Intestinal Fibrosis Is Reduced by Early Elimination of Inflammation in a Mouse Model of IBD: Impact of a "Top-Down" Approach to Intestinal Fibrosis in Mice

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Background: The natural history of Crohn's disease follows a path of progression from an inflammatory to a fibrostenosing disease, with most patients requiring surgical resection of fibrotic strictures. Potent antiinflammatory therapies reduce inflammation but do not appear to alter the natural history of intestinal fibrosis. The aim of this study was to determine the relationship between intestinal inflammation and fibrogenesis and the impact of a very early "top-down" interventional approach on fibrosis in vivo.

Methods: In this study we removed the inflammatory stimulus from the *Salmonella typhimurium* mouse model of intestinal fibrosis by eradicating the *S. typhimurium* infection with levofloxacin at sequential timepoints during the infection. We evaluated the effect of this elimination of the inflammatory stimulus on the natural history of inflammation and fibrosis as determined by gross pathology, histopathology, mRNA expression, and protein expression.

Results: Fibrogenesis is preceded by inflammation. Delayed eradication of the inflammatory stimulus by antibiotic treatment represses inflammation without preventing fibrosis. Early intervention significantly ameliorates but does not completely prevent subsequent fibrosis.

Conclusions: This study demonstrates that intestinal fibrosis develops despite removal of an inflammatory stimulus and elimination of inflammation. Early intervention ameliorates but does not abolish subsequent fibrosis, suggesting that fibrosis, once initiated, is self-propagating, suggesting that a very early top-down interventional approach may have the most impact on fibrostenosing disease.

(Inflamm Bowel Dis 2012;18:460–471)

Key Words: Crohn's disease, intestinal fibrosis, colitis, top-down interventional approach, *Salmonella typhimurium*

C rohn's disease (CD) is characterized by repeated cycles of intestinal inflammation (flares) and healing. Despite the increasing use of potent antiinflammatory therapies which effectively treat inflammatory flares, a majority of CD patients ultimately develop fibrostenotic disease, necessitating surgical intervention at substantial personal and economic costs.[1–4] Indeed, some antiinflammatory therapies are associated with an increased risk of stenosis, stricture, or obstruction.[5] The persistence of intestinal fibrosis, and the failure of effective control of inflam-

mation to alter the natural history of CD, suggests that intestinal fibrosis can pass a "point of no return," after which the fibrotic process becomes autopropagative and fails to respond to antiinflammatory interventions.[6]

In this study we used an experimental mouse model of fibrosing colitis to examine the impact of eliminating the inflammatory stimulus on the development of fibrosis. In the *Salmonella typhimurium* model, inflammation is driven by persistent *S. typhimurium* colonization of the cecum. As described by Grassl *et al*.,[7] chronic *S. typhimurium* infection, which can persist up to 70 days, induces colitis and severe tissue fibrosis mainly in the cecum, characterized by markedly reduced cecal size, transmural tissue fibrosis, and a Th1/Th17 cytokine profile that recapitulates human CD.[7,8] In addition, the *S. typhimurium* model reproduces the bacterial antigen stimulus of CD while readily allowing for the rapid removal of the inflammatory stimulus with antibiotic treatment. Thus, the model affords the opportunity to evaluate how removal of inflammation at different timepoints impacts the natural history of intestinal fibrosis.

We describe the first reported manipulation of the *S. typhimurium* colitis model by intervention with levofloxacin,

a fluoroquinolone antibiotic with bactericidal activity against intracellular bacteria. Very early antibiotic intervention (a "top-down" approach) with levofloxacin in *S. typhimurium*-infected mice repressed *S. typhimurium*-induced inflammation and subsequent fibrosis. However, late (day 8) eradication of *S. typhimurium* did not alter the development of intestinal fibrosis, suggesting that fibrosis, once initiated, is self-propagative.

MATERIALS AND METHODS

Reagents

Levofloxacin (levaquin in 5% dextrose, Hospira, Austin, TX) was obtained from the University of Michigan Hospital pharmacy.

Mice

Female 8–12-week-old CBA/J mice (Jackson Laboratories, Bar Harbor, ME) received 20 mg streptomycin in 0.1 M Hank's buffered salt solution (HBSS) to eradicate the commensal microbiota 24 hours prior to infection with 3×10^6 colony-forming units (cfu) *S. typhimurium* strain SL1344 in 100 λ 0.1 M HEPES buffer (pH 8.0) by oral gavage. Control mice received 100 λ 0.1 M HEPES by oral gavage.

In the first endpoint analysis experiment we examined the effect of eradication of *S. typhimurium* at different timepoints after the infection (Fig. 1A). Mice were infected with *S. typhimurium* as described above. To determine the natural progression of inflammation and fibrogenesis in this model, *S. typhimurium*-infected mice (halting groups) were euthanized at day 2, 4, 8, or 21 postinfection. Mice in the *S. typhimurium*-infected levofloxacin treatment groups (intervention groups) received 0.5 mg/mL levofloxacin ad libitum in drinking water starting at day 2, 4, or 8 postinfection and continuing until euthanasia at day 21 postinfection.

In the second time course comparison experiment, which was designed to determine how early eradication of *S. typhimurium* impacted inflammation and fibrosis at early and late timepoints (Fig. 1B), mice were infected with *S. typhimurium* as described above. Half the infected animals then received 0.5 mg/mL levofloxacin starting at day 3 postinfection through day 21. Animals in the *S. typhimurium*/levofloxacin group and matched *S. typhimurium*-infected animals were euthanized at day 4, 12, or 21 postinfection. Uninfected control animals were divided into two groups, of which half received 0.5 mg/mL levofloxacin in drinking water through day 21. Uninfected control animals (treated with or without levofloxacin) were euthanized at day 21 of the study. All animal experiments were conducted with the approval and oversight of the University of Michigan UCUCA (University Committee on Use and Care of Animals).

Bacterial Strains

Salmonella typhimurium strain SL1344 (a gift from M. O'Riordan, University of Michigan, Ann Arbor, MI), which is
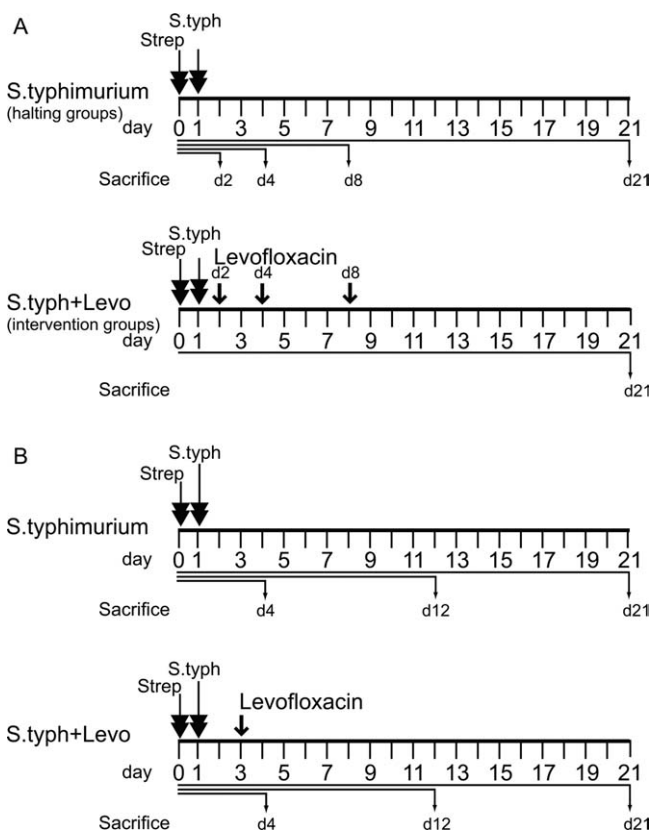

FIGURE 1. Treatment schematic of levofloxacin endpoint analysis and time course comparison experiments. (A) Treatment schematic of the endpoint analysis experiment. Mice in the *S. typhimurium* infection halting groups (upper panel) were treated with streptomycin (strep, double arrows) 1 day prior to infection with *S. typhimurium* (S. typh, double arrows) and sacrificed at day 2, 4, 8, or 21 postinfection. In the levofloxacin intervention groups (lower panel), mice received streptomycin and *S. typhimurium* on the same schedule. Levofloxacin intervention (single arrows) began day 2, 4, or 8 post-*S. typhimurium* infection and continued until sacrifice at day 21 postinfection. (B) Treatment schematic of the time course comparison experiments. Mice in the *S. typhimurium* infection groups (upper panel) were treated with streptomycin (strep, double arrows) 1 day prior to infection with *S. typhimurium* (S. typh, double arrows) and sacrificed day 4, 12, or 21 postinfection. In the levofloxacin intervention time course (lower panel) mice received streptomycin and *S. typhimurium* on the same schedule. Levofloxacin intervention (arrow) began day 3 post-*S. typhimurium* infection and continued until sacrifice at day 4, 12, or 21 postinfection. In all experiments five animals were used per experimental group.

naturally resistant to streptomycin, was grown in LB broth containing 100 μg/mL streptomycin at 37°C.

Gross Pathology and Tissue Collection

Cecum and distal colon were collected at sequential timepoints post-*S. typhimurium* infection, photographed, measured, and weighed. Cecal area was determined from digital photographs using ImageJ ROI (NIH, Bethesda, MD) to delineate the cecum. To control for differences in focal distance, cecal area was normalized against a 1-cm marker in the photographic image.

Tissues were snap-frozen in liquid nitrogen and stored at −80°C prior to molecular analysis. Cecal contents were collected and serially diluted before plating onto LB-streptomycin plates to determine bacterial titers.

Stool Cultures

A fresh stool was collected, suspended in 500 μL sterile phosphate-buffered saline (PBS), streaked onto LB plates containing 10 μg/mL streptomycin, and cultured overnight at 37°C.

Histology

Formalin-fixed and paraffin-embedded tissues were stained with Masson's trichrome by the University of Michigan CCGC Research Histology and Immunoperoxidase Laboratory (Ann Arbor, MI). Digital photomicrographs of tissue sections were taken with an Olympus BX microscope (University of Michigan Microscopy and Image Analysis Laboratory). Tissue measurements were quantitated with ImageJ analysis software.

Histological Scoring

Histological scoring was performed in a blinded manner by a pathologist (D.M.). Cecal inflammation was determined using a Wirtz Scale 0 to 4 point scoring system as previously described.[9,10] Cecal fibrosis was scored separately as follows: (0) no fibrosis; (1) mild fibrosis (focal mucosal/submucosal collagen deposition without architectural distortion); (2) moderate fibrosis (significant mucosal/submucosal collagen deposition with modest distortion of mucosal/submucosal architecture but without obscuring of the mucosal/submucosal border); and (3) severe fibrosis (extensive mucosal submucosal collagen deposition with marked architectural distortion obscuring the mucosal/submucosal border).

Immunoblotting

Immunoblotting was utilized for the detection of α-smooth muscle actin (αSMA). Whole tissue was pulverized under liquid nitrogen and lysed in ice-cold RIPA buffer with a cocktail of proteinase inhibitors (Roche, Nutley, NJ). Protein content was determined using a modified Bradford assay kit (BioRad, Hercules, CA). Total protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to PVDF membranes (Amersham Biosciences, Piscataway, NJ). Membranes were blocked in 5% milk solution for 1 hour at room temperature or overnight at 4°C. αSMA was detected by incubating the membrane overnight at 4°C with mouse antihuman monoclonal antibody (Sigma, St. Louis, MO) at 1:5000 dilution in 5% milk/TBST. As a loading control, a mouse antibody for GAPDH (Chemicon, Temecula, CA) was used after a stripping procedure. Secondary antibody antimouse IgG+ horseradish peroxidase (HRP) (Amersham, Piscataway, NJ) was incubated for 1 hour at room temperature and the signal was detected by the Pierce detection system (Pierce, Rockford, IL). Autoradiographs were scanned and quantitated using Image J analysis software.

Real-time Quantitative Polymerase Chain Reaction (PCR)

RNA was extracted from the cecum using the RNeasy kit (Qiagen, Valencia, CA). Reverse transcription of 2 μg of total RNA was performed with the Superscript First Strand RT kit (Invitrogen, La Jolla, CA). Quantitative real-time PCR (qRT-PCR) of IL-1β (Mm01336189_m1), TNFα (Mm00443258_m1), IL-17 (Mm00439619_m1), IL-6 (Mm99999064_m1), IGF-1 (Mm00439560_m1), TGFβ (Mm00441726_m1), IL-12p40 (Mm01288993_m1), CTGF (Mm01192933_g1), and GAPDH (4352932E) was performed with the TaqMan assays-on-demand primer/probe gene expression assays (ABI, Foster City, CA). qRT-PCR was performed using a Stratagene Mx3000P real-time PCR system (Stratagene, La Jolla, CA). Cycling conditions were 95°C 10 minutes, followed by 40 cycles of 95°C 15 seconds and 62°C 60 seconds. $\Delta\Delta$Ct values were calculated from GAPDHs expression.

Statistical Analysis

Comparisons between several groups of mice and associated tissues or RNA expression were analyzed with analysis of variance (ANOVA), while pairwise comparisons of two groups were performed with Student's t-test.

RESULTS

Inflammation Precedes Fibrosis in the S. typhimurium Mouse Model of Intestinal Fibrosis

To determine the relationship between inflammation and fibrosis, mice were infected with *S. typhimurium* and sacrificed at day 2, 4, 8, or 21 postinfection (halting groups). Disease activity was assessed by gross pathology, histopathology, inflammatory and profibrotic cytokine expression, and αSMA protein expression. *S. typhimurium* heavily colonized the mouse cecum at 1.7×10^7 cfu/mL by day 2 postinfection and persisted through day 21 (3.4×10^7 cfu/mL, data not shown). By day 2 postinfection, the gross cecal pathology demonstrated a striking reduction in cecal size that persisted and was virtually indistinguishable from the gross pathology following a chronic 21-day infection (Fig. 2A). The gross pathology was consistent with quantification of cecal area, which showed a significant 2-fold reduction at day 2 that was indistinguishable from the chronic day-21 group (Fig. 2B).

To determine the onset of intestinal inflammation, cecal tissue sections were scored for inflammation on the Wirtz scale (0 to 4).[9,10] Early inflammatory changes were seen at day 2 postinfection with a significant increase in histological inflammatory score compared to uninfected animals (1.0 vs. 0.0, $P < 0.001$) (Fig. 2C). Inflammation progressed during the course of infection through day 21. The early gross phenotypic and inflammatory changes in the cecum were associated with a rapid and dramatic 12–120-fold induction of proinflammatory Th1 cytokines IL-1β and TNFα by day 2 postinfection which peaked by
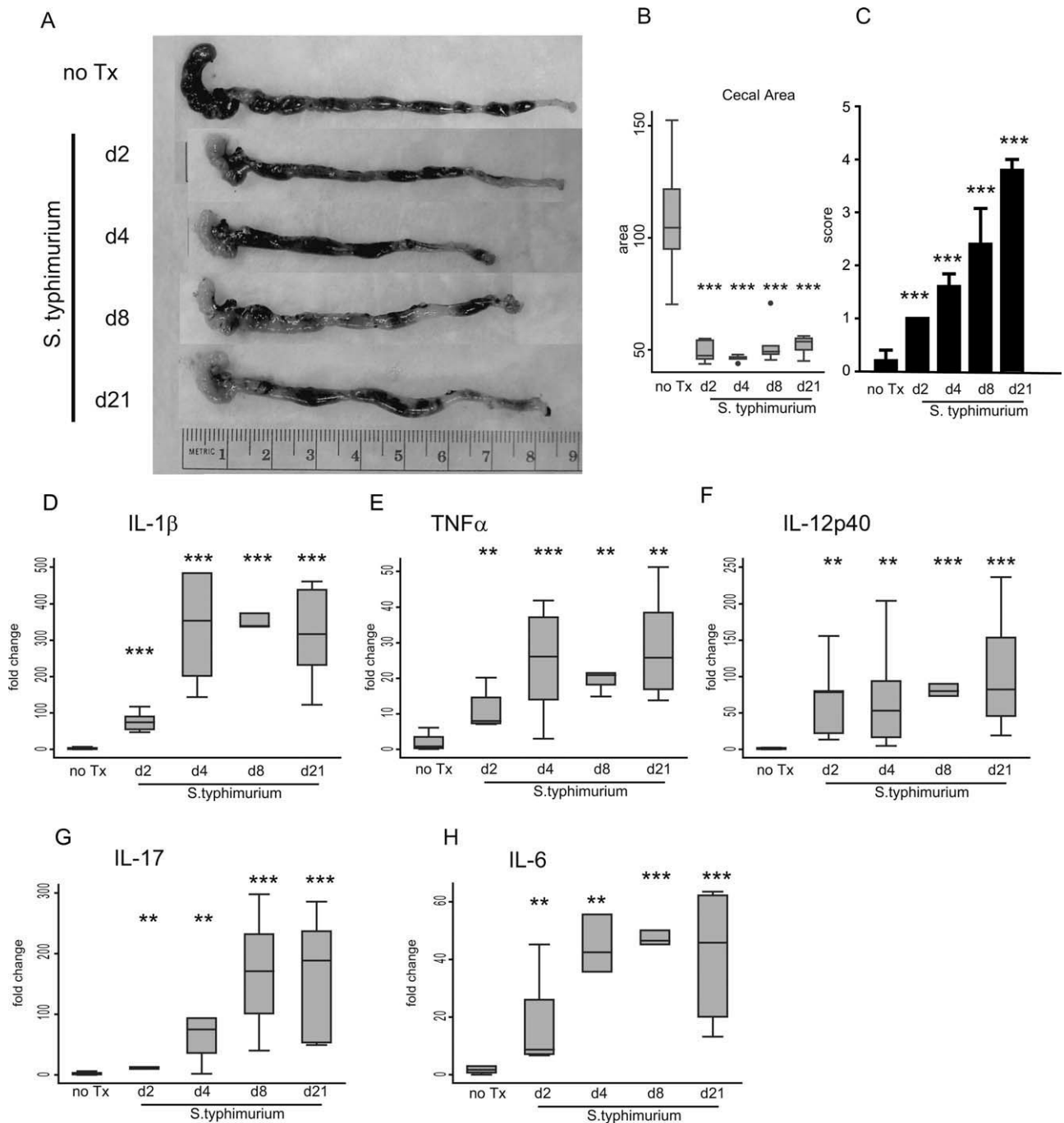
FIGURE 2. Inflammation precedes fibrosis in the *S. typhimurium* mouse model of intestinal fibrosis. (A) Representative gross appearance of the cecum and distal colon of *S. typhimurium*-infected mice day 2 (d2), day 4 (d4), day 8 (d8), or day 21 (d21) postinfection compared to uninfected (no Tx) against a 1-cm reference ruler. (B) Cecal area calculated from uninfected mice (no Tx) compared to *S. typhimurium*-infected mice day 2, 4, 8, or 21 postinfection. (C) Blinded inflammatory scoring of cecal sections using the 0–4 point Wirtz scale. (D–H) qRT-PCR gene expression of Th1 and inflammatory cytokines in the cecum of uninfected (no Tx) compared to *S. typhimurium*-infected mice day 2, 4, 8, or 21 postinfection. Gene expression was normalized to GAPDH expression. Statistical comparisons between uninfected controls (no Tx) and the infected groups are denoted with asterisks. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

day 4 and persisted through day 21 (Fig. 2D,E). Similarly, IL-12p40, a cytokine involved in both the Th1 and Th17 responses in the pathogenesis of CD,[11] was markedly induced by day 2 postinfection (Fig. 2F). *S. typhimurium*

infection also elicited an early Th17 response, characterized by 17–19-fold induction of IL-17 and IL-6 cytokines by day 2 postinfection which increased over the time course of infection (Fig. 2G,H).

The induction of fibrosis followed the inflammatory phase, with significant increases in TGFβ, IGF-1, and CTGF expression observed at days 4–8 (Fig. 3A–C). In contrast to the inflammatory cytokines, which peaked at day 4, the fibrogenic markers showed a progressive increase through day 21 (Fig. 3A–C). Histopathological analysis of trichrome-stained cecal tissue demonstrated mild but significant fibrotic changes as early as day 2 postinfection (1.2 vs. 0, $P = 0.04$), which progressed to extensive tissue fibrosis with architectural distortion by day 21. (Fig. 3D,E). An independent quantification of trichrome staining for collagen revealed a similar trend, with a modest but significant increase in collagen staining at day 2 postinfection with maximal collagen deposition by day 21 (data not shown). αSMA protein expression, a marker of myofibroblast activation, was not observed until day 8 and continued to increase with a significant 4-fold increase in protein expression between day 8 and day 21 (Fig. 3F,G).

Intervention Reduces the S. typhimurium-induced Inflammatory (Th1) Response

To determine whether the inflammatory response to *S. typhimurium* infection persists without continued inflammatory stimulus (bacterial colonization), an endpoint analysis experiment was performed. Mice were infected with *S. typhimurium* and treated with oral doses of levofloxacin beginning at day 2, 4, or 8 postinfection and sacrificed at day 21 (Fig. 1A). Within 24 hours of levofloxacin administration, 100% of the animals were negative for *S. typhimurium* as determined by stool cultures (data not shown). Gross pathology, histopathology, cytokine expression, and protein expression were compared between animals in the levofloxacin intervention groups, uninfected animals, and animals infected with *S. typhimurium*. Both early (day 2) and later (days 4, 8) intervention reduced cecal contraction and tissue weight at day 21 (Fig. 4A,B).

Histological analysis of inflammation in the cecal tissue sections determined that early intervention reduced tissue inflammation. Inflammation was significantly repressed in the day 2, 4, and 8 intervention groups at day 21 compared to the infected group (0.6, 1.6, 1.6 vs. 3.8, $P < 0.0001$) (Fig. 4C). However, inflammation scores were still significantly higher at day 21 in the day 2, 4, and 8 levofloxacin intervention groups compared to uninfected animals (0.6, 1.6, 1.6 vs. 0.0, $P = 0.04$, $P = 0.004$, $P < 0.001$).

Molecular analysis of the cecal tissue revealed a more profound antiinflammatory effect of early intervention. Early (day 2) intervention repressed the induction of proinflammatory cytokines IL-1β, TNFα, and IL12p40 at day 21 to levels indistinguishable from uninfected controls (Fig. 4D–F). Although intervention at later timepoints failed to completely abolish the induction of IL-1β, TNFα, IL12p40, and IL-6 (Fig. 4H), the expression of these cyto-

kines was still markedly suppressed in comparison to the untreated/*S. typhimurium*-infected animals. In contrast, both early and late intervention repressed IL-17 (Fig. 4G).

Early Intervention Ameliorates But Does Not Completely Prevent Fibrosis

Given that fibrosis develops in response to inflammation, removal of the inflammatory stimulus would be expected to limit fibrosis. In mice treated with levofloxacin at day 2 or day 4 postinfection, expression of TGFβ and CTGF cytokines in the cecum at day 21 was indistinguishable from the expression in uninfected animals at day 21 postinfection. In contrast, in the day 4 levofloxacin group IGF-1 was still significantly elevated, reflecting a heterogeneous fibrogenic response to day 4 intervention (Fig. 5A–C). However, day 8 levofloxacin intervention did not alter the expression of the fibrotic markers at day 21 postinfection. TGFβ, IGF-1, and CTGF expression were significantly induced compared to uninfected animals and did not differ from *S. typhimurium*-infected animals at day 21 postinfection (Fig. 5A–C).

Similarly, histological scoring of fibrosis revealed that day 2 or 4 levofloxacin intervention reduced tissue fibrosis at day 21 to levels indistinguishable from uninfected animals (0.4, 0.6 vs. 0.0, $P = 0.35$, $P = 0.35$, respectively) (Fig. 5D). The later (day 8) levofloxacin intervention group had significant tissue fibrosis at day 21 that was similar to the infected group at day 21 (1.8 vs. 2.8, $P = 0.36$) and significantly greater than the fibrosis in uninfected controls (1.8 vs. 0.0, $P < 0.001$).

In contrast, αSMA protein expression at day 21 postinfection was significantly induced despite early intervention with levofloxacin (Fig. 5E,F). Although αSMA protein expression in the early (day 2 and day 4) levofloxacin groups was 3–5-fold lower than the day 21 *S. typhimurium*-infected mice, αSMA expression in all three intervention groups was increased compared to uninfected controls at day 21. During the course of infection, αSMA protein expression was not induced until day 8 postinfection. However, the persistence of αSMA protein expression at day 21 in the infected/levofloxacin-treated groups indicates that the fibrogenic process and myofibroblast activation is initiated early and persists despite removal of the inflammatory stimulus and eradication of the inflammatory response.

Early Intervention Represses Gross Pathological Changes and Inflammation

As demonstrated above, the fibrogenic process is initiated between day 4 and day 8 with maximum fibrosis at day 21 postinfection. Therefore, in a second time course comparison experiment we investigated the effect of early removal of the inflammatory stimulus by treatment with levofloxacin at day 3 upon the development of fibrosis over the next 18 days (Fig. 1B). Following infection, *S. typhimurium*
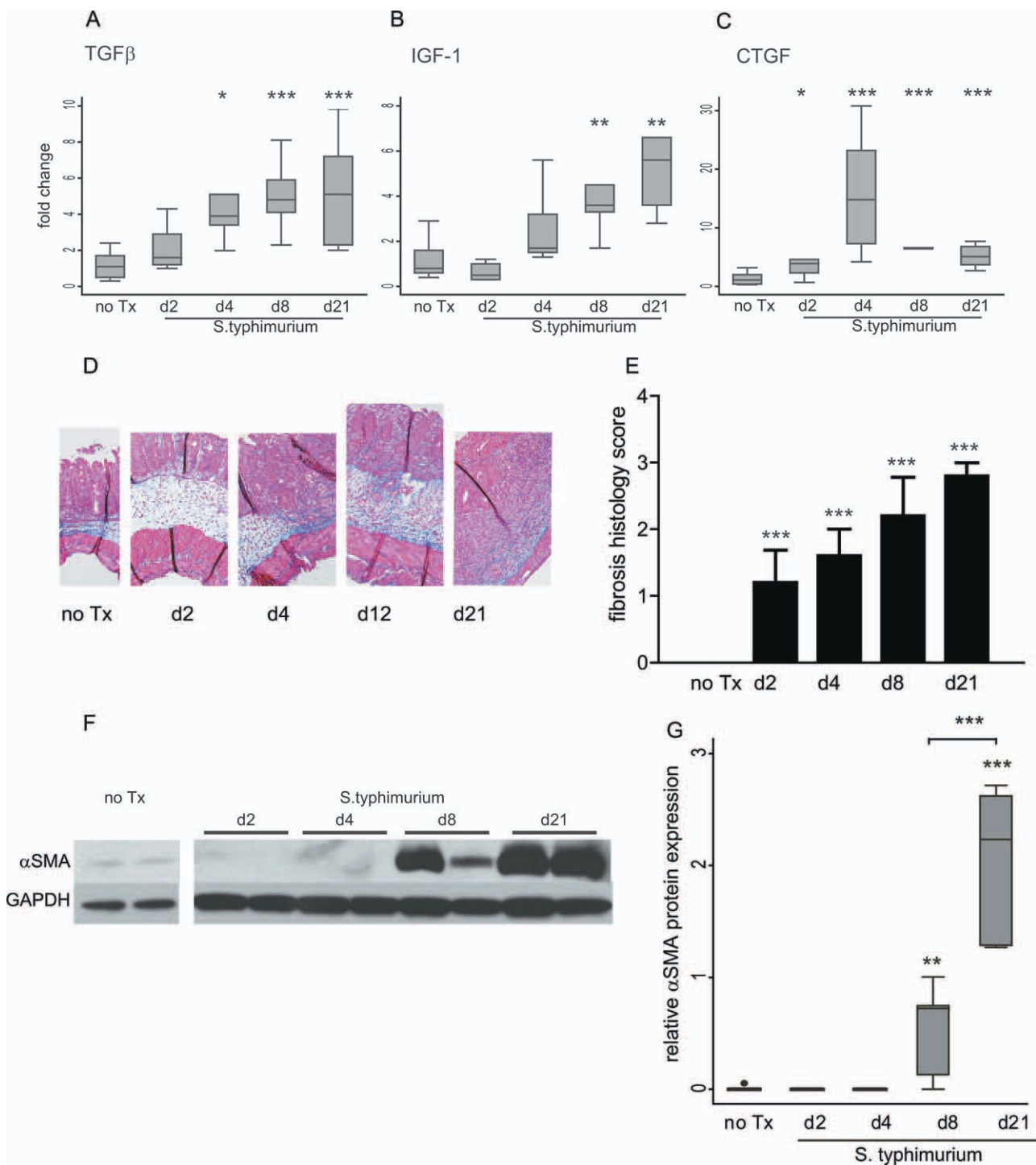
FIGURE 3. Development of fibrosis in the *S. typhimurium* mouse model of fibrosis. (A–C) qRT-PCR expression of fibrotic genes in cecum from uninfected mice (no Tx) compared to *S. typhimurium*-infected mice day 2, 4, 8, or 21 postinfection. Gene expression was normalized to GAPDH expression. (D) Representative trichrome histological sections (100× magnification). (E) Blinded fibrosis scoring of cecal sections was determined from trichrome-stained cecal sections. (F) Representative western blot of αSMA protein expression in *S. typhimurium* mice days 2, 4, 8, or 21 post-infection compared to uninfected controls. The samples shown were run on the same protein gel. The image has been cut to rearrange the loading order for clarity. GAPDH expression was used as a loading control. (G) Quantitation of αSMA protein expression in cecum from uninfected mice (no Tx) compared to *S. typhimurium*-infected mice day 2, 4, 8, or 21 postinfection. Western blots were digitally scanned and quantitated using ImageJ. Relative expression was normalized to GAPDH protein expression. Statistical comparisons between uninfected controls (no Tx) and the infected groups are denoted with asterisks. Brackets represent statistical comparisons between *S. typhimurium*-infected groups. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
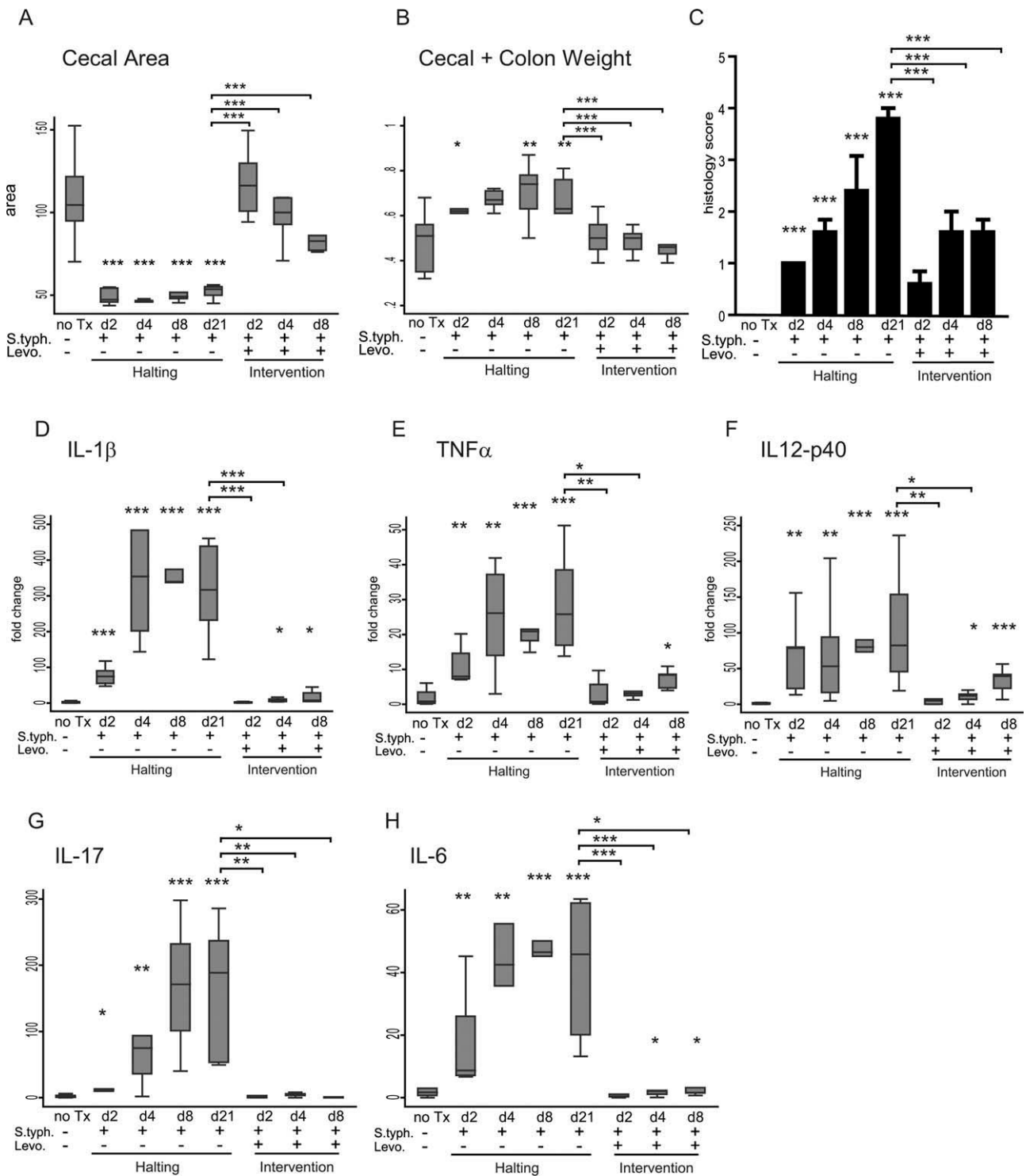
FIGURE 4. Intervention represses the inflammatory response. (A) Cecal area from uninfected mice (no Tx) compared to *S. typhimurium*-infected mice day 2, 4, 8, or 21 postinfection, and *S. typhimurium*-infected mice treated with levofloxacin day 2, 4, or 8 postinfection and sacrificed day 21 postinfection. (B) Combined cecal + colon weight. (C) Blinded inflammatory scoring of cecal sections using the 0–4 point Wirtz scale. (D–H) qRT-PCR expression of IL-1β (D), TNFα (E), IL-12p40 (F), IL-17 (G), and IL-6 (H) in mice infected with *S. typhimurium*-infected mice day 2, 4, 8, or 21 postinfection, and *S. typhimurium*-infected mice treated with levofloxacin day 2, 4, or 8 postinfection and sacrificed day 21 postinfection compared to uninfected mice (no Tx). Statistical comparisons between the infection groups or intervention groups and the uninfected controls are denoted with asterisks. Statistically significant comparisons between the *S. typhimurium*-infected and levofloxacin intervention groups at day 21 postinfection are denoted in brackets. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
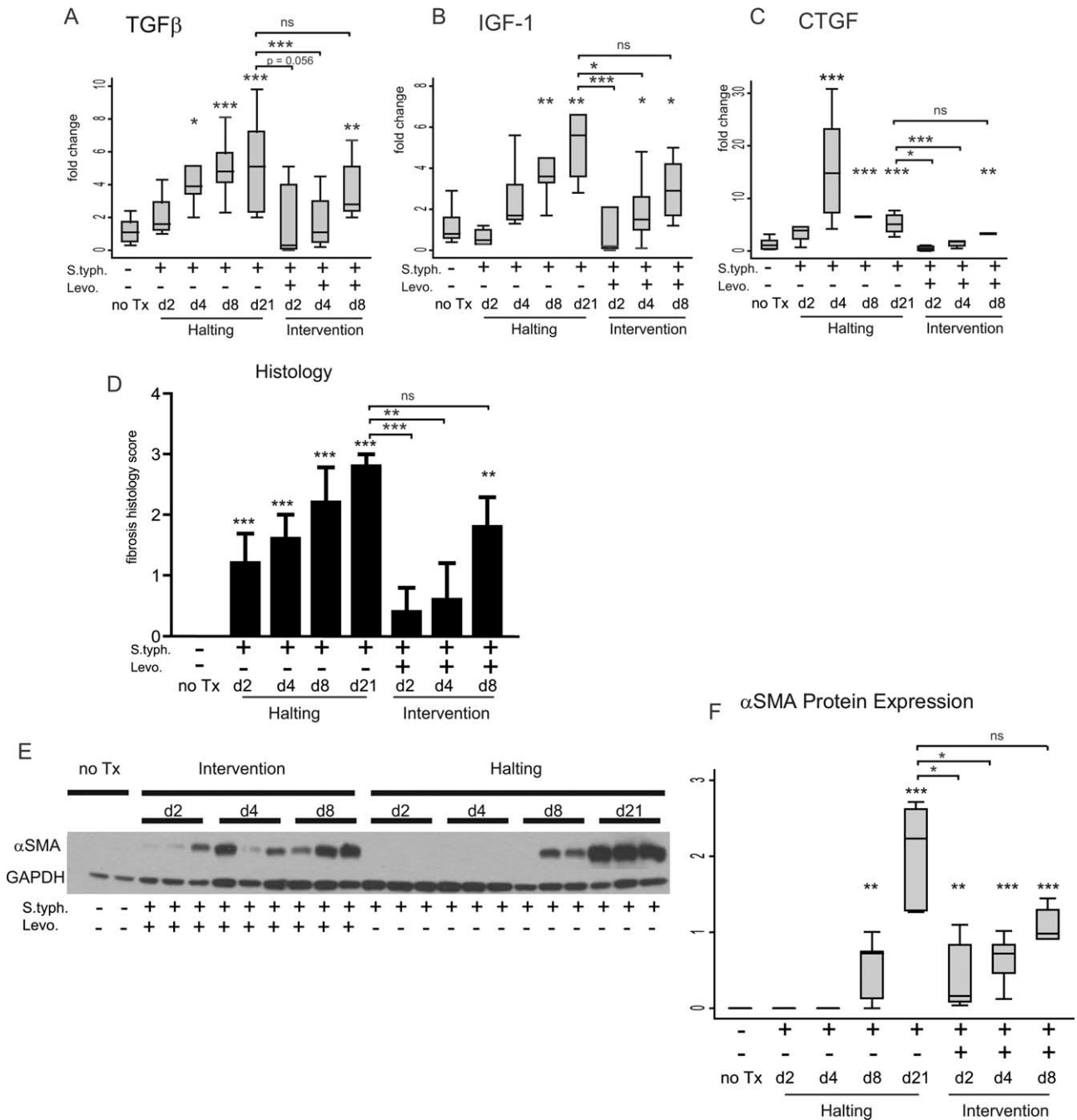
FIGURE 5. Early intervention ameliorates but does not completely prevent fibrosis. (A–C) qRT-PCR expression of fibrotic markers TGFβ (A), IGF-1 (B), and CTGF (C) in mice infected with *S. typhimurium* day 2, 4, 8, or 21 postinfection, and *S. typhimurium*-infected mice treated with levofloxacin day 2, 4, or 8 postinfection and sacrificed day 21 postinfection compared to uninfected mice (no Tx). (D) Blinded fibrosis scoring of cecal sections was determined from trichrome-stained cecal sections. (E) Representative western blot illustrating αSMA protein expression in the cecum of uninfected mice (no Tx) compared to mice infected with *S. typhimurium* and treated with levofloxacin 2, 4, or 8 postinfection and sacrificed day 21 postinfection and mice infected with *S. typhimurium*-infected mice day 2, 4, 8, or 21 postinfection. GAPDH protein expression was used as a loading control. (F) Quantitation of αSMA protein expression in the cecum. Western blots were digitally scanned and quantitated using ImageJ. Relative expression was normalized to GAPDH protein expression. Statistical comparisons between the infection groups or intervention groups and the uninfected controls are denoted with asterisks. Statistically significant comparisons between the *S. typhimurium*-infected and levofloxacin intervention groups at day 21 postinfection are denoted in brackets. ns = not statistically significant, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

was eradicated in half the animals by treatment with levofloxacin day 3 postinfection (during the inflammatory phase of infection but before the fibrogenic phase). Mice from both the untreated and levofloxacin intervention groups were sacrificed at day 4, 12, or 21 postinfection to determine the effect of eradication on inflammation and fibrosis and compared to uninfected animals that received either levofloxacin treatment or no intervention.

Twenty-four hours after initiating levofloxacin therapy, all animals in the intervention group had cleared the *S. typhimurium* as determined by stool culture (data not shown). In the day 4 postinfection group, this clearance was associated with a concurrent change in gross pathology with a small (17%) but significant increase in cecal area compared to matched day 4 *S. typhimurium*-infected animals. (Fig. 6A) Similar improvement in cecal + colon weight and length were also observed (data not shown). By day 12 postinfection the gross pathology of the levofloxacin intervention group was indistinguishable from uninfected controls.

Histological analysis of cecal tissue sections identified significant inflammation at both day 4 and day 12 in the levofloxacin-treated group compared to uninfected controls (inflammation score 2.4 vs. 0.4 at day 4, $P = 0.035$, 2.2 vs. 0.4 at day 12, $P < 0.001$) (Fig. 6B). Compared to the matched *S. typhimurium*/untreated animals, inflammatory scores were indistinguishable between the day 4 groups (2.4 vs. 2.8, $P = 0.25$). Early Levofloxacin intervention did significantly repress inflammation by day 12 and day 21 (2.2 vs. 3.8, $P < 0.001$ and 1.2 vs. 3.4, $P < 0.001$), although the inflammatory score in the levofloxacin intervention group at day 12 remained significantly higher than uninfected controls (1.2 vs. 0.4, $P = 0.035$).

The histological inflammatory response was reflected by a similar pattern of change in proinflammatory cytokine gene expression. Cecal expression of proinflammatory cytokines IL-1β, TNFα, and IL12p40 were significantly induced and did not differ in between the *S. typhimurium*-infected and levofloxacin-treated mice at day 4 postinfection compared to uninfected controls (Fig. 6C–E). However, by day 12 postinfection IL-1β, TNFα, and IL12p40 expression was significantly reduced in levofloxacin-treated compared to day 12 infected and untreated mice.

Early intervention with levofloxacin at day 3 exerted a profound and immediate effect on the Th17 cytokine response. At day 4 postinfection, IL-17 and IL-6 were repressed 4-fold in the levofloxacin intervention group compared to the *S. typhimurium* group (Fig. 6F,G).

Early Intervention Represses Inflammation But Does Not Prevent Fibrosis

Early intervention with levofloxacin at day 3 postinfection repressed the induction of profibrotic genes TGFβ, IGF-1, and CTGF. Although infection induced TGFβ >3-

fold at day 4 postinfection, treatment with levofloxacin at day 3 repressed TGFβ to levels indistinguishable from uninfected controls (Fig. 7A). A similar trend occurred for CTGF (Fig. 7C). Although IGF-1 was not induced until later in the time course, levofloxacin treatment significantly repressed IGF-1 induction (Fig. 7B).

Histologic scoring of cecal fibrosis identified a significant reduction in fibrosis in the levofloxacin intervention groups by day 12 and day 21. At day 12 the fibrosis score was significantly lower in the intervention group compared to the untreated group (2.0 vs. 3.0, $P < 0.001$) (Fig. 7D). By day 21, fibrosis had resolved in the levofloxacin intervention group to levels similar to uninfected controls (1.0 vs. 0.6, $P = 0.35$).

αSMA protein expression was significantly increased at day 12 postinfection in both the levofloxacin intervention and *S. typhimurium* groups compared to uninfected controls (Fig. 7E,F). While overall cecal αSMA protein expression was lower in the levofloxacin intervention group, this was not statistically significant at day 12 compared to those without levofloxacin treatment. By day 21 postinfection, αSMA protein expression was significantly repressed by 2.6-fold in the levofloxacin intervention ($P < 0.0001$ vs. *S. typhimurium*-infected mice). Although αSMA protein expression was attenuated at day 21 in the levofloxacin intervention group compared to *S. typhimurium*-infected mice, αSMA protein was still significantly higher compared to uninfected controls, suggesting early intervention attenuated but did not completely resolve myofibroblast activation and fibrogenesis.

DISCUSSION

CD, despite available potent antiinflammatory therapies, largely progresses to a complicated fibrostenosing phenotype. Current therapies induce and maintain remission of inflammation but have a poor record in preventing fibrostenotic complications, with a majority of CD patients inexorably progressing towards fibrostenotic disease and subsequent surgical intervention.[1–3,12,13] In a small recent study, ≈20% of CD patients with fibrostenotic disease treated with biologics required surgical intervention within 2 to 5 years.[14] Current therapies that target inflammation do not alter the natural history of CD, suggesting that intestinal fibrosis, once initiated, becomes irreversible.[15]

To better understand why antiinflammatory therapy fails to alter the natural history of intestinal fibrosis, we examined the temporal relationship between inflammation and fibrosis and the consequences of removal of inflammation from the *S. typhimurium* murine model of fibrosis. Although other murine colitis models (PG-PS, TNBS, DSS) are commonly used to study IBD, these have limitations for elucidating the pathobiology of fibrosis due to inherent murine resistance to fibrotic disease, incomplete disease penetrance, and high mortality.[8]
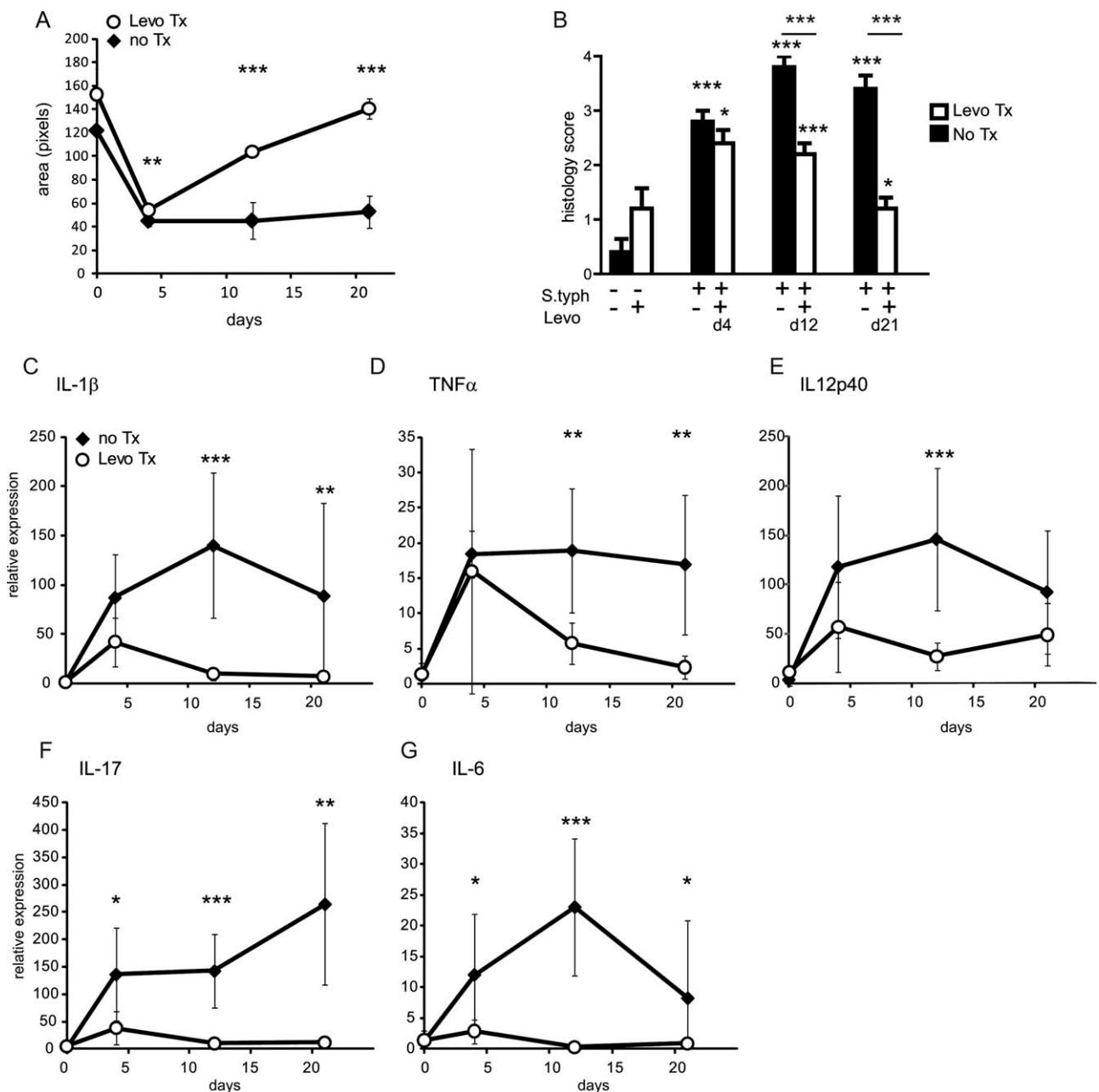
FIGURE 6. Early intervention represses inflammation. (A) Levofloxacin intervention reverses cecal contraction in response to *S. typhimurium* infection. Cecal area of mice infected with *S. typhimurium* (solid diamonds) compared to *S. typhimurium*-infected animals treated with levofloxacin day 3 postinfection (open circles) at day 4, 12, or 21 postinfection. (B) Blinded inflammatory scoring of cecal sections using the 0–4 point Wirtz scale. (C–G) qRT-PCR expression of IL-1β, TNFα IL-12p40, IL-17, and IL-6 in the cecum day 4, 12, or 21 postinfection. Statistical comparisons between the infection and intervention groups are denoted with asterisks. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

In addition, none are particularly amenable to experimental manipulation of inflammation. Our study, the first systematic manipulation inflammation in a rodent colitis model, demonstrates that eradication of the inflammatory stimulus is not sufficient to prevent progression of fibrosis. As we demonstrated in the infection time course comparison experiments, inflammatory changes occur early (day 2) and precede fibrogenesis

(day 4 to day 8). Treatment of *S. typhimurium*-infected mice with levofloxacin at days 2, 4, and 8 postinfection reversed inflammatory changes in gross pathology, histopathology, and repressed the Th1/Th17 inflammatory cytokine gene expression at day 21 postinfection. However, levofloxacin intervention at day 4 or day 8 failed to prevent fibrogenesis at day 21 postinfection, as evidenced by increased profibrotic gene
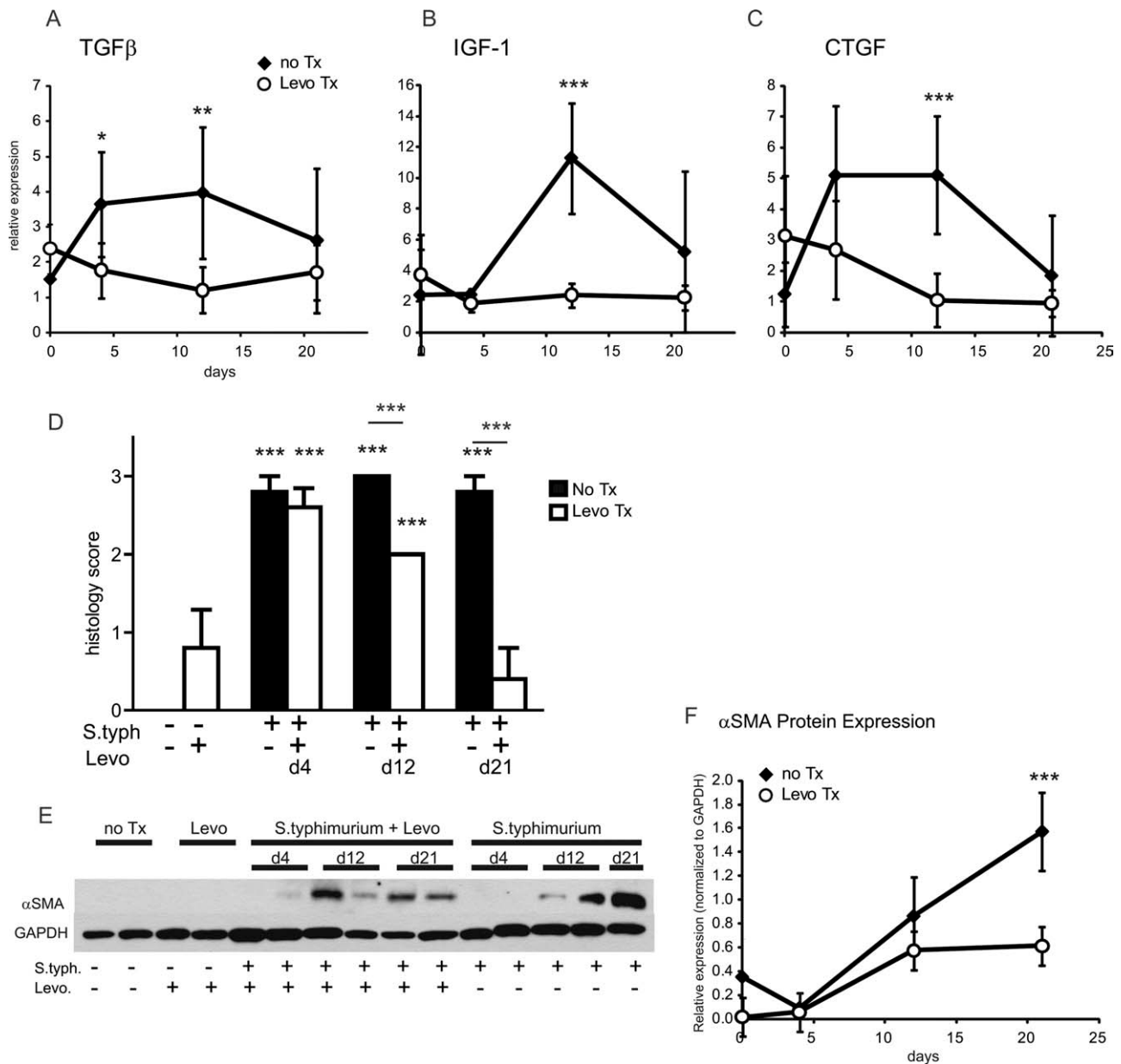
FIGURE 7. Early intervention represses inflammation but does not prevent fibrosis. (A–C) qRT-PCR expression of fibrotic markers TGFβ (A), IGF-1 (B), and CTGF (C) in mice infected with *S. typhimurium* (solid diamonds) compared to *S. typhimurium*-infected mice treated with levofloxacin day 3 postinfection (open circles) day 4, 12, or 21 postinfection. (D) Blinded fibrosis scoring of cecal sections was determined from trichrome stained cecal sections. (E) Representative western blot of cecal αSMA protein expression in mice infected with *S. typhimurium* compared to mice infected with *S. typhimurium* and treated with levofloxacin, uninfected mice (no Tx), and levofloxacin-treated uninfected mice (Levo). GAPDH was used as a control for protein loading. (F) αSMA protein expression in the cecum in response to *S. typhimurium* infection or *S. typhimurium* infection and levofloxacin intervention. Relative αSMA expression was normalized to GAPDH expression. Statistically significant comparisons between the infection and intervention groups are denoted with asterisks. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

expression, increased tissue fibrosis, and increased αSMA protein expression. Strikingly, fibrosis not only persisted, but actually progressed even after eradication of the inflammatory stimulus, suggesting that established fibrosis is not readily reversible, even with eradication of the inciting inflammatory stimulus. Further, this suggests that established intestinal fibrogenesis is, to some extent, a self-sustaining process.

Current CD treatment paradigms follow a "step-up" approach in which increasing disease severity (flares) or treatment failure drives therapeutic escalation.[16] However, recent clinical studies suggest an early, aggressive, or "top-down" approach may improve patient outcomes.[17,18] To determine whether a top-down approach alters the natural history of intestinal fibrosis, we treated *S. typhimurium*-infected

mice with levofloxacin at day 3 postinfection and compared the impact on progression of fibrosis with infected mice at days 4, 12, and 21 postinfection. Consistent with a potential benefit to the top-down approach, early intervention improved tissue pathology as determined by gross pathology and histopathology, and repressed the induction of inflammatory and profibrogenic genes. In addition, early intervention suppressed, yet did not completely abrogate, induction of αSMA protein expression, suggesting partial myofibroblast activation. In contrast, late eradication of the inflammatory stimulus did not prevent fibrotic progression.

In summary, our study demonstrates that by manipulating a rodent model of fibrosing colitis we can examine biologically and clinically important questions concerning the interaction between inflammation and fibrosis. Late removal of the inflammatory stimulus does not prevent subsequent tissue fibrosis, suggesting a "point of no return" at which fibrogenesis becomes autopropagative. This finding is similar to studies demonstrating that liver fibrosis is reversible if the activating stimulus is suppressed at an early timepoint.[19] While no human study has yet addressed the connection between very early use of biologics (antiinflammatory therapy within a week of diagnosis) and the development of fibrostenotic disease, we demonstrate here that very early therapeutic intervention can ameliorate the late endpoint of fibrosis. These findings support the potential benefits of a very early top-down treatment approach,[17] and suggest that the benefits may wane as the time between the start of symptoms and therapeutic intervention increases.

CD is chronic and progressive, with a natural history filled with complications. Currently, we cannot reliably stratify patients by their risk of fibrostenotic disease.[20] Given that current therapies target inflammation, no effective antifibrotics exist, and that once initiated, and that fibrosis can autopropagate even in the absence of inflammation, very early intervention or a top-down approach may have the greatest impact on the natural history of CD.

ACKNOWLEDGMENT

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