MyD88 signaling promotes both mucosal homeostatic and fibrotic responses during *Salmonella*-induced colitis

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Månsson LE, Montero M, Zarepour M, Bergstrom KS, Ma C, Huang T, Man C, Grassl GA, Vallance BA. MyD88 signaling promotes both mucosal homeostatic and fibrotic responses during *Salmonella*-induced colitis. Am J Physiol Gastrointest Liver Physiol 303: G311–G323, 2012. First published June 7, 2012; doi:10.1152/ajpgi.00038.2012.—*Salmonella enterica* serovar Typhimurium is a clinically important gram-negative, enteric bacterial pathogen that activates several Toll-like receptors (TLRs). While TLR signaling through the adaptor protein MyD88 has been shown to promote inflammation and host defense against the systemic spread of *S. Typhimurium*, curiously, its role in the host response against *S. Typhimurium* within the mammalian gastrointestinal (GI) tract is less clear. We therefore used the recently described *Salmonella*-induced enterocolitis and fibrosis model: wild-type (WT) and MyD88-deficient (MyD88−/−) mice pretreated with streptomycin and then orally infected with the ΔaroA vaccine strain of *S. Typhimurium*. Tissues were analyzed for bacterial colonization, inflammation, and epithelial damage, while fibrosis was assessed by collagen quantification and Masson’s trichrome staining. WT and MyD88−/− mice carried similar intestinal pathogen burdens to postinfection day 21. Infection of WT mice led to acute mucosal and submucosal inflammation and edema, as well as significant intestinal epithelial damage and proliferation, leading to widespread goblet cell depletion. Impressive collagen deposition in the WT intestine was also evident in the submucosa at postinfection days 7 and 21, with fibrotic regions rich in fibroblasts and collagen. While infected MyD88−/− mice showed levels of submucosal inflammation and edema similar to WT mice, they were impaired in the development of mucosal inflammation, along with infection-induced epithelial damage, proliferation, and goblet cell depletion. MyD88−/− mice also had fewer submucosal fibroblasts and 60% less collagen. We noted that cyclooxygenase (Cox)-2 expression was MyD88-dependent, with numerous Cox-2-positive cells identified in fibrotic regions of WT mice at postinfection day 7, but not in MyD88−/− mice. Treatment of WT mice with the Cox-2 inhibitor rofecoxib (20 mg/kg) significantly reduced fibroblast numbers and collagen levels without altering colitis severity. In conclusion, MyD88 and Cox-2 signaling play roles in intestinal fibrosis during *Salmonella*-induced enterocolitis.

*Salmonella* species are facultative intracellular gram-negative bacteria that cause a wide array of illnesses, including systemic disease and enterocolitis, in a multitude of hosts (reviewed in Ref. 30). While these pathogens possess numerous virulence factors, their ability to cause disease is also dependent on the host and its response to these microbes, as well as the host’s relative resistance to infection. Possessing many pathogen-associated molecular patterns, *Salmonella* has been shown to activate a number of Toll-like receptors (TLRs) in vitro and in vivo. Moreover, the adaptor protein MyD88 plays a critical role in innate signaling by the TLR and the IL-1 receptor superfamily. Curiously, although *Salmonella enterica* serovar Typhimurium is an enteric bacterial pathogen, murine infection with *Salmonella Typhimurium* has been used predominantly to model human typhoid (caused by *Salmonella enterica* serovar Typhi), rather than enterocolitis. In fact, many of these studies have demonstrated that innate signaling by specific TLRs, or by MyD88, plays important roles in triggering host inflammatory responses that limit *S. Typhimurium* proliferation at systemic sites such as the liver and spleen (15, 33, 36, 40).

Although *S. Typhimurium* is an enteric pathogen, it is only recently that studies have begun to assess how innate signaling impacts host defense and the resulting disease when this pathogen infects its host’s intestines (15, 23). This is an important question, since innate immune signaling in the gastrointestinal (GI) tract appears to have unique functions compared with its roles at other sites in the host. In particular, recent studies have found that innate signaling through the adaptor protein MyD88 can prove surprisingly critical in protecting the host from lethal colitis by promoting mucosal homeostatic responses following intestinal tissue damage. Specifically, MyD88 signaling limits mucosal damage in the dextran sodium sulfate (DSS)-induced colitis model, preventing colonic ulceration by promoting epithelial cell proliferation and repair in response to innate recognition of commensal bacteria (2, 29). Moreover, using the attaching/effacing bacterial pathogen *Citrobacter rodentium*, we recently confirmed this tissue protective role for MyD88 signaling. During infection, MyD88-deficient (MyD88−/−) mice developed severe cecal and colonic ulceration and carried significantly heavier pathogen burdens, leading to rapid morbidity and mortality (12).

Questions regarding innate immune responses in the GI tract have been difficult to answer with respect to *S. Typhimurium*, because this pathogen generally causes little intestinal pathology in mice; rather, its infection mimics human typhoid (3). However, a recently characterized model of infectious cecal inflammation in mice provides an alternate model for the study of *S. Typhimurium*-induced intestinal disease in humans. After oral administration of streptomycin, mice challenged with *S. Typhimurium* display signs of intestinal inflammatory pathology with many histopathological similarities to human disease, including severe inflammation in the large bowel with little or no inflammatory pathology in the ileum (3). Typically, this infection model has been limited to very acute studies, since mice lacking a functional *nramp1* gene, including C57BL/6
mice, are highly susceptible to wild-type (WT) S. Typhimu-
rium infection, succumbing in only a few days (37). Consid-
ering that most TLR-deficient mouse strains are generated on
this genetic background, studies using these mice have been
limited to very acute experiments. However, we recently re-
ported that infection of C57BL/6 mice with attenuated S.
Typhimurium strains or infection of resistant mouse strains that
carry a functional *nramp1* gene can cause a chronic infection of
the intestine leading to a novel model of the intestinal fibrosis
with features similar to those in the GI tissues of some patients
with Crohn’s disease (13). Infected mice suffer extensive
transmural collagen deposition in the gut wall, in concert with
the accumulation of numerous fibroblasts in the intestinal
submucosa and mucosa (14).

To address the impact of MyD88 on the colitic and fibrotic
responses in this model, we infected mice with the attenuated
ΔaroA strain of *S. Typhimurium*. Significant mucosal damage,
including epithelial pathology and proliferation and inflamma-
tory cell recruitment, along with submucosal pathology, in-
cluding edema, inflammation, fibroblast proliferation, and
collagen deposition, was noted in infected WT C57BL/6 mice.

Immunostaining for the cyclooxygenase (Cox)-2 enzyme was
also strongly elevated during infection. In contrast, although
submucosal inflammation and edema were similarly elevated in
the MyD88−/− mice, the development of mucosal inflam-
mination, epithelial pathology, and fibrogenesis was significantly
attenuated in MyD88−/− mice. Moreover, we found that up-
regulation of Cox-2 [posttranscriptional gene silencing (Ptgs)-2]
gene expression was largely MyD88-dependent, and through the
inhibition of Cox-2 by the inhibitor rofecoxib, we found that
Cox-2 contributed to the resulting fibrosis, but not colitis, identi-
fying a possible mechanism underlying MyD88-dependent intes-
tinal fibrosis.

**MATERIALS AND METHODS**

**Mice and Cell Lines**

C57BL/6 mice were purchased from the National Cancer Institute
(Frederick, MD), while congenic MyD88−/− mice (backcrossed ≥10
times onto a C57BL/6 background) were bred in our animal facilities.
Mice were kept in sterilized, filter-topped cages, handled in tissue
culture hoods, and fed autoclaved food and water under specific
pathogen-free conditions. At 8–12 wk of age, mice were given 20 mg
of streptomycin by oral gavage 24 h prior to infection. Mice were
infected with 3 × 10^6 ΔaroA S. Typhimurium in 100 μl of PBS buffer
(pH 7.2) by oral gavage. Mice were gavaged with a daily dose (100
μl) of the Cox-2 inhibitor rofecoxib (20 mg/kg) in 4% carboxymeth-
ylcellulose (carrier) or the carrier alone. The protocols were approved
by the University of British Columbia’s Animal Care Committee and
were in direct accordance with guidelines of the Canadian Council
on the Use of Laboratory Animals. Mouse macrophage (RAW 264.7) and
fibroblast (NIH 3T3) cell lines were purchased from the American
Type Culture Collection.

**Bacterial Strain and Enumeration**

*Salmonella enterica* serovar Typhimurium strain SL1344 ΔaroA
(9) was grown, with shaking, at 37°C (200 rpm) in Luria-Bertani (LB)
broth supplemented with 100 μg/ml streptomycin. For bacterial enu-
meration, tissues were collected into 800 μl of sterile PBS on ice and
homogenized with a MixerMill 301 (Retsch, Newtown, PA). Serial
homogenate dilutions were plated on LB-agar plates (100 μg/ml
streptomycin).

**Histology**

Tissues were fixed in 10% neutral buffered formalin overnight and
then placed into 70% ethanol. Fixed tissues were embedded in paraffin
and cut into 5-μm sections. Tissues were stained with hematoxylin-
eosin or Masson’s trichrome according to standard techniques by the
University of British Columbia Histology Laboratory or Wax-it Hist-
ology Services (Vancouver, BC, Canada).

**Ki67 Quantification**

Formalin-fixed tissues were stained for the proliferation marker
Ki67. Images of the mucosal region were taken at ×63 magnification
and used for quantification of epithelial Ki67-positive cells. Ten im-
ages were taken for each tissue for quantification.

**Tissue Pathology Scoring**

Tissue pathology and crypt height measurements in colic tissues
removed from ΔaroA Typhimurium-infected mice were scored using
hematoxylin-eosin-stained sections, as previously described by Bar-
thel et al. (3) with the following modification: instead of scoring
mucosal and submucosal damage together, we scored them separately.

Briefly, two observers blinded to the experimental conditions scored
colic tissues for mucosal pathology, including polymorphonuclear
leukocyte (PMN) infiltration (0–4), goblet cell numbers/depletion
(0–3), and epithelial integrity (0–3), as previously outlined (3). For
submucosal pathology, edema (0–3) was measured as outlined by
Barthel et al., while PMNs and mononuclear cells in the submucosa
were enumerated separately in 10 high-power fields (×400 magnifi-
cation, 420-μm field diameter), and the average number of cells per
high-power field was calculated. The scores were defined as follows:

0 cultivated cells/high-power field, 1 5–20 cells/high-power field, 2 21–60 cells/high-power field, 3 61–100 cells/high-power field, and
4 100 cells/high-power field. Crypt lengths were measured by
micrometry, as previously described (21), by two observers blinded to
the experimental condition, with 10 measurements taken in the distal
colon for each mouse. Only well-oriented crypts were measured.

**Immunohistochemistry**

Paraformaldehyde- or formalin-fixed sections (5 μm) were depar-
affinized, rehydrated, or treated with antigen retrieval as necessary.
Immunostaining was carried out using the following antibodies:
anti-F4/80 rat antibody (AbD Serotec; 1:2,000 dilution), anti-my-
eloperoxidase rabbit polyclonal antibody (Thermo Scientific; 1:100
dilution), anti-vimentin rabbit polyclonal antibody (MBL; 1:50
dilution), anti-collagen I rabbit polyclonal antibody (RDI Research Diag-
nostics; 1:20 dilution), anti-fibroblast-specific protein-1 rabbit poly-
clonal antibody and anti-Cox-2 rabbit polyclonal antibody (Cayman
Chemical; 1:250 dilution), and anti-Ki67 (Thermo Scientific; 1:200
dilution). The following secondary antibodies were used: Alexa Fluor
568-conjugated goat anti-rabbit IgG (Molecular Probes; 1:2,000 dilution), Alexa Fluor 568-conjugated goat anti-rat IgG (Molecular
Probes; 1:2,000 dilution), and Alexa Fluor 488-conjugated goat anti-
rabbit IgG (Molecular Probes; 1:2,000 dilution). Tissues were
mounted using ProLong Gold Antifade (Molecular Probes) containing
4',6- diamidino-2-phenylindole for DNA staining. Images were obtained
using a Zeiss AxioImager microscope equipped with an AxioCam HRm
camera operating through AxioVision software.

**RNA Isolation, cDNA Synthesis, and Real-Time PCR**

Mice were euthanized at indicated time points, and a 2-mm length
of the cecal tip was excised and immediately submerged in RNAAlater
(Qiagen, Mississauga, ON, Canada). RNA was extracted using an
RNaseasy Plus Mini kit (Qiagen) according to the manufacturer’s
instructions. RNA concentration was determined using a NanoDrop
ND-1000 (NanoDrop Technologies, Wilmington, DE), and reverse
transcription was performed with the Omniscript RT kit (Qiagen) with 1 μg of RNA used as starting material. Final CDNA was diluted 1:5, and 5 μl of diluted cDNA were used for PCR. Real-time PCR was performed using qi SYBR Green Supermix (Qiagen) and primers for β-actin (4), collagen type I and TGF-β1 (14), monocyte chemotactic protein-1 (MCP-1) (21), and Cox-2/Ptgs-2 (11). PCR was performed on an Opticon 2 (Bio-Rad).

Collagen Assay

Pepsin-soluble collagen levels were determined with the Sircol collagen assay (Accurate Chemical, Westbury, NY) according to the manufacturer’s instructions. Briefly, 0.5-cm2 sections of cecum were cut into small pieces and digested overnight in 500 μl of pepsin solution (10 mg/ml in 0.5 M acetic acid) with vigorous shaking at room temperature. Collagen was quantified using a standard curve made with collagen supplied by the manufacturer.

In Vitro Cell Assay

Conditioned medium preparation. Mouse macrophage RAW 264.7 cells cultured in DMEM supplemented with 10% FBS were seeded at 4 × 105 cells/well in six-well plates. On the next day, cells were infected at multiplicity of infection 10. Before infection, ΔaroA S. Typhimurium was opsonized with mouse serum for 15 min at 37°C. For infection, the cell medium was replaced by 1 ml of the DMEM-bacteria mix, and the cells were incubated for 30 min at 37°C. The medium was aspirated, and the cells were washed three times with 1× PBS. Macrophages were further incubated for 90 min at 37°C in DMEM with 10% FBS and 100 μg/ml gentamicin; then the medium was replaced with medium supplemented with 15 μg/ml gentamicin. Infected cells were grown for 18 h, and the culture supernatants from infected and uninfected macrophages were collected, centrifuged, and filtered using a 0.2-μm filter. These two samples are designated conditioned medium (CM) after infection (CM-I) and CM of uninfected macrophages (CM-UI).

Fibroblast cultured in CM. Murine NIH 3T3 fibroblasts were grown in DMEM supplemented with 10% FCS. The cells were seeded in six-well plates at a final concentration of 4 × 105 cells/well. On the next day, the medium was substituted with macrophage CM. Fibroblasts were also grown in the presence of 10 ng/ml LPS (Sigma) and multiplicity of infection 2:1 of heat-killed ΔaroA S. Typhimurium bacteria. After 24 h, the cell lysate was collected for RNA isolation.

Statistical Analysis

Student’s t-test was performed using a 95% confidence interval with Prism version 5.0 (GraphPad Software, San Diego, CA). Values are means ± SE.

RESULTS

MyD88 Signaling Limits Systemic, but not Intestinal, S. Typhimurium Burdens

Previous studies examining the role of MyD88 signaling in the Salmonella colitis model examined tissues at 2–3 days postinfection (15), largely because MyD88−/− mice (on a C57BL/6 background) are highly susceptible to WT S. Typhimurium, rapidly succumbing to infection. In an attempt to address whether MyD88 affects the host response over a longer time course, we infected WT and MyD88−/− mice with S. Typhimurium, as previously described (14). Infected mice were followed until postinfection day 7, with additional mice euthanized at postinfection day 3 for quantification of S. Typhimurium burdens and tissue collection for histology (Fig. 1A). We found that WT and MyD88−/− mice survived until postinfection day 7 and beyond (data not shown). Between 106 and 109 S. Typhimurium were recovered from the cecum of infected WT and MyD88−/− mice throughout the course of infection, with a modest trend toward more bacteria in the MyD88−/− mice that did not reach significance (Fig. 1B). In contrast, MyD88−/− mice carried increased systemic S. Typhimurium burdens, with an average of 108 bacteria in their spleens at postinfection day 7 compared with <105 bacteria in WT mice (Fig. 1C), a finding that agrees with previous studies (15, 40). These data demonstrate that although MyD88 signaling limits systemic S. Typhimurium burdens, it has little impact on the burden of S. Typhimurium that resides within the host’s intestines.

Mucosal Pathologies During S. Typhimurium-Induced Colitis Are MyD88-Dependent

Since intestinal S. Typhimurium burdens were similar in the two mouse strains, we next assessed the intestinal pathology of these mice. Cecal tissues collected from mice euthanized at postinfection days 3 and 7 were studied using hematoxylin-eosin-stained tissue sections (Fig. 1A) and a scoring system that assessed mucosal pathology separately from submucosal pathology. Figure 2 shows pathology scores for the mucosa, which included PMN cell infiltration, goblet cell depletion, and loss of epithelial cell integrity. By postinfection day 3, we noticed infiltrating PMNs in the mucosa of WT and MyD88−/− mice; however, the numbers of neutrophils were significantly higher in the WT than the MyD88−/− mice [scores of 2.3 ± 0.2 (n = 5) and 1.6 ± 0.3 (n = 8), respectively]. A similar trend was observed at postinfection day 7, with the score for WT mice remaining at 2.3 ± 0.3 (n = 5), while the MyD88−/− mouse score was 1.4 ± 0.4 (n = 6). Goblet cell depletion was also noted in WT and MyD88−/− mice; however, the depletion was more severe in the WT mice at postinfection days 3 and 7. Interestingly, a very similar trend was observed for damage to epithelial integrity, where a lower score was found in the MyD88−/− than WT mice at postinfection days 3 and 7, indicating that MyD88 signaling plays an important role in the induction of mucosal injury and inflammation during S. Typhimurium infection.

We then analyzed colitic parameters reflecting inflammation and tissue damage within the submucosa (Fig. 2). A number of PMNs were found infiltrating the submucosal region of WT mice at postinfection day 3, and a similar infiltration was found in the submucosa of the MyD88−/− mice. A similar trend was also observed regarding the presence of mononuclear cells in the submucosa, with PMNs and mononuclear cells modestly increasing in both mouse strains at postinfection day 7. When scoring for submucosal edema, we found that the edema was greater in the WT mice at postinfection day 3; however, by day 7 the edema in the MyD88−/− mice had increased to levels similar to the WT mice (Fig. 2). Taken together, these data show that although inflammation and epithelial-dependent changes in the mucosa during S. Typhimurium infection are MyD88-dependent, concurrent submucosal responses, including inflammation and edema, appear to be MyD88-independent.

MyD88 Plays a Critical Role in S. Typhimurium-Induced Epithelial Cell Proliferation

MyD88 signaling has previously been shown to contribute to increased epithelial cell proliferation in the DSS and C. rodentium models of colitis. On the basis of the MyD88 dependence of the crypt hyperplasia in these models, we addressed whether epithelial cell proliferation was also increased in the S. Typhimurium...
colitis model and whether it was MyD88-dependent. To quantify epithelial cell proliferation, we stained tissues for Ki67, a marker of proliferation. As expected, WT and MyD88−/− mice showed similar levels of proliferation measured by Ki67 quantification, with Ki67 reaching $9.9 \pm 0.8$ and $12.1 \pm 1.1$ positive cells/crypt, respectively, under uninfected conditions (Fig. 3A). In contrast, by postinfection day 7, the proliferating epithelial cell numbers had significantly increased in the WT mice, reaching $39.0 \pm 5.0$ Ki67-positive cells/crypt, whereas proliferation was less elevated than at baseline in MyD88−/− mice ($23.4 \pm 1.6$ Ki67-positive cells/crypt; Fig. 3B). Moreover, dramatic crypt hyperplasia (increase in crypt lengths) was observed in infected WT mice compared with infected MyD88−/− mice (Fig. 3C). Taken together, these data indicate that colitis-induced epithelial cell proliferation likely contributes to the MyD88-dependent mucosal pathology in this model.

**S. Typhimurium-Induced Fibrosis During Chronic Infection Is MyD88-Dependent**

To investigate whether MyD88 signaling contributes to the fibrosis development in this model, we extended the duration of the infection, assessing mice at postinfection days 7 and 21. During the course of infection, WT and MyD88−/− mice survived until postinfection day 21, and the intestinal *S. Typhimurium* burdens were similar at this time in the two mouse strains, with WT and MyD88−/− mice reaching $8.9 \pm 3.2 \times 10^4$ ($n = 8$) and $1.2 \pm 0.6 \times 10^4$ ($n = 6$) colony-forming units, respectively. Assessing the ceca from uninfected and infected WT and MyD88−/− mice for collagen levels, we found profound collagen deposition within the C57BL/6 ceca at postinfection day 7, and this 10-fold induction over baseline persisted at postinfection day 21 (Fig. 4A). Interestingly, infected MyD88−/− mice displayed only a one- to twofold increase in collagen, such that their collagen levels were significantly lower than those of C57BL/6 mice at postinfection days 7 and 21 (Fig. 4A). We also assessed the expression of the profibrotic cytokines MCP-1 (27) and TGF-β1 and found a significant upregulation in MCP-1 gene expression in C57BL/6 and MyD88−/− mouse ceca at postinfection day 7, but this upregulation in the MyD88−/− mice was significantly reduced compared with that in C57BL/6 mice (Fig. 4B). Moreover, TGF-β1 expression was only significantly upregulated over baseline in infected C57BL/6 mice.
The disparity in collagen levels between infected WT and MyD88\(^-/-\) mouse tissues was confirmed by Masson’s trichrome and collagen staining (Fig. 4, C and D, respectively). Masson’s trichrome staining within the WT tissues was widely and transmurally distributed, although, as previously outlined, staining was strongest in the submucosa. The areas staining positively for collagen in infected MyD88\(^-/-\) tissues were significantly smaller than those in C57BL/6 mice.

S. Typhimurium-induced fibroblast accumulation is attenuated in MyD88\(^-/-\) mice. By immunofluorescence staining, we investigated whether differences in fibroblast accumulation in the ceca of C57BL/6 vs. MyD88\(^-/-\) mice could explain the differential collagen levels, as fibroblasts are the major collagen-producing cells in this model (14). Our staining revealed that collagen-rich areas also contained high densities of vimentin-positive fibroblasts. We found that S. Typhimurium infection led to a massive accumulation of fibroblasts within the cecal mucosa and particularly in the submucosa of C57BL/6 mice by postinfection day 7, and the presence of these fibroblasts was maintained or even increased at postinfection day 21 (Fig. 4E). In contrast, the number of fibroblasts identified within the cecal tissues of infected MyD88\(^-/-\) mice was dramatically attenuated at postinfection days 7 and 21, suggesting that limitations in fibroblast accumulation in the infected MyD88\(^-/-\) mice were the basis for their reduced collagen levels.

Increased Cox-2 Expression During S. Typhimurium Infection Is MyD88-Dependent

Our data indicate that MyD88 signaling plays a critical role in epithelial cell proliferation and intestinal fibrosis, two distinct forms of intestinal tissue repair (5, 39). We next assessed the expression of the enzyme Cox-2, since it has been frequently associated with mucosal tissue repair (5, 11) and shown to be induced in a MyD88-dependent manner. Interestingly, using quantitative PCR, we found that gene expression levels of Cox-2 (also known as Ptgs-2) were significantly induced by postinfection day 3 in WT mice and progressed to a sixfold increase by postinfection days 7 and 14; however, this bacterial-induced increase was almost completely abrogated in the MyD88\(^-/-\) mice at postinfection days 3 and 7 (Fig. 5A). Interestingly, we noted a modest, but significant, increase in Cox-2/Ptgs-2 gene expression at postinfection day 14 in the MyD88\(^-/-\) mice, suggesting some form of compen-
satory response at the late stages of infection. To address the cellular sources of Cox-2, we immunostained infected tissues and, as shown in Fig. 5, 

At postinfection day 7, we found few, if any, positive cells in the MyD88−/− mouse tissues (Fig. 5C). By postinfection day 21, a few patches of Cox-2-expressing cells were detected in the MyD88−/− mucosa, but the numbers were still far fewer than in the C57BL/6 mice. These results indicate a temporal MyD88-dependent upregulated expression of Cox-2 upon bacterial infection.

Cox-2 Contributes to S. Typhimurium-Driven Intestinal Fibrosis

To investigate whether Cox-2 contributed to the pathologies seen in this model, we inhibited Cox-2 by treating WT mice daily with an oral dose of 20 mg/kg rofecoxib, a Cox-2-specific inhibitor (25), during a 7-day infection, while a control group only received the carrier. As assessed at postinfection day 7, rofecoxib treatment was unable to significantly reduce epithelial cell proliferation (not shown). However, the rofecoxib-treated group displayed a 50% reduction in collagen levels compared with the control group (Fig. 6A), suggesting a role for Cox-2 in intestinal fibrosis. The decrease in collagen deposition in rofecoxib-treated ceca was also confirmed by Masson’s trichrome (Fig. 6D) and collagen I (not shown) staining of tissue sections. To investigate if the reduced fibrosis was linked to a Cox-2-dependent reduction in tissue damage, hematoxylin-eosin-stained tissue slides (Fig. 6C) were examined by pathology scoring. Interestingly, no significant differences in pathology were found between the two groups in the mucosa (Fig. 6B). Moreover, no significant differences in the pathological scoring of the submucosal regions were determined between carrier- and rofecoxib-treated animals. The levels of edema in WT and MyD88−/− mice (scores of 0.9 ± 0.0 and 0.8 ± 0.1, respectively, n = 5) were similar. In addition, we did not observe significant differences in mononuclear cell infiltration (scores of 2.7 ± 0.2 and 2.5 ± 0.1 for WT and MyD88−/−, respectively, n = 6) and PMN infiltration (scores of 1.2 ± 0.1 and 1.1 ± 0.2 for WT and MyD88−/−, respectively, n = 6). These results indicate a selective role for Cox-2 in controlling fibrosis-related collagen deposition but not colitis development. To determine whether the accumulation of fibroblasts was affected by the Cox-2 inhibition, immunofluorescence staining with the fibroblast marker vimentin was performed. We found significantly reduced numbers of

Fig. 3. Epithelial cell proliferation in C57BL/6 and MyD88−/− mice upon S. Typhimurium ΔaroA infection. A: Ki67 immunostaining shows proliferating cells (red) in the mucosa, with nuclei [4′,6′-diamidino-2-phenylindole (DAPI)] shown in blue. UI, uninfected. Original magnification: ×63; scale bars: 20 μm. B: quantification of Ki67-positive epithelial cells per crypt in uninfected and postinfection day 7 cecal tissues. C: crypt length, where individual data points are calculated as average length of ~15 crypts from different sites in each cecum. *P = 0.01–0.05; **P < 0.0005.
fibroblasts in rofecoxib-treated tissues, suggesting that Cox-2 contributes to fibrosis through a role in the recruitment of fibroblasts to the infected cecum (Fig. 6, E and F).

**Cox-2 Is Expressed by Fibroblasts in Fibrotic Regions**

To address the cellular source of the Cox-2 in this model, a series of immunofluorescence stainings were performed. A majority of the Cox-2-stained cellular patches (Fig. 7A, left) overlapped with areas containing dense fibroblast (vimentin-positive) populations (Fig. 7A, right). Only rarely were Cox-2-positive cells (Fig. 7B, left) found in areas with neutrophils (myeloperoxidase-positive; Fig. 7B, right) or in the epithelial layer (determined by location). Double staining for Cox-2 and macrophages (F4/80-positive) revealed an occasional macrophage that stained positively for Cox-2 (not shown); however, as seen in Fig. 7C, the vast majority of macrophages in the cecae, although often in proximity to Cox-2-positive cells, did not themselves co-stain with Cox-2, thus indicating the presence of additional Cox-2-expressing cell types in this enterocolitis/fibrosis model. Therefore, Cox-2 was found localized predominantly to fibrotic areas with a high density of fibroblasts, and these fibroblasts, as well as other, yet to be defined,
cell types are likely important sources of Cox-2 in this infection model.

To further address the importance of fibroblasts and possible interactions between these cell types and macrophages in the induction of Cox-2 expression, mouse macrophage (RAW 264.7) and fibroblast (NIH 3T3) cell lines were examined in vitro. Supernatant from macrophages infected with S. Typhimurium ΔaroA, as well as the media from uninfected macrophages, was used to prepare CM. Growing the murine fibroblasts in the macrophage CM for 24 h triggered a 240-fold increase in Cox-2 gene transcription, while LPS or heat-killed bacteria did not lead to an increase (Fig. 7D). Also, macrophages themselves responded to the infection by an upregulation (140-fold) of Cox-2 expression. These data confirm the Cox-2 expression by fibroblasts and suggest that such expression may occur in response to macrophage-derived mediators. Thus future studies with this model should address interactions between macrophages and fibroblasts, two cell types commonly found in fibrotic tissues.

**DISCUSSION**

In this study, we show that MyD88-dependent signaling plays mucosal homeostatic and profibrotic roles in the GI tract during infection by S. Typhimurium. By using the attenuated ΔaroA strain of S. Typhimurium, we were able to investigate the role of MyD88 signaling using genetically deficient mice backcrossed onto the highly susceptible C57BL/6 genetic background. Inflammatory signaling through MyD88, as well as by specific TLRs, has been well characterized as limiting S.
Typhimurium proliferation and spread at sites such as the liver and spleen (31, 34). While we noted significantly increased S. Typhimurium burdens in the spleens of infected MyD88−/− mice, we were surprised to find no significant impact of MyD88 on intestinal burdens. In contrast, we noted that MyD88 signaling played a dramatic causal role in most of the epithelial changes during S. Typhimurium infection, including increased epithelial cell proliferation, goblet cell depletion, and loss of epithelial integrity. While mucosal inflammation was also reduced in infected MyD88−/− mice, submucosal inflammation and tissue damage as measured by edema were similar in the infected WT and MyD88−/− mice, indicating that the underlying inflammatory mechanisms differ between the mucosa and submucosa. The submucosal damage in this model may reflect the ability of S. Typhimurium to translocate across the intestinal epithelium, reaching deeper tissues within the gut as well as systemic tissues, including the spleen (8, 31).

While a recent study showed that MyD88 plays a role in inducing Th17 responses very early during the S. Typhimurium enterocolitis model (20), this is the first demonstration that innate signaling in this model also plays a role in promoting mucosal homeostasis. MyD88 signaling has been previously...
described to play a mucosal homeostatic role in DSS- and C. rodentium-induced colitis models (12, 29). In these models, MyD88 signaling was found to promote epithelial cell proliferation and repair (29) and to protect epithelial barrier function, such that colitic MyD88−/− mice were unable to respond appropriately to intestinal damage, thereby suffering widespread mucosal ulcerations and high mortality rates. While we noted a similar role for MyD88 in this model, the MyD88−/− mice readily survived the infection and showed only modest epithelial damage, suggesting that host factors, rather than S. Typhimurium ΔaroA-infected C57BL/6 mice at postinfection day 7. A: Cox-2-positive cells (red) and fibroblasts (vimentin, red) on adjacent tissue sections. Original magnification: ×20; scale bars: 50 μm. Arrows indicate areas where Cox-2 is expressed and vimentin-positive cells are found in the same region. B: Cox-2-positive cells (green) and neutrophils [myeloperoxidase (MPO), red] on adjacent tissue sections. Original magnification: ×10; scale bars: 100 μm. Arrowhead indicates area where Cox-2 is expressed but no neutrophils are present. C: Cox-2-positive cells (green) and macrophage (F4/80 marker)-positive cells (red). Arrowhead indicates Cox-2-expressing cells (Cox-2-positive, F4/80-negative); arrow indicates macrophages (F4/80-positive, Cox-2-negative). Original magnification: ×60; scale bars: 50 μm. D: quantitative RT-PCR analysis of Cox-2/Ptgs-2 expression in in vitro stimulated mouse macrophage (RAW 264.7) and fibroblast (NIH 3T3) cell lines. Supernatants from infected (I) or uninfected (UI) macrophages were used to prepare conditioned media (CM) to stimulate fibroblasts. Fibroblasts were also tested under conditions where they not stimulated (un-stim) or treated with 10 ng/ml LPS or with heat-killed bacteria (HKB).
Typhimurium, cause the intestinal epithelial damage in this model. Considering the impact of MyD88, these data suggest that the S. Typhimurium colitis model could prove useful in further assessment of the impact of TLRs and other innate pathways in regulating mucosal integrity.

Intestinal fibrosis also develops as a result of chronic S. Typhimurium infection (14). In our study, C57BL/6 and MyD88−/− mice continued to be colonized by S. Typhimurium until 3 wk postinfection and beyond, confirming chronic infection. At postinfection days 7 and 21, the cecal walls of infected mice showed significant inflammation and widespread collagen deposition in the submucosa and mucosa. In this study, we provide the first evidence that intestinal fibrosis is MyD88-dependent. The decreased mucosal homeostatic and fibrotic responses in the MyD88−/− mice were not the result of impaired bacterial colonization, as these mice carried similar intestinal pathogens in their ceca compared with WT mice. Interestingly, when Hapfelmeier et al. (15) studied the role of MyD88 in the S. Typhimurium-induced colitis model, they found a MyD88 dependence of bacterial clearance but no difference in cecal pathology between C57BL/6 and MyD88−/− mice. We believe this discrepancy reflects the fact that Hapfelmeier et al. used the more virulent wild-type SL1344 S. Typhimurium strain, which causes more severe pathology and 100% mortality in MyD88−/− mice within the first few days of infection.

Mesenchymal cells in the intestine can be broadly classified as fibroblasts, smooth muscle cells, or myofibroblasts, and these are the main producers of extracellular matrix components, such as collagen, which are deposited during fibrosis. A recent review on fibrogenesis in Crohn’s disease concluded that even though fibrosis development likely is a result of the actions of all three cell types, fibroblasts are likely the main players in intestinal fibrogenesis (6). This conclusion readily agrees with our previous findings using this model, where large accumulations of fibroblasts were found in areas with extensive collagen deposition (14). Adding to this observation, our present data suggest that the fibroblast accumulation itself appears to be partly MyD88-dependent, as the accumulation of vimentin-positive (also fibroblast-specific protein-1-positive; not shown) cells was greatly reduced in the tissues of infected MyD88−/− mice compared with C57BL/6 mice. Considering that most fibroblast accumulation, as well as collagen deposition, was observed in the submucosa, yet inflammation and edema at this site were MyD88-independent, these data suggest that fibrosis is not simply a secondary effect of local inflammation and tissue damage. Instead, the accumulation of fibroblasts within the submucosa likely reflects a more complicated process, potentially linked to MyD88-dependent mucosal homeostatic processes.

With this in mind, a recent study by Johnson et al. (18), using our model, found that it is the host response, rather than the chronic presence of S. Typhimurium, that is required for development of intestinal fibrosis. Once infection and the resulting inflammatory response were established, antibiotic treatment and removal of the bacterial pathogen had little impact on the subsequent development of intestinal fibrosis. These unexpected results suggest that intestinal fibrosis does not require persistent microbial stimulation of the immune system but is, instead, a consequence of, and presumably an attempt to repair, the severe mucosal and submucosal damage suffered during the initial stages of infection.

Fukata et al. (11) showed that intestinal injury by DSS caused MyD88-dependent Cox-2 expression in macrophages and epithelial cells. They found this response to be essential for tissue repair processes, such as inducing epithelial cell proliferation and limiting apoptosis. In contrast, using the same colitis model, Brown et al. (5) did not find Cox-2/Ptgs-2 expression to be regulated by injury or to be MyD88-dependent. Instead, they reported a MyD88-dependent repositioning of Cox-2-expressing stromal cells of unknown identity toward colonic epithelial progenitor cells located at the base of colonic crypts. In a recent study, this group determined that these colonic stromal Cox-2-positive cells are mesenchymal stem cells (38). This repositioning was required to maintain epithelial cell proliferation in response to injury. Knowing the conflicting data regarding injury-induced expression of Cox-2 in the intestine, we were interested to find that Cox-2/Ptgs-2 was induced by S. Typhimurium infection and that its upregulation by postinfection day 7 was strictly MyD88-dependent. By day 14, however, Cox-2/Ptgs-2 gene expression was significantly elevated over baseline in infected MyD88−/− mice but still significantly lower than in infected WT mice, suggesting some form of compensatory response in the absence of MyD88.

Considering the role attributed to Cox-2 in promoting mucosal tissue repair, we were eager to address whether Cox-2 played a role in fibrosis development and, if so, whether it was a consequence of the inflammatory qualities of Cox-2 or, rather, a direct, inflammation-independent, Cox-2 pathway leading to intestinal fibrosis. To our knowledge, no previous studies have addressed this question, but several studies have used Cox-2 inhibitors in clinical trials (22) or in different animal models of colitis. The results from these studies range from Cox-2 inhibitors protecting from intestinal damage and colitis (10, 24) to worsening the pathology and delaying the healing process (26, 35). A similar range of conclusions was reported from studies addressing the role of Cox-2 in liver and lung fibrosis. Using Cox-2-deficient mice or Cox-2 inhibitors, the conclusions range from Cox-2/Ptgs-2 deficiency or inhibition resulting in an exaggerated fibrotic response in the lung (16, 19) to no role in the lung (7) or liver (41) or an inhibitory effect on lung (1) and liver (17, 28) fibrosis. In the cases where Cox-2 was suggested to have an inhibitory effect, it was generally explained as secondary to decreased tissue inflammation.

When we addressed this in our model of colitis and intestinal fibrosis, we found that, upon treatment of the infected mice with the Cox-2-specific inhibitor rofecoxib, the collagen deposition was reduced by ~50%. Interestingly, the tissue pathology scores were similar in the rofecoxib- and carrier-treated groups, demonstrating that Cox-2 is not critical for the development of colitis in this model, instead suggesting a direct role for Cox-2 in fibrosis development. While additional studies are needed, we also identified an overt reduction in fibroblast accumulation following rofecoxib use, suggesting that Cox-2 contributes to fibrosis in this model by playing a role in fibroblast accumulation in the infected cecum. By combining our tissue staining with in vitro studies, we confirmed Cox-2 expression by fibroblasts. Furthermore, our data showed Cox-2 expression by fibroblasts in response to mediators secreted from infected macrophages, pinpointing the importance of these cell types in this mouse model of intestinal fibrosis.

Interestingly, production of Cox-2 and TGF-β1, both induced

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in our study, has been suggested to act in a negative-feedback loop balancing the level of fibrosis formation during skeletal muscle healing (32). The role of Cox-2 in promoting intestinal fibrosis, as well as the mechanisms underlying its induction, will be important subjects for further studies.

Taken together, our studies show that the ΔaroA strain of S. Typhimurium can be used to address the role of specific host factors in mice as broadly immunodeficient as MyD88−/− mice, showing the broad potential for this mouse model of colitis and intestinal fibrosis. More specifically, we provide the first evidence that MyD88-dependent signaling plays a role in intestinal fibrosis, through MyD88-driven induction of Cox-2, where Cox-2 represents a potential therapeutic target for prevention of intestinal fibrosis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

L. E. M., M. M., and B. A. V. are responsible for conception and design of the research. L. E. M., M. M., C. Ma, and T. H. performed the experiments; L. E. M., M. M., and B. A. V. drafted the manuscript; L. E. M., M. M., and B. A. V. revised the manuscript, approved the final version of the manuscript.

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