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

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**ABSTRACT**

*Salmonella enterica* serovar Typhimurium is a clinically important gram-negative, enteric bacterial pathogen that activates several Toll-like receptors (TLR). 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 GI tract is less clear. We therefore used the recently described salmonella induced enterocolitis and fibrosis model, pre-treating wildtype (WT) and MyD88 deficient (-/-) mice with streptomycin followed by oral infection with the ΔaroA vaccine strain of *S*. Typhimurium. Tissues were analyzed for bacterial colonization, inflammation and epithelial damage as well as collagen quantification and Masson's trichrome staining. WT and MyD88 -/- mice carried similar intestinal pathogen burdens out to day 21 post-infection. 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 day 7 and 21 post-infection, with fibrotic regions rich in fibroblasts and collagen. While infected MyD88 -/- mice showed similar levels of submucosal inflammation and edema to WT mice, they were impaired in the development of mucosal inflammation, along with infection-induced epithelial damage, proliferation and goblet cell depletion. MyD88 -/- mouse tissues also had fewer submucosal fibroblasts and 60% less collagen in their tissues. We noted that cyclo-oxygenase (Cox)-2 expression was MyD88 dependent with numerous Cox-2 positive cells identified in fibrotic regions of WT mice 7 days post
infection, but not in MyD88 -/- mice. Treating 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 both play roles in intestinal fibrosis during *Salmonella*-induced enterocolitis.
**INTRODUCTION**

*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 (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 (PAMPs), *Salmonella* has been shown to activate a number of TLRs both *in vitro* and *in vivo*. Moreover, the adaptor protein MyD88 plays a critical role in innate signaling by the Toll-like receptor (TLR) and the interleukin 1 receptor (IL-1R) superfamily. Curiously, despite *Salmonella enterica* serovar Typhimurium being 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 play 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).

In contrast, although *S.* Typhimurium is an enteric pathogen, it is only recently that studies have begun to assess how innate signaling impacts on host defense and the resulting disease when this pathogen infects its hosts’ intestines (15, 23). This is an important question since innate immune signaling in the gastrointestinal (GI) tract appears to have unique functions, as compared to 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 DSS chemical colitis model, preventing colonic ulceration by promoting epithelial cell proliferation and repair in response to innate recognition of commensal bacteria (2, 29). Moreover, we recently confirmed this tissue protective role for MyD88 signaling using the attaching/effacing bacterial pathogen Citrobacter rodentium. During infection, MyD88 deficient mice developed severe cecal and colonic ulceration and carried significantly heavier pathogen burdens leading to rapid morbidity and mortality (12).

So far, 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. Following 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 wildtype S. Typhimurium infection, succumbing in only a few days (37). Considering that most TLR or MyD88 deficient mouse strains are generated on this genetic background, studies using these mice have been limited to
very acute experiments. However, we recently described 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 seen in the gastrointestinal
tissues of some patients with Crohn’s disease (CD) (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 inflammatory cell
recruitment were noted in infected WT C57BL/6 mice, along with submucosal pathology
including edema, inflammation, fibroblast proliferation and collagen deposition.
Immunostaining for the cyclo-oxygenase (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 inflammation,
epithelial pathology and fibrogenesis were all significantly attenuated in MyD88 -/- mice.
Moreover, we found that upregulation of Cox-2 (Ptgs-2) gene expression was largely
MyD88 dependent, and through the inhibition of Cox-2 by using the inhibitor rofecoxib,
we found that Cox-2 contributed to the resulting fibrosis but not colitis, identifying a
possible mechanism underlying MyD88 dependent intestinal fibrosis.
MATERIALS AND METHODS

Mice and cell lines

C57BL/6 mice were purchased from the National Cancer Institute, Frederick, Maryland, USA, while congenic MyD88 −/− mice (backcrossed at least 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. 8-12 week old mice were given 20 mg of streptomycin by oral gavage 24 hours prior to infection. Mice were infected with 3x10^6 ΔaroA S. Typhimurium in 100 µl PBS buffer (pH 7.2) by oral gavage. As indicated, mice were gavaged with a daily dose (100 µl) of the Cox-2 inhibitor rofecoxib (20 mg/kg) in 4% Carboxymethylcellulose (carrier), or the carrier alone. The protocols employed 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 (RAW264.7) and fibroblast (NIH 3T3) cell lines were purchased from the American Type Culture Collection (ATCC).

Bacterial strain and enumeration

Salmonella enterica serovar Typhimurium strain SL1344 ΔaroA (9) was grown, shaking, at 37°C (200 rpm) in Luria-Bertani (LB) broth supplemented with 100 µg/ml streptomycin. For bacterial enumeration, tissues were collected into 800 µl of sterile PBS on ice and homogenized with a MixerMill 301 (Retsch, Newtown, PA, USA). 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 Haematoxylin-eosin (H&E) or Masson’s trichrome using standard techniques by the UBC Histology lab or Wax-it Histology Services (Vancouver, BC).

Ki67 quantification

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

Tissue pathology scoring

Tissue pathology and crypt height measurements in cecal tissues removed from S. Typhimurium infected mice were scored using H&E stained sections as previously described by Barthel et al (3) with the modification that instead of scoring mucosal and submucosal damage together, we scored them separately. In brief, two observers, blinded to the experimental conditions scored caecal tissues for mucosal pathology including 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 magnification; field diameter of 420 µm), and the average number of cells/high-power field was calculated. The scores were defined as follows: 0 = < 5 cells/high-power field; 1 = 5 to 20 cells/high-power field; 2 =
21 to 60/high-power field; 3 = 61 to 100/high-power field; and 4 = >100/high-power field. Crypt lengths were measured by micrometry, as previous 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**

5 µm paraformaldehyde or formalin sections were deparaffinized, rehydrated or treated with antigen retrieval as necessary. Immunostaining was carried out using following antibodies: anti-F4/80 rat antibody (AbD Serotec, 1: 2000), anti-myeloperoxidase (MPO) rabbit polyclonal antibody (Thermo Scientific, 1:100), anti-Vimentin rabbit polyclonal antibody (MBL; 1:50), anti-Collagen 1 rabbit polyclonal antibody (RDI Research Diagnostics 1: 20), anti-Fibroblast Specific Protein-1 (FSP) rabbit polyclonal antibody and anti- Cox-2 rabbit polyclonal antibody (Cayman chemical, 1: 250) and anti-Ki67 (Thermo Scientific, 1:200). The following secondary antibodies were used: AlexaFluor 568-conjugated goat anti-rabbit IgG (Molecular Probes; 1:2000), AlexaFluor 568-conjugated goat anti-rat IgG (Molecular Probes; 1:2000) and AlexaFluor 488-conjugated goat anti-rabbit IgG (Molecular Probes; 1:2000). Tissues were mounted using ProLong Gold Antifade (Molecular Probes) that contains 4’,6’-diamidino-2-phenylindole (DAPI) 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 polymerase chain reaction**
Mice were sacrificed at indicated time points and 2 mm of the caecal tip were excised and immediately submerged in RNAlater (Qiagen, Mississauga, ON, Canada). RNA was extracted using RNeasy Plus Mini kit (Qiagen) according to manufacturer’s instructions. RNA concentration was determined using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA) and reverse transcription was performed with the Omniscript RT kit (Qiagen) using 1 μg RNA as starting material. Final cDNA was diluted 1:5 and 5 μl of diluted cDNA was used for PCR reactions. Real time PCR was performed using iQ SYBR-Green Supermix (Qiagen) and primers for β-actin (4), Collagen-I and TGF-β1 (14), 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, USA) according to manufacturer’s instructions. Briefly, 0.5 cm sections of caecum 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 media preparation. Mouse macrophage RAW 264.7 cultured in DMEM supplemented with 10% FBS were seeded at 4x10^5 cells/well in 6 well plates. The next day, cells were infected at multiplicity of infection (MOI) 10. Before infection ∆aroA S.
Typhimurium was opsonized with mouse sera for 15 min at 37 °C. For infection, the cell media was replaced by 1ml of the DMEM-bacteria mix and incubated for 30 minutes at 37 °C. The media was aspirated and the cells were washed three times with 1x PBS. Macrophages were further incubated for 90 minutes at 37 °C in DMEM with 10% FBS and containing gentamicin at 100 μg/ml, after which the media was replaced with media supplemented with 15μg /ml of gentamicin. Infected cells were grown for 18 hours and the culture supernatants from infected and uninfected macrophages were collected, centrifuged and filtered using 0.2 μm filter. These two samples are then designated as conditioned media (CM) after infection (CM-I) and conditioned media of uninfected macrophages (CM-UI).

Fibroblast cultured in conditioned media. Murine NIH 3T3 fibroblasts were grown in DMEM supplemented with 10% of FCS. The cells were seeded in 6 well plates at a final concentration of 4x10⁵ cells/well. The next day the media was substituted with macrophage conditioned media. Fibroblasts were also grown in the presence of 10 ng/ml LPS (SIGMA) and MOI 2:1 of heat killed ΔaroA S. Typhimurium bacteria (HKB). Twenty-four hours later the cell lysate was collected for RNA isolation.

Statistical analysis
Student’s t test was performed using a 95% confidence interval with GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). Results are expressed as the mean value with standard error of the mean (SEM). P values: * = 0.01-0.05; ** = 0.005-0.01; *** = <0.0005.
RESULTS

MyD88 signaling limits systemic but not intestinal S. Typhimurium burdens

Previous studies examining the role of MyD88 signaling in the *Salmonella* colitis model have examined tissues at 2-3 days post-infection (pi) (15), largely because MyD88 -/- mice (on a C57BL/6 background) are highly susceptible to WT *S. Typhimurium*, rapidly succumbing to infection. To try and address whether MyD88 impacts on the host response over a longer time course, we infected wildtype and MyD88 -/- mice with *S. Typhimurium* as previously described (14). Infected mice were followed out until day 7 post-infection (pi), with additional mice euthanized at day 3 pi for quantification of *S. Typhimurium* burdens and tissue collection for histology (Fig. 1A). Notably, we found that both WT and MyD88 -/- mice survived until day 7 post-infection and beyond (data not shown). Between $10^6$ and $10^9$ *S. Typhimurium* were recovered from the caeca of infected C57BL/6 and MyD88 -/- mice throughout the course of infection, with a modest trend towards 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 $10^5$ bacteria in their spleens at day 7 pi, compared to fewer than $10^3$ 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 reside 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 suffered by these mice. Caecal tissues collected from mice euthanized at 3 and 7 days pi were studied using H&E stained tissue sections (Fig. 1A) and a scoring system was used that assessed mucosal pathology separately from submucosal pathology. Fig 2 (left panel) shows pathology scores for the mucosa, which included polymorphonuclear (PMN) cell infiltration, goblet cell depletion and loss of epithelial cell integrity. By day 3 pi, we noticed the presence of infiltrating PMNs in the mucosa of C57BL/6 (WT) and MyD88 -/- mice, however, the numbers of neutrophils present at this time point were significantly higher in the WT mice (score of 2.3 ± 0.2, N=5) compared to the MyD88 -/- mice (score of 1.6 ± 0.3, N=8). A similar trend was observed at day 7 pi with the score for WT mice staying at similar values of 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 both WT as well as MyD88 -/- mice, however the depletion was more severe in the WT mice at day 3 and 7 pi. Interestingly, a very similar trend was observed for damage to epithelial integrity, where a lower score was found in the MyD88 -/- mice at day 3 and 7 pi compared to WT mice, 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 (right panel)). A number of PMNs were found infiltrating the submucosal region of WT mice at day 3 pi, 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 both PMNs and mononuclear cells modestly increasing in both mouse strains at day 7 pi. When scored for submucosal edema, we found that the observed edema was greater in the WT mice at day 3 pi, 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 identify 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. Based on the MyD88 dependence of the crypt hyperplasia seen 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 measure by Ki67 quantification with Ki67 reaching values of 9.9 ± 0.8 and 12.1 ± 1.1 positive cells/crypt respectively under uninfected (UI) conditions (Fig 3A). In contrast, by day 7 pi, the proliferating epithelial cell numbers had significantly increased in the WT mice reaching values of 39.0 ± 5.0 Ki67 positive cells per crypt, whereas proliferation was less elevated compared to baseline in MyD88 -/- mice, with values of 23.4 ± 1.6 Ki67 positive cells per crypt (Fig. 3B). Moreover, dramatic crypt hyperplasia (increase in crypt lengths) was observed in infected WT mice.
in comparison with MyD88 -/- infected mice (Fig. 3C). Taken together, these data indicate that colitis induced epithelial cell proliferation likely contributes to the MyD88 dependent mucosal pathology seen in this model.

S. Typhimurium induced fibrosis during chronic infection is MyD88 dependent.

To investigate whether MyD88 signaling contributes to the fibrosis development seen in this model, we extended the duration of the infection, assessing mice at day 7 and day 21 pi. During the course of infection both WT and MyD88 -/- mice survived until day 21 pi, and the intestinal S. Typhimurium burdens were similar at this time in the two mouse strains with WT mice reaching CFU values of 8.9 ± 3.2 x10^4, N=8 and MyD88 -/- mice reaching CFU of 1.2 ± 0.6 x10^4, N=6, respectively. Assessing the caeca from uninfected and infected WT and MyD88 -/- mice for collagen levels, we found profound collagen deposition within the C57BL/6 caeca at 7 days pi, and this 10-fold induction over baseline persisted at day 21 pi (Fig 4A). Interestingly, infected MyD88 -/- mice displayed only a 1-2 fold increase in collagen, such that their collagen levels were significantly lower than C57BL/6 mice at both day 7 and 21 pi (Fig 4A). We also assessed the expression of the profibrotic cytokines, monocyte chemoattractant protein 1 (MCP-1) (27) and transforming growth factor (TGF-β1) and found a significant up-regulation in MCP-1 gene expression in both C57BL/6 and MyD88 -/- mouse caeca at day 7 pi, but the up-regulation in the MyD88 -/- mice was significantly reduced compared with that in C57BL/6 mice (Fig 4 B). Moreover, TGF-β1 expression was only significantly up-regulated 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 staining and by collagen-I staining (Fig. 4C and D, respectively). The Masson’s trichrome staining within the WT tissues was found to be 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 compared to those seen 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 caeca of C57BL/6 mice versus 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 caecal mucosa and particularly in the submucosa of C57BL/6 mice by day 7 pi, and the presence of these fibroblasts was maintained or even increased at day 21 pi (Fig. 4E). In contrast, the number of fibroblasts identified within the caecal tissues of infected MyD88-/- mice was dramatically attenuated at both day 7 and 21 pi, suggesting that limitations in fibroblast accumulation in the infected MyD88-/- mice was 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 both 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 qPCR we found gene expression levels of Cox-2 (also known as Ptgs-2) to be significantly induced by 3 days pi in WT mice, which progressed to a 6-fold increase by 7 and 14 days pi; however, this bacterial-induced increase was almost completely abrogated in the MyD88 -/- mice at both day 3 and 7 pi (Fig. 5A). Interestingly, we noted a modest but significant increase in Cox-2/Ptgs-2 gene expression at day 14 pi in the MyD88 -/- mice suggesting some form of compensatory response at the late stages of infection. To address the cellular sources of Cox-2, we immunostained infected tissues and as shown in Figure 5B and C, at day 7 and 21 pi, patches of Cox-2-positive cells (green, see arrows) were identified in the mucosa and submucosa of WT mice. In contrast, at 7 days pi few if any positive cells were found in the MyD88 -/- mouse tissues (Fig. 5C). By day 21 pi, 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 day 7 pi, rofecoxib treatment was unable to significantly reduce epithelial cell proliferation (not shown). However, the rofecoxib
treated group displayed a 50% reduction in collagen levels as compared to the control
group (Fig. 6A), suggesting a role for Cox-2 in intestinal fibrosis. The decrease in
collagen deposition in rofecoxib treated caeca was also confirmed by Masson’s
Trichrome (Fig. 6D) and collagen-I staining (not shown) of tissue sections. To investigate
if the reduced fibrosis was linked to a Cox-2 dependent reduction in tissue damage, H&E
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 versus rofecoxib treated animals.
The levels of edema in WT (score of 0.9 ± 0.1, N=5) and MyD88 -/- mice (score of 0.8 ±
0.1, N=5) were similar. In addition, we did not observe any significant differences in
mononuclear cell infiltration (WT score of 2.7 ± 0.2, N=6 and MyD88 -/- score of 2.5 ±
0.1, N=6) and PMN infiltration (WT score of 1.2 ± 0.1, N=6 and MyD88 -/- score of 1.1
± 0.2 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 that rofecoxib-treated tissues had
significantly reduced numbers of fibroblasts, suggesting that Cox-2 contributes to fibrosis
through a role in the recruitment of fibroblasts to the infected cecum (Fig. 6 E, 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
panel) overlapped with areas containing dense fibroblast (vimentin positive) populations (Fig. 7A – right panel). Only rarely were Cox-2 positive cells (Fig 7B – left panel) found in areas with neutrophils (MPO positive) (Fig. 7B – right panel) 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 (red - arrow) found in the caeca, although often in proximity to Cox-2 positive cells, did not themselves co-stain with Cox-2 (green – arrowhead), 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, in vitro examination using mouse macrophage (RAW264.7) and fibroblast (NIH 3T3) cell lines was performed. Supernatant from macrophages infected (I) with S. Typhimurium ΔaroA, as well as the media from uninfected (UI) macrophages were used to prepare conditioned media (CM). Growing the murine fibroblasts in the macrophage CM for 24 hours triggered a 240-fold increase in Cox-2 gene transcription; while LPS or heat killed bacteria (HKB) did not lead to any increase (Fig. 7D). Also macrophages themselves responded to the infection by an up-regulation of Cox-2 expression (140-fold). This data confirms the Cox-2 expression by fibroblasts, and suggests 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.
In this study, we show that MyD88 dependent signaling plays both mucosal homeostatic and pro-fibrotic 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 have 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, there was surprisingly 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 seen 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 seen 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 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 both DSS and *C. rodentium* induced colitis models (12, 29). In these models, MyD88 signaling was found to promote epithelial cell proliferation and repair (29) as well as 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 it is host factors, rather than the *S. Typhimurium* that causes the intestinal epithelial damage in this model. Considering the impact of MyD88, these data suggest 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, both C57BL/6 and MyD88 -/- mice stayed colonized by *S. Typhimurium* until 3 weeks post infection and beyond, confirming chronic infection. At day 7 and 21 pi, the caecal 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 caeca as compared to WT mice. Interestingly, when Hapfelmeier *et al.* studied the role of MyD88 in the *S. Typhimurium* induced colitis model, they found a MyD88 dependence of bacterial clearance, but found
no difference in caecal pathology in C57BL/6 versus MyD88 -/- mice (15). We believe this discrepancy reflects Hapfelmeier et al.’s use of 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 (ECM) components, such as collagen, which are deposited during fibrosis. A recent review on fibrogenesis in CD 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 suggests the fibroblast accumulation itself appears to be partly MyD88 dependent, as the accumulation of vimentin positive (also FSP-1 positive – not shown) cells was greatly reduced in the tissues of infected MyD88 -/- mice, when compared to 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 was 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., using this model found that it is the host response rather than the chronic presence of *S. Typhimurium* that is required for intestinal fibrosis to develop (18). Once infection and the resulting inflammatory response was 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.

In a study by Fukata et al. intestinal injury by DSS was shown to cause MyD88 dependent Cox-2 expression in macrophages and epithelial cells (11). They found this response to be essential for tissue repair processes such as inducing epithelial cell proliferation and limiting apoptosis. In contrast, Brown et al., using the same colitis model, did not find Cox-2/Ptgs-2 expression to be regulated by injury or to be MyD88 dependent (5). Instead they reported a MyD88-dependent repositioning of Cox-2 expressing stromal cells of unknown identity, towards colonic epithelial progenitor cells located at the base of colonic crypts. In a recent study from this group they 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, it was interesting to find Cox-2/Ptgs-2 to be induced by *S. Typhimurium* infection, and that its up-regulation by day 7 of infection 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
wildtype 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, was it 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 there are no
previous studies addressing 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 either Cox-2 -/- 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 having no role in the lung (7) or liver (41), or to having an inhibitory
effect on lung (1) and liver fibrosis (17, 28), In the cases where Cox-2 was suggested to
have an inhibitory effect, the effect 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
treating the infected mice with the Cox-2 specific inhibitor rofecoxib, the collagen
deposition was reduced by approximately 50%. Interestingly, the tissue pathology scores
were similar in the rofecoxib-treated 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 in our study, have 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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REFERENCES


FIGURE LEGENDS

Figure 1. Histology and colonization of S. Typhimurium ΔaroA infected C57BL/6 wild-type and MyD88 -/- mouse caecum.

(A) H&E stained tissues (magnification 5x, scale bar: 200 µm) of C57BL/6 and MyD88 -/- mice under non-infected conditions as well as at day 3 and 7 pi. (B) S. Typhimurium ΔaroA colonization of caeca of wildtype C57BL/6 and MyD88 -/- mice. (C) S. Typhimurium ΔaroA colonization of spleens of wildtype C57BL/6 and MyD88 -/- mice. (L = lumen). Each point represents the S. Typhimurium CFU from the respective tissues, while mean ± SEM is shown.

Figure 2. Tissue pathology in C57BL/6 mice and MyD88 -/- mice upon S. Typhimurium ΔaroA infection.

Tissue pathology scores. Left panel shows pathology found in the mucosal region and the right panel shows pathology in the submucosal region. Mucosal pathology scoring includes infiltration of PMNs, goblet cell depletion and loss of epithelial integrity, whereas submucosal pathology included scoring PMN infiltration, mononuclear cell infiltration and submucosal edema. (Each bar represents the average of 5-7 tissues).

Figure 3. Epithelial cell proliferation in C57BL/6 mice and MyD88 -/- mice upon S. Typhimurium ΔaroA infection.

(A) Ki67 immunostaining shows proliferating cells (red) in the mucosa, with nuclei (DAPI) shown in blue, (magnification 63x, scale bar: 20 µm). (B) Quantification of Ki67 positive epithelial cells/crypt in uninfected and day 7 post-infection caecal tissues. (C)
Crypt length where the individual data points are calculated as the average length of approximately 15 crypts taken at different sites in each caecum.

Figure 4. Infection induced collagen deposition and fibroblast accumulation by *S. Typhimurium* \(\Delta aroA\) infection in C57BL/6 mice compared to MyD88 \(-/-\) mice.

(A) Collagen levels (ug/5mm tissue) in the caecal wall measured by Sircol collagen assay. Each bar represents the average of 5-10 *S. Typhimurium* \(\Delta aroA\) infected C57BL/6 and MyD88 \(-/-\) mice at 0, 7 and 21 days pi. (B) qRT-PCR analysis of the induction of MCP-1 and TGF-\(\beta\)1 gene expression in the caecum during *S. Typhimurium* \(\Delta aroA\) infection in C57BL/6 and MyD88 \(-/-\) mice. (C) Masson’s Trichrome stained tissues, note extracellular matrix stains blue (original magnification 5x, scale bar: 200 \(\mu\)m). (D) Collagen-1 (red) and nuclei (DAPI, blue) stained tissues (magnification 10x, scale bar: 100 \(\mu\)m) (E) Fibroblasts (vimentin, red) accumulation within cecal tissues and host cell nuclei (DAPI, blue).

Figure 5. MyD88 dependence of Cox-2/Ptgs-2 expression.

(A) qRT-PCR analysis of the induction of Cox-2/Ptgs-2 in the caecum during *S. Typhimurium* \(\Delta aroA\) infection in C57BL/6 and MyD88 \(-/-\) mice at 0, 3, 7 and 14 days pi. (B and C) IHC staining of Cox-2 (green) with (B) overview of Cox-2 expression in C57BL/6 mice at 7 days pi (magnification 5x, scale bar: 200 \(\mu\)m); and (C) comparison of location (see arrows) in wild-type C57BL/6 mice compared to MyD88 \(-/-\) mice over the course of infection (0, 7 and 21 days pi) (magnification 20x, scale bar: 50 \(\mu\)m). (L = lumen, m = mucosa sm = submucosa)
Figure 6. Role of Cox-2 in *S. Typhimurium* ΔaroA induced fibrosis and colitis.

(A) Collagen levels in the caeca of mice 7 days pi, treated with the Cox-2 inhibitor rofecoxib compared to placebo (carrier), as determined by Sircol collagen assay. Each bar represents the average of 4-7 mice. (B) Tissue pathology scores showing the average score of 4-5 mice. (C) H&E and (D) Masson’s Trichrome stained caeca, from non-infected and day 7 post-infected carrier and rofecoxib treated mice (magnification 5x, scale bar: 200 µm). (E-F) Mucosal fibroblast accumulation in mucosal tissues. (E) Vimentin (red), alpha-SMA (green) stained caecum (magnification 63x, scale bar: 20 µm). (L = lumen). (F) Quantification of vimentin positive cells within the mucosa per high power field.

Figure 7. Identity of cells expressing Cox-2.

(A-D) IHC staining of *S. Typhimurium* ΔaroA infected C57BL/6 caeca 7 days pi. (A) Cox-2 positive cells (red) and fibroblasts (Vimentin, red) on adjacent tissue sections (magnification 20x, scale bar: 50 µm). Arrows are pointing to areas where Cox-2 is expressed and vimentin positive cells are also found in the same region. (B) Cox-2 positive cells (green) and Neutrophils (MPO, red) on adjacent tissue sections (magnification 10x, scale bar: 100 µm). Arrowhead is pointing to 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 is pointing to Cox-2-expressing cells (Cox-2-positive, F4/80-negative), and arrow is pointing to macrophages (F4/80 positive, Cox-2 negative). (magnification 60x, scale bar: 50 µm, L = lumen, m =
mucosa, sm = submucosa). (D) qRT-PCR analysis of Cox-2/Ptgs-2 expression in *in vitro*
stimulated mouse macrophage (RAW264.7) and fibroblast (NIH 3T3) cell line.

Supernatants from infected (I) or un-infected (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).
A

Non-infected 3 day post infection 7 days post infection

C57BL/6

MyD88 -/-

B

CFU per whole cecum

Days pi: 0 3 7

C57BL/6 MyD88 -/-

C

CFU per whole spleen

Days pi: 0 3 7

C57BL/6 MyD88 -/-
A

C57Bl/6 vs. MyD88 -/-

UI

Day 7

B

Ki67 positive cells/intact crypt

Day 7 pi: C57Bl/6 vs. MyD88 -/-

C

Crypt Length (μm)

Days pi: 0, 3, 7

C57Bl/6 vs. MyD88 -/-