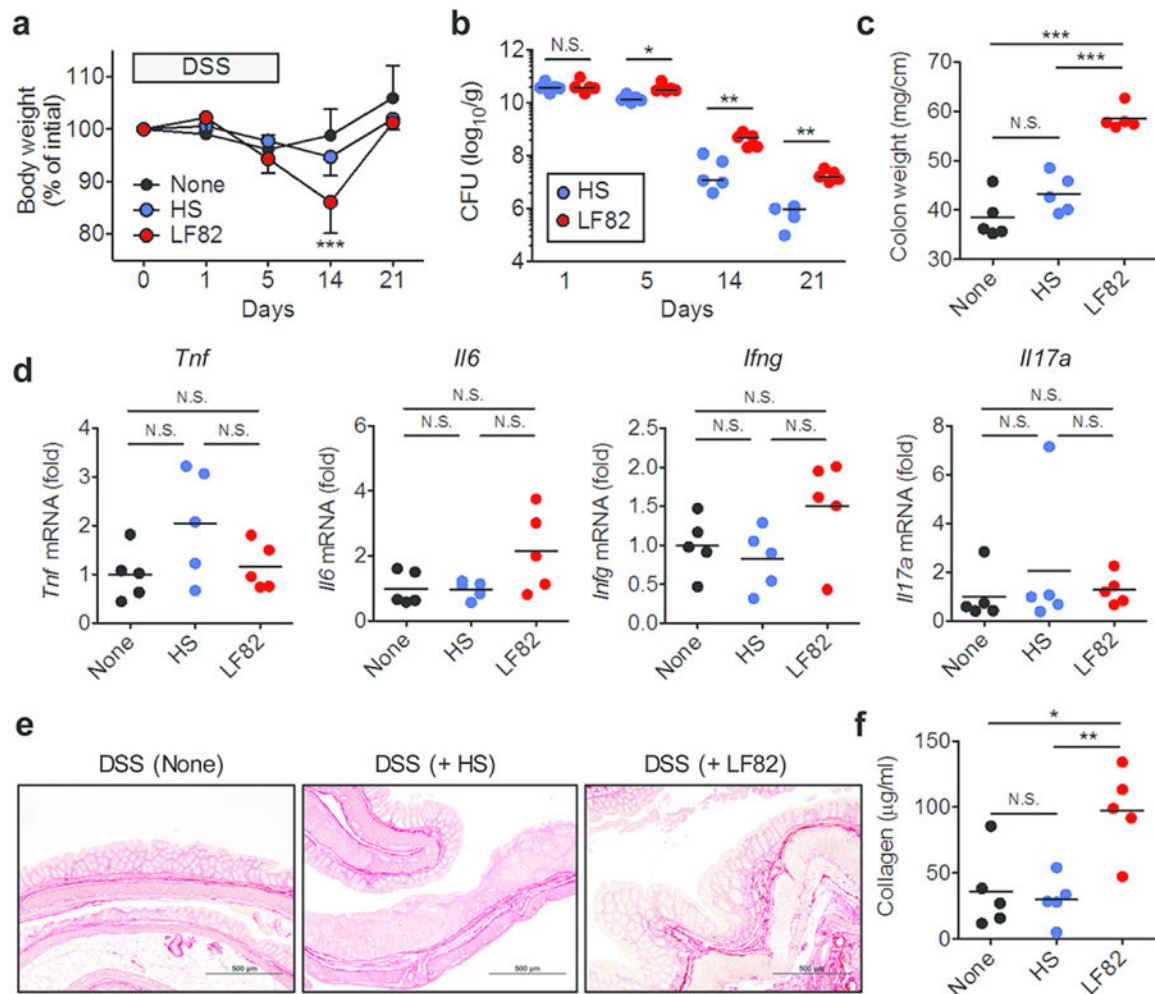


Figure 3. AIEC colonization promotes intestinal fibrosis in DSS-induced colitis mice.

SPF C57BL/6 mice were pre-treated with 20mg of streptomycin (Strep) 1 day prior to *E. coli* inoculation. Strep-treated mice were then inoculated with human commensal *E. coli* strain HS or human AIEC strain LF82 (1×10^9 CFU each). *E. coli*-colonized mice were then treated with 3.0% DSS for 5 days followed by regular water. Mice were sacrificed 21 days post DSS administration. **(a)** Body weight change. Data shown are mean \pm SD (N=5). ***; $P < 0.001$ by 2-Way ANOVA with Bonferroni post-hoc test (vs None). **(b)** Intestinal colonization (feces) of *E. coli* strains. Dots represent individual mice. Bars represent median. N.S.; not significant, *, $P < 0.05$, **, $P < 0.01$ by Man-Whitney *U* test. **(c)** Colon weight (per length) at day 21. **(d)** The mRNA expression of pro-inflammatory cytokines in the colonic mucosa. **(e, f)** The accumulation of collagen was examined by Picosirius red staining on day 21 **(e)** and collagen assay **(f)**. **(c-f)** Dots represent individual mice (mean \pm SD). N.S.; not significant, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ by 1-Way ANOVA with Bonferroni post-hoc test.

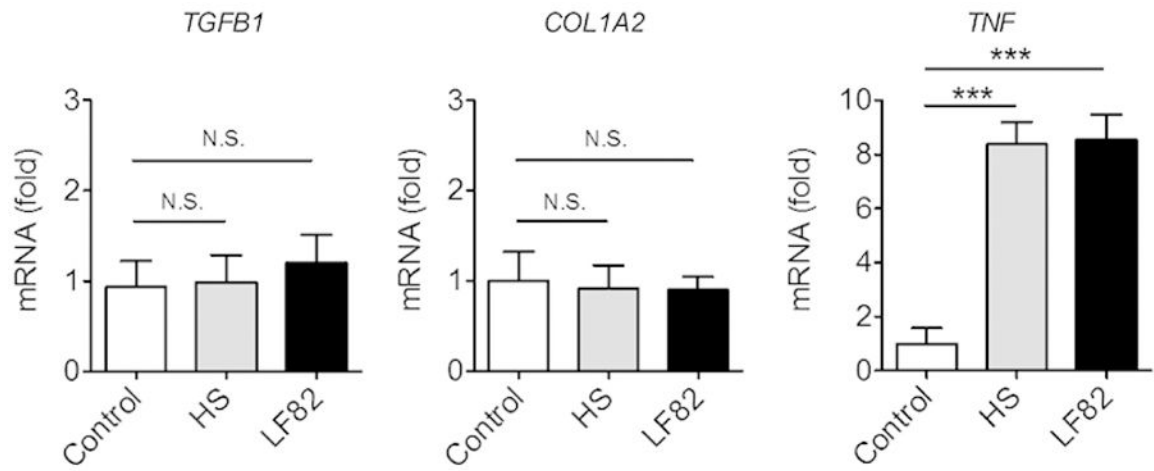


Figure 4. AIEC does not directly promote collagen deposition in colonic fibroblasts.

Human primary colonic fibroblasts (1×10^4 cells/500 μ l/well; 24 well plate) were stimulated with *E. coli* HS or LF82 at MOI=10 for 3 hr followed by 15 hrs of additional culture in the presence of gentamycin (100 μ g/ml) to prevent bacterial overgrowth. The expression of fibrosis-related mediators and a pro-inflammatory cytokine was quantified by qPCR. Data shown are mean \pm SD (N=6). *, P < 0.05 by 1-Way ANOVA with Bonferroni post-hoc test.

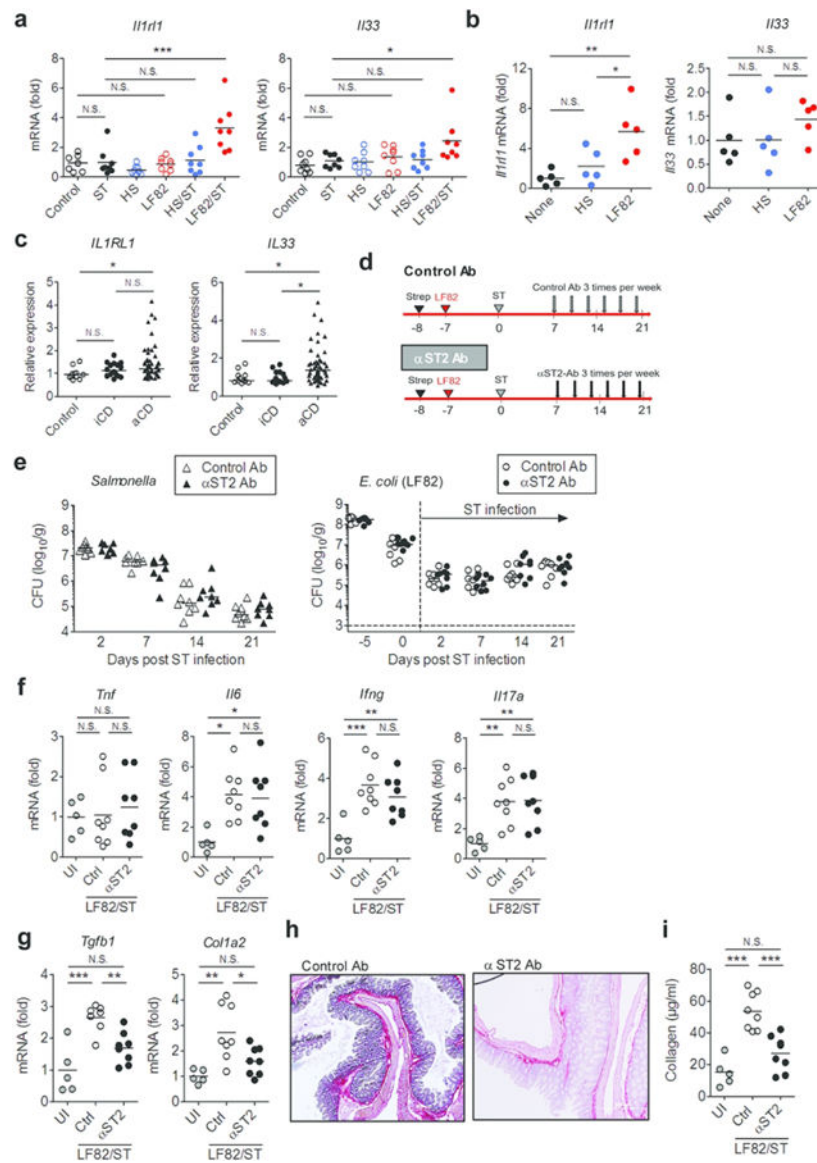


Figure 5. AIEC colonization promotes intestinal fibrosis via the activation of IL-33-ST2 signaling.

(a, b) The mRNA expression of IL-33 (*Ii33*) and its receptor ST2 (*Ii1rl1*) in the colonic mucosa *Salmonella/E.coli* co-colonization (day 21) (a) and DSS + *E. coli* (day 21) (b) models. (c) mRNA expression of ST2 (*IL1RL1*) and IL-33 (*IL33*) genes in the ileal tissue from control subjects (N=11), patients with inactive CD (N=16) and active CD (N=51). Data were derived from Gene Expression Omnibus (GEO) dataset GSE75214. Bars represent median. N.S.; not significant, *, P < 0.05, **, P < 0.01, ***, P < 0.001 by Kruskal-Wallis test. (d) Mice pre-colonized with AIEC strain LF82 were infected with *S. Typhimurium spiA* (ST). Anti-ST2 blocking antibody and isotype control IgG (50µg/mouse) were injected intra-peritoneally 3 times per week starting on day 7 post *Salmonella* infection (6 times total). Mice were sacrificed 21 days following ST infection. (e) Intestinal colonization (feces) of *S. Typhimurium spiA* (left) and pre-colonized *E. coli* LF82 (right) on indicated days following ST infection. (f) mRNA expression of indicated cytokines 21 days post ST

infection. **(g)** Expression of fibrosis-related genes in the colonic mucosa (day 21). **(h, i)** Accumulation of collagen was quantified by Picro-sirius red staining **(h)** and collagen assay **(i)**. Dots represent individual mice. Bars represent median **(e)** or mean **(f, g, i)**. N.S.; not significant, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ by 1-Way ANOVA with Bonferroni post-hoc test.

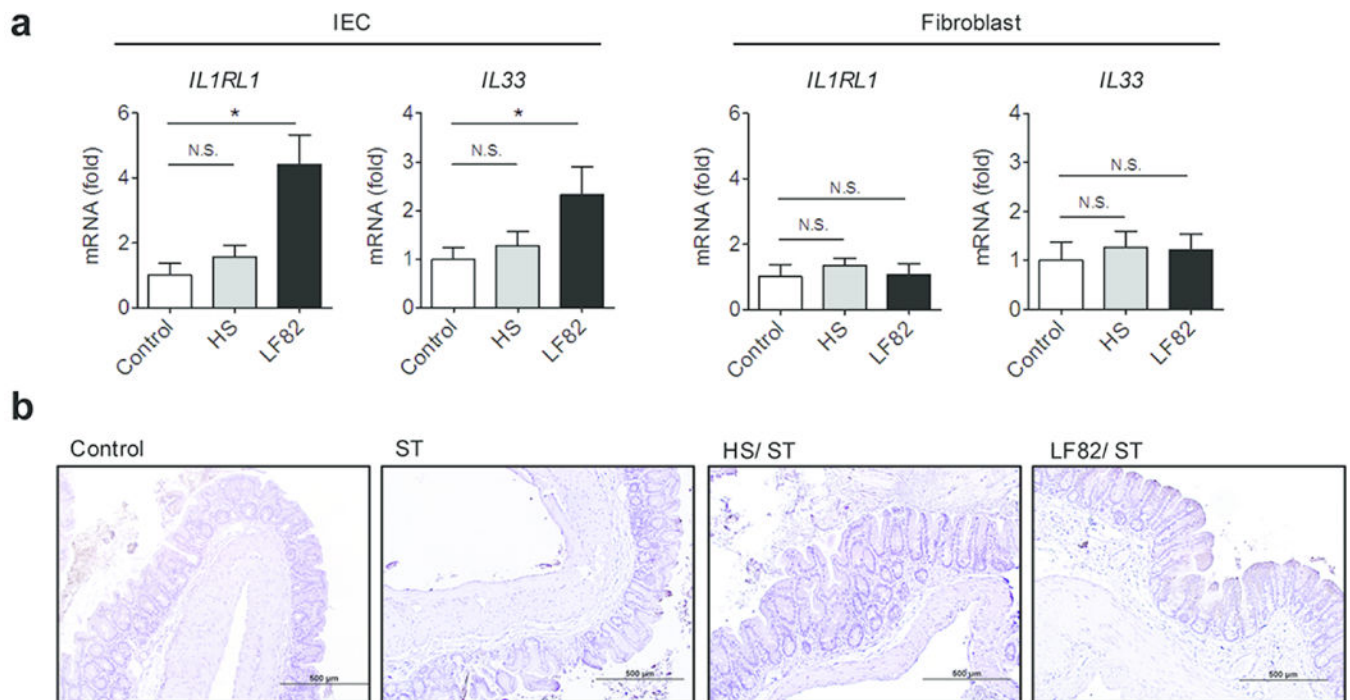


Figure 6. CD-associated *E. coli* strains induce ST2 expression in IECs.

(a) Human IEC T84 cells and primary colonic fibroblasts (1×10^5 cells/500 μ l/well; 24 well plate) were stimulated with *E. coli* HS or LF82 at MOI=10 for 3 hr followed by 15 hrs of additional culture in the presence of gentamycin (100 μ g/ml) to prevent bacterial overgrowth. mRNA expression of IL-33 (*IL33*) and ST2 (*IL1RL1*) was quantified by qPCR. Data shown are mean \pm SD (N=6). N.S.; not significant, *, $P < 0.05$ by 1-Way ANOVA with Bonferroni post-hoc test. (b) Strep-treated mice were then inoculated with human commensal *E. coli* strain HS or human AIEC strain LF82 (1×10^9 CFU). The control and ST groups only received PBS. Seven days following *E. coli* colonization, mice were infected with 2×10^7 CFU of *S. Typhimurium spiA* (ST). At day 21 post ST infection, the colonic tissues were harvested and ST2 was stained. Representative images of ST2 immunohistochemistry are shown.

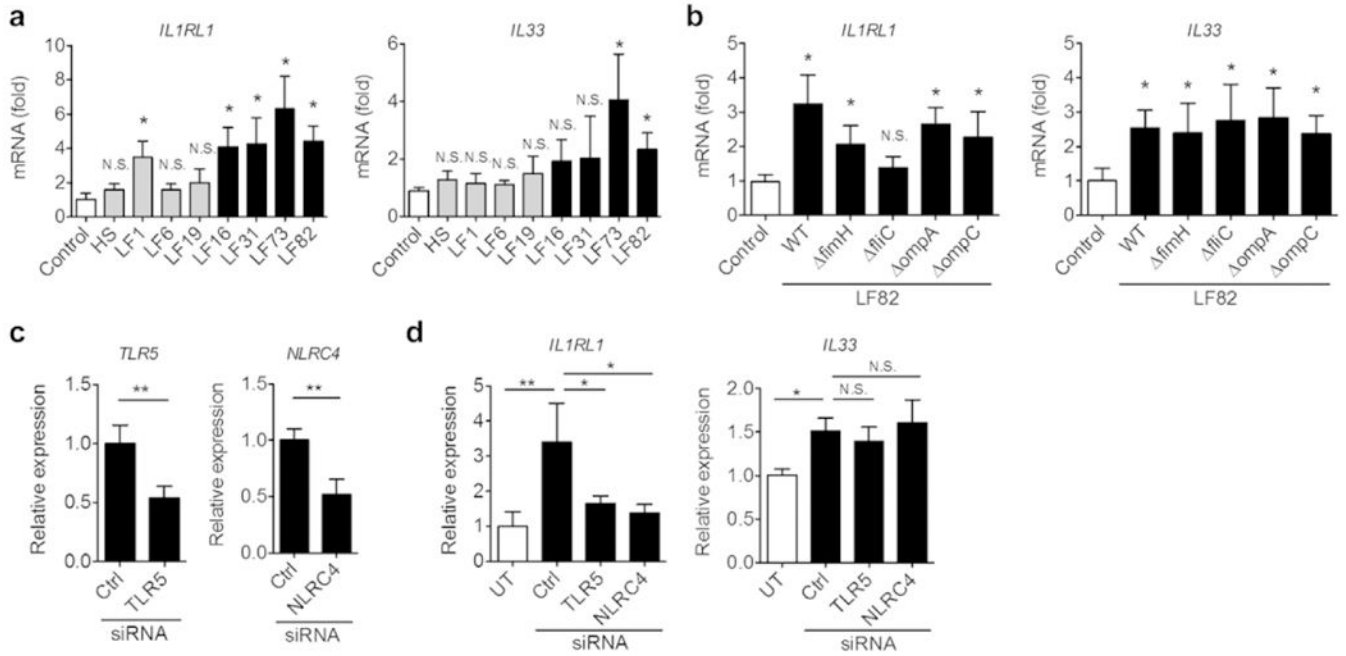


Figure 7. The induction of ST2 by AIEC is mediated by flagellin

(a) T84 cells were stimulated with human commensal *E. coli* strain HS or *E. coli* strains isolated from CD patients. Gray bars indicate non-AIEC strains and black bars indicate AIEC strains based on adhesion/invasion assay. (b) T84 cells were stimulated with AIEC LF82 or various mutants of LF82 at MOI=10 for 3 hr followed by 15 hrs of additional culture in the presence of gentamycin (100 μg/ml) to prevent bacterial overgrowth. mRNA expression of IL-33 (*IL33*) and ST2 (*IL1RL1*) was quantified by qPCR. Data shown are mean ± SD (N=6). N.S.; not significant, *, P < 0.05 by Dunnett test (vs control). (c) *TLR5* and *NLRC4* mRNA were knocked-down in T84 cells using siRNA specific to human *TLR5* or *NLRC4*. Relative expression to Ctrl Non-targeting pool siRNA are shown. Data shown are mean ± SD (N=3). **, P < 0.01 by Student-t test. (d) *TLR5* and *NLRC4* mRNA were knocked-down in T84 cells using siRNA. Cells were then stimulated with AIEC LF82 at MOI=10 or medium (untreated; UT) for 3 hr followed by 15 hrs of additional culture in the presence of gentamycin (100 mg/ml) to prevent bacterial overgrowth. mRNA expression of ST2 (*IL1RL1*) and IL-33 (*IL33*) was quantified by qPCR. Data shown are mean ± SD (N=3). N.S.; not significant, *, P < 0.05, **, P < 0.01 by 1-Way ANOVA with Bonferroni post-hoc test.

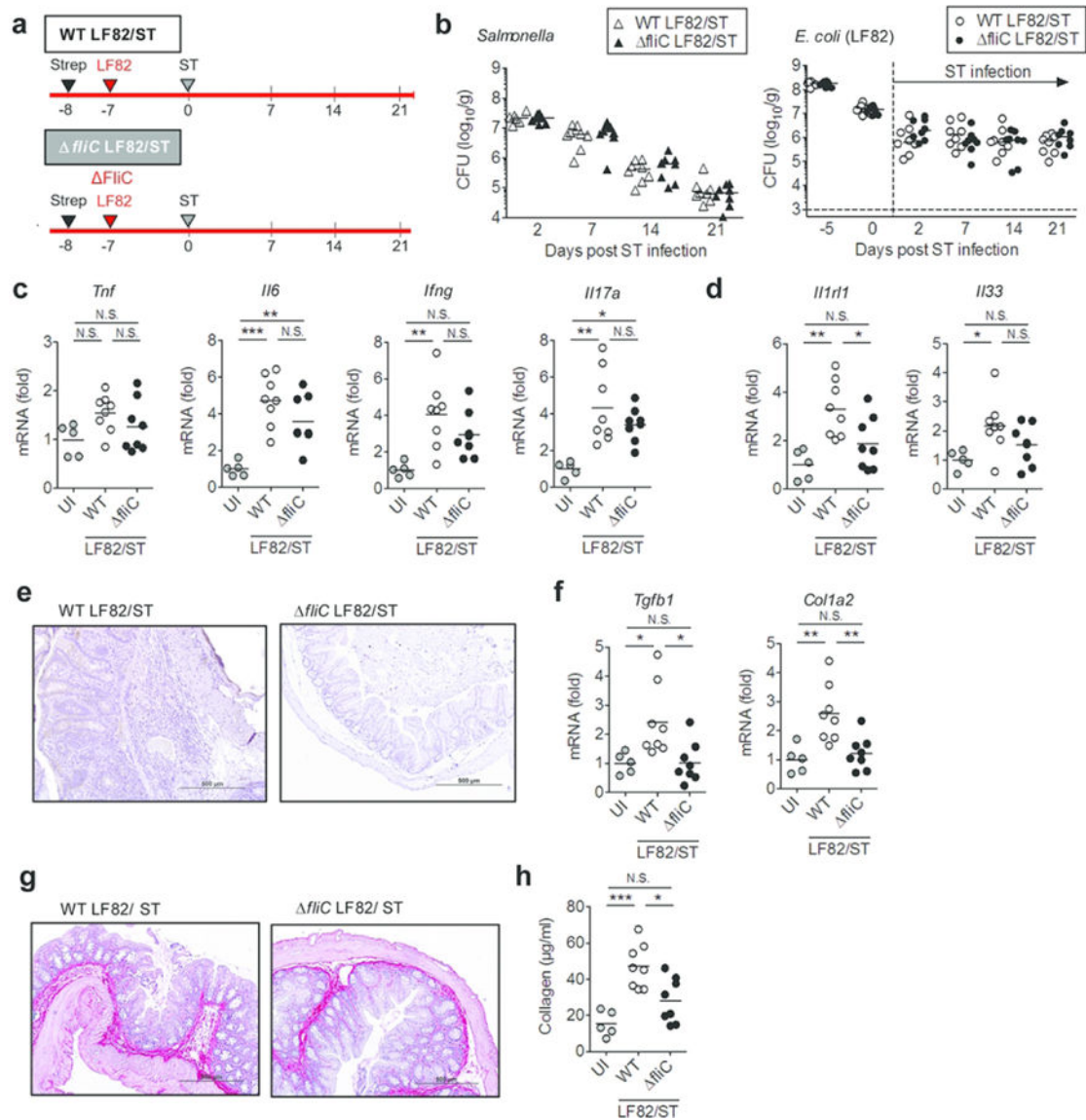


Figure 8. Flagellin is required to promote intestinal fibrosis by a CD-associated *E. coli*. (a) SPF C57BL/6 mice were pre-treated with 20mg of streptomycin (Strep) 1 day prior to *E. coli* inoculation. A CD-associated AIEC strain LF82 (WT or *fliC* mutant) strains (1×10^9 CFU) were then inoculated to Strep-treated mice. Seven days after AIEC colonization, mice were infected with 2×10^7 CFU of *Salmonella typhimurium spiA* (ST). (b) Intestinal colonization (in feces) of ST and AIEC LF82 (WT or *fliC* mutant) at indicated days post ST infection. Dots represent individual mice. Bars represent median. (c) Expression of inflammation-related genes in the colonic mucosa (day 21 ST). (d) The mRNA expression of IL-33 (*Il33*) and its receptor ST2 (*Il1r1*) in the colonic mucosa. (e) Representative images of ST2 immunohistochemistry (day 21). (f) Expression of fibrosis-related genes in the colonic mucosa (day 21 ST). (g, h) Accumulation of collagen was examined by Sirius red staining (g) and collagen assay (h). Dots represent individual mice. Bars represent mean.

N.S.; not significant, *; $P < 0.05$, **; $P < 0.01$, ***; $P < 0.001$ by 1-Way ANOVA with Bonferroni post-hoc test.

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