MyD88 signalling plays a critical role in host defence by controlling pathogen burden and promoting epithelial cell homeostasis during Citrobacter rodentium-induced colitis

D. L. Gibson, C. Ma, K. S. B. Bergstrom, J. T. Huang, C. Man and B. A. Vallance*  
Division of Gastroenterology, BC Children’s Hospital, University of British Columbia, Vancouver, British Columbia, Canada.

Summary

Myeloid differentiation factor (MyD)88, an adaptor protein shared by the Toll-interleukin 1 receptor superfamily, plays a critical role in host defence during many systemic bacterial infections by inducing protective inflammatory responses that limit bacterial growth. However, the role of innate responses during gastrointestinal (GI) infections is less clear, in part because the GI tract is tolerant to commensal antigens. The current study investigated the role of MyD88 following infection by the murine bacterial pathogen, Citrobacter rodentium. MyD88-deficient mice suffered a lethal colitis coincident with colonic mucosal ulcerations and bleeding. Their susceptibility was associated with an overwhelming bacterial burden and selectively impaired immune responses in colonic tissues, which included delayed inflammatory cell recruitment, reduced iNOS and abrogated production of TNF-α and IL-6 from MyD88-deficient macrophages and colons cultured ex vivo. Immunostaining for Ki67 and BrDU revealed that MyD88 signalling mediated epithelial hyper-proliferation in response to C. rodentium infection. Thus, MyD88-deficient mice could not promote epithelial cell turnover and repair, leading to deep bacterial invasion of colonic crypts, intestinal barrier dysfunction and, ultimately, widespread mucosal ulcerations. In conclusion, MyD88 signalling within the GI tract plays a critical role in mediating host defence against an enteric bacterial pathogen, by controlling bacterial numbers and promoting intestinal epithelial homeostasis.

Introduction

Toll-like receptors (TLRs) play a central role in the initiation of innate cellular immune responses to invading microbial pathogens. A simplified view of this process is that once TLRs, aside from TLR3, interact with their ligands, they subsequently associate and then activate the adaptor protein myeloid differentiation factor (MyD)88 (Kawai and Akira, 2006; Oda and Kitano, 2006). MyD88 is also shared by the interleukin-1 receptor (IL-1R1) as TLRs, IL-1R and MyD88 contain the Toll/IL-1 receptor (TIR) cytosolic domain involved in signal transduction (O’Neill, 2003). MyD88 activation triggers a cascade of signalling events leading to the nuclear localization of NF-κB, along with other inflammatory responses. While TLRs, aside from TLR9, can also signal independently of MyD88 (Colonna, 2007), the MyD88-dependent pathway appears to be the primary means by which TLRs trigger inflammation affording critical host defence against a number of systemic bacterial infections (Mancuso et al., 2004; Skerrett et al., 2004; Albiger et al., 2005).

While other studies have previously found MyD88-dependent innate immune responses to be important during mucosal infection to various bacterial pathogens (Skerrett et al., 2004; Hapfelmeier et al., 2005; Weiss et al., 2005; Watson et al., 2007), it is unclear whether TLR-dependent signalling provides host defence against non-invasive bacterial pathogens in the gastrointestinal (GI) tract. This is in part because the GI tract is constantly exposed to a large load of luminal commensal bacteria, which somehow leave the intestinal immune system relatively tolerant to their products. Even so, recent studies have clarified that TLR activation by commensal bacteria does occur and is critical in maintaining colonic homeostasis, rather than inducing inflammation (Rakoff-Nahoum et al., 2004; Araki et al., 2005; Brown et al., 2007; Cario et al., 2007). While loss of TLR signalling does not cause spontaneous disease, it does aggravate the colitis suffered when mice are exposed to dextran sodium sulfate (DSS) in their drinking water. In fact, mice lacking the TLR adaptor protein MyD88 develop severe mucosal ulcerations, pronounced colonic bleeding and high mortality rates in responses to DSS (Rakoff-Nahoum et al., 2004;...
Araki et al., 2005). The mechanisms underlying this susceptibility are unclear, but it appears that activation of the innate immune system protects the colonic epithelium from damage and/or promotes repair of the damage induced by DSS (Rakoff-Nahoum et al., 2004; Brown et al., 2007; Rakoff-Nahoum and Medzhitov, 2007). In fact, new reports indicate that NF-κB activation within the colonic epithelium is essential for the regulation of epithelial integrity, thereby preventing chronic intestinal inflammation (Nenci et al., 2007).

While these findings suggest a novel role for innate immunity in the GI tract, no studies have yet addressed whether the innate immune system is required to maintain mucosal homeostasis during infection by clinically important enteric bacterial pathogens, such as the minimally invasive microbes enteropathogenic Escherichia coli (EPEC) and enterohaemorrhagic E. coli (EHEC). This is a key question because the GI tract is the site where most bacterial infections occur, and responses that help maintain mucosal integrity could dictate the pathology, morbidity and mortality suffered during these infections. While the attaching/effacing (A/E) pathogens EPEC and EHEC are human-specific and do not cause relevant disease in mice, we and others are now using Citrobacter rodentium, a Gram-negative A/E mucosal pathogen that rapidly infects the colonic epithelium of mice in a fashion identical to that utilized by EPEC and EHEC. Moreover, C. rodentium-infected mice develop a colitis characterized by inflammatory cell infiltration, crypt cell hyperplasia, goblet cell depletion and significant intestinal barrier disruption (Luperchio and Schauer, 2001; Eckmann, 2006; Ma et al., 2006).

Considering the luminal location of A/E pathogens, until recently, it was unclear whether TLRs responded effectively to these microbes in vivo. However recent studies by our group have shown that the inflammatory response elicited during C. rodentium infection is at least partially TLR4 mediated (Khan et al., 2006). Still, TLR4-deficient mice did not show defects in host defence, leaving it unclear whether TLR-driven inflammatory responses are actually protective during this infection. Because C. rodentium products also activate TLR2 (Gibson et al., 2007) and TLR4, with TLR4-dependent responses contributing to both inflammation and tissue damage but not to host defence (Khan et al., 2006). Because MyD88 is a common adapter protein for all TLRs with the exception of TLR3, we wanted to investigate whether loss of MyD88 signalling would have an impact on host defence against C. rodentium infection. C57BL/6 and MyD88–/– mice were infected with C. rodentium, and their resulting morbidity and mortality were measured. While few differences were observed between the two mouse strains up to and including day 3 post infection (p.i.), by day 4 p.i., ~40% of MyD88–/– mice began to pass bloody stool, and by day 6 p.i., blood was observed in the stool and anal regions of 80–100% of infected MyD88–/– mice observed from independent infections. The presence of occult blood in their stool was confirmed by Hemoccult® (Beckman Coulter, Mississauga, ON) assay kits (data not shown). In contrast, blood was not obvious in the stool passed by C57BL/6 mice, and in three separate infections, only 0–20% of C57BL/6 mice tested positive for occult blood.

Signs of lethargy and piloerection accompanied the passing of bloody stool by infected MyD88–/– mice. These signs of morbidity increased over the course of the infection such that 60–80% of MyD88–/– mice required euthanization between days 5 and 8 p.i. (Fig. 1A), and the remainder succumbed or required euthanization by day 10 p.i. (data not shown). In contrast, C57BL/6 mice rarely required euthanization (0–15%), and only between days 10 and 14 p.i. (Fig. 1A). While weight loss in infected C57BL/6 mice was transient, evident at day 1 p.i. followed by recovery, infected MyD88–/– mice suffered significantly greater weight loss, lasting until the mice were euthanized by day 8 p.i. (Fig. 1B). Taken together, these results suggest that MyD88–/– mice are highly suscep-
MyD88–/– mice develop severe mucosal injury during C. rodentium infection

To determine the basis for the bleeding and high rate of mortality suffered by infected MyD88–/– mice, we evaluated colonic tissues macroscopically at various time points during infection. By day 8 p.i., in C57BL/6 mice, C. rodentium infection typically caused a thickening of the colonic mucosa, and a shortening in the length of the colon (Fig. 3A); however, little other overt damage was seen. While MyD88–/– mice developed these changes in colonic morphology, they also exhibited large bleeding ulcers throughout the distal colon (1–3 cm from the anal verge) and throughout most of the caecum.

We next assessed infection-induced tissue pathology through microscopy. During C. rodentium infection, C57BL/6 mice normally suffer moderate inflammation and colonic pathology characterized by inflammatory cell infiltration, increased colonic crypt heights, disruption of normal colonic architecture and goblet cell depletion. This pathology is generally focused within the last 2 cm (distal region) of the colon, with minimal damage found in other regions of the lower bowel (Luperchio and Schauer, 2001;
MyD88-deficient mice carry increased \textit{C. rodentium} burdens. C57BL/6 and MyD88–/– mice were inoculated by oral gavage with media containing $2.5 \times 10^8$ cfu of \textit{C. rodentium} and imaged or sacrificed for bacterial quantification.

A. \textit{In vivo} bioluminescent images of mice and their lower GI tracts following infection with bioluminescent \textit{C. rodentium} for 7 days show the relative signal intensity visualized at a given anatomical location within the mouse or tissue. MyD88–/– mice had significantly more intense signals localized to the caecum and throughout the entire colon. The colour bar is displayed on the right, where red corresponds to the highest signal intensity and blue corresponds to the lowest signal intensity with corresponding logarithmic units of light measurement (photons per sec cm$^{-2}$ per sr).

B. \textit{C. rodentium} cfu were recovered from the MLN, spleen, caecum, distal, mid- and proximal regions of the colon from C57BL/6 (□) and MyD88–/– (■) mice and enumerated at 7 days p.i. Asterisks denote the recovery of significantly more \textit{C. rodentium} from the MyD88–/– mice with indicated $P$-values (*$P < 0.5$, Mann–Whitney $T$-test) and lines represent the mean.

C. Distal colonic tissues from infected C57BL/6 and MyD88–/– mice were stained for the presence of LPS (shown in red) and DAPI-stained for host cell nuclei (shown in blue) (200× magnification). While \textit{C. rodentium} were localized to the colonic surface epithelium and lumen in C57BL/6 mice, they were found in larger numbers throughout the colonic tissue, including the submucosa and blood vessels (white arrow) in MyD88–/– mice. In contrast to infected C57BL/6 mice, where crypts remained intact, crypts were destroyed in infected MyD88–/– mice (panels showing 400× magnification of area in adjacent image marked by ‘+’).
MacDonald et al., 2003; Ma et al., 2006). As shown in Fig. 3B, C57BL/6 developed the above-described modest injuries but, in marked contrast, MyD88−/− mice developed severe and gross ulcerations both in the distal colon (Fig. 3B) and in caecum (data not shown). This damage was accompanied by the complete destruction of many crypts in the distal colon and caecum (Fig. 3B; arrows point to crypts shown in high-magnification images), while those crypts that remained were stunted and filled with bacteria.

Quantitatively, MyD88−/− mice suffered significantly more tissue damage than C57BL/6 mice, scoring $3.7 \pm 0.33$ versus $1.75 \pm 0.1$ for loss of epithelial integrity, $3.0 \pm 0.06$ versus $1.8 \pm 0.26$ for goblet cell depletion and $1.75 \pm 0.06$ versus $0.8 \pm 0.15$ for submucosal oedema (Fig. 3C). As expected, the colons of C57BL/6 mice displayed significantly increased crypt heights (hyperplasia) in response to 6 days of infection (−20% increase), with crypts increasing in height up to 120% thereafter (data not shown) as previously described (Vallance et al., 2002a). Interestingly, under uninfected conditions, MyD88−/− mice possessed shorter crypts (−20% shorter) than those measured in uninfected C57BL/6 mice, and during infection, the crypt heights of MyD88−/− mice did not significantly increase. No increase in crypt heights was detected even at day 8 p.i., as assessed from the few remaining intact crypts in regions that were not severely ulcerated (Fig. 3C). These results reveal that MyD88−/− mice exhibit increased susceptibility to severe colonic injury and are impaired in the development of C. rodentium-induced crypt hyperplasia. To ensure that the susceptibility of the MyD88−/− mice was
due to the loss of MyD88 signalling, and not to differences in mouse facilities we also infected heterozygous MyD88−/+ mice, raised as littermates with the MyD88−/− mice. We observed pathology in the heterozygous mice similar to that found in the C57BL/6 mice, suggesting that the susceptibility of the MyD88−/− mice was genetic and not environmental (data not shown).

MyD88−/− mice suffer impaired inflammatory responses

Our previous studies found that TLR4−/− mice suffered impairment in the generation of inflammatory responses during C. rodentium infection (Khan et al., 2006). To assess whether MyD88−/− mice suffered similar impairments, we assayed inflammatory cell recruitment as well as the expression of chemokines and cytokines known to be upregulated during C. rodentium infection. As shown in Fig. 4A, many F4/80-positive macrophages and myeloperoxidase (MPO)-positive neutrophils were recruited to the submucosa in infected C57BL/6 mice at day 6 p.i., while not many inflammatory cells were identified in the colons of MyD88−/− mice at day 6 p.i. By day 8 p.i., a modest increase in inflammatory cell recruitment was observed in the C57BL/6 mice; however, by this time point, massive ulcers had formed in the MyD88−/− mice, and select areas near or beneath these ulcers were found to contain large numbers of macrophages and neutrophils (Fig. 4A). Interestingly, the expression of chemokines previously linked to this inflammatory cell influx (Khan et al., 2006), MCP-1 (macrophage chemokine) and MIP-2 (neutrophil chemokine), were similar in infected C57BL/6 and MyD88−/− mice (Fig. 4B), revealing that macrophages and neutrophils are not recruited solely by these chemokines. Furthermore, while inducible nitric oxide synthase (iNOS) expression was strongly induced during infection of C57BL/6 mice, this response was abrogated

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in infected MyD88−/− tissues throughout the course of infection (day 6 p.i. shown; Fig. 4B). These results indicate that recruitment of inflammatory cells at early stages of infection, as well as the induction of iNOS expression, is dependent on MyD88 signalling. Moreover, we have previously found that iNOS expression plays a role in controlling C. rodentium numbers (Vallance et al., 2002b), suggesting that the impairment of this response may have contributed to the increased pathogen burden associated with the MyD88 deficiency. Notably, we did not find defective β-defensin 1–3 or mCRAMP transcript expression in MyD88−/− mice under control or infected conditions, indicating that at least these other antimicrobial factors were not MyD88-dependent and thus less likely to be responsible for the increased pathogen burden carried by the MyD88−/− mice.

We have previously found that IL-6 but not TNF-α production in C. rodentium-infected bone marrow-derived macrophages (BMDM) and cultured colons was dependent on TLR2 signalling and associated with impaired barrier function (Gibson et al., 2007). Both TNF-α and IL-6 have also been previously linked to intestinal cytoprotection (Rakoff-Nahoum et al., 2004), while TNF-α is known to be important in controlling C. rodentium pathogen load (Goncalves et al., 2001). Therefore, we assessed the production of these cytokines in the absence of MyD88. We stimulated wild-type and MyD88-deficient BMDM with C. rodentium and measured TNF-α and IL-6 in supernatants after 24 h. In the absence of MyD88, BMDM were severely impaired in their expression of TNF-α and IL-6 (Fig. 5A). To determine whether TNF-α and IL-6 production was also reduced in the colons of MyD88−/− mice, we cultured distal colonic tissues from MyD88−/− and C57BL/6 mice at day 6 p.i. and measured cytokine production by enzyme-linked immunosorbent assay (ELISA). As shown in Fig. 5B, in comparison with uninfected C57BL/6 mice, 6 days of infection led to an increase in TNF-α and IL-6 levels. In contrast, MyD88 deficiency resulted in a significant impairment of both of these cytokines throughout the infection (day 6 shown; Fig. 5B). These results reveal that MyD88 signalling is required for TNF-α and IL-6 production in response to C. rodentium.

MyD88 activation is critical for mucosal integrity

We and others have found that C. rodentium infection leads to intestinal epithelial barrier dysfunction (Ma et al., 2006). We also recently determined that in the absence of TLR2 signalling, this barrier dysfunction is exaggerated, although the mechanisms underlying this role remain unclear (Gibson et al., 2007). To evaluate whether MyD88-dependent signalling might be involved, we orally administered FITC-dextran to mice and assessed barrier function by measuring translocated FITC-dextran in serum and colonic tissues. As expected, C. rodentium infection led to significantly higher serum levels of FITC-dextran in C57BL/6-infected mice, indicative of barrier dysfunction (Fig. 6A). In comparison, MyD88−/− showed even higher levels (2.5-fold increase) of FITC-dextran in the serum compared with C57BL/6-infected mice. This reveals that MyD88 maintains barrier function during C. rodentium infection. Consistent with increased serum levels of FITC-dextran, fluorescent microscopic analysis showed deeper infiltration of FITC-dextran into the distal colonic crypts of infected MyD88−/− mice than infected C57BL/6 mice (Fig. 6B). As seen in Fig. 6, although not statistically significant, uninfected MyD88−/− also showed
increased serum levels of FITC-dextran relative to uninfected C57BL/6 mice, and similarly, uninfected MyD88–/– mice appeared to have more FITC-dextran infiltrating their crypts compared with the control C57BL/6 mice. Taken together, these results suggest that MyD88–/– mice suffer impaired intestinal barrier function, even under control conditions, and this dysfunction is exacerbated during mucosal assault by *C. rodentium*. Thus, MyD88-mediated processes play an essential role in maintaining intestinal mucosal integrity.

**MyD88 activation promotes intestinal epithelial cell homeostasis**

Considering the widespread ulcers, and heavily infected and stunted colonic crypts seen in the infected MyD88–/– mice, we wondered whether regulation of epithelial cell growth and repair might be responsible for their susceptibility to mucosal injury. Recently, it was shown that MyD88 controls homeostasis in the intestine during DSS-induced colitis (Rakoff-Nahoum et al., 2004; Brown et al., 2007) by regulating epithelial cell proliferation. To determine whether MyD88 also plays a role in cellular proliferation during *C. rodentium*-induced colitis, control and infected MyD88–/– and C57BL/6 mice were injected i.p. with 5′-bromo-2′-deoxyuridine (BrDU) and sacrificed 2 h later. The lower GI tract was excised and immunostained for BrDU as well as for Ki67, a nuclear factor that marks cellular proliferation. Analysis of the steady-state levels of Ki67 and BrDU-positive intestinal epithelial cells revealed that the number of proliferating cells in MyD88–/– mice was 2-fold that found in C57BL/6 mice (Fig. 7). While the proliferative zone of the colonic epithelium is usually limited to the bottom third of crypts, in the absence of MyD88, the proliferative zone was expanded, with proliferating cells identified in the mid- and even upper regions of crypts. These results indicate that MyD88 is critical for epithelial cell homeostasis under uninfected conditions, as has previously been reported (Rakoff-Nahoum et al., 2004). During *C. rodentium* infection in C57BL/6 mice, the number of Ki67 and BrDU-positive cells significantly increased (~3.5-fold increase), with the proliferative zone expanding to include the middle region of the crypt. In contrast, infection of MyD88–/– mice did not cause any increase in the number of proliferating cells compared with littermate controls, and the proliferative zone did not change (Fig. 7). Correspondingly, there was a significant increase in the total number of cells per crypt during *C. rodentium* infection in C57BL/6 mice but not in MyD88–/– mice. Moreover, Ki67 and BrDU incorporation, as well as crypt heights, continued to increase in C57BL/6 mice at later time points (days 8–10 p.i.), suggesting that these host responses are important for defence against *C. rodentium*. In contrast, by days 7–8 p.i., BrDU and Ki67 staining started to decrease in the MyD88–/– mice, coinciding with the loss of crypts and the development of ulcers. These results reveal that MyD88 plays a key role in epithelial cell homeostasis by promoting epithelial cell proliferation and crypt hyperplasia in response to *C. rodentium* infection.

**Discussion**

The innate immune system and the specific innate receptors through which it acts are recognized as important factors in the development of host immune responses against many bacterial pathogens, aiding in their control or clearance from infected hosts. Unfortunately, these pro-
Protective roles have only been rigorously assessed in vitro or in systemic infections, with little known about the roles of TLRs in mediating host responses to enteric bacterial pathogens within the GI tract. Unlike most sites in the body where sterility aids in the detection of invasive pathogens, the GI tract is constantly exposed to vast numbers of commensal microbes, resulting in a mucosal immune system that is tolerant to their antigens. This tolerance is undoubtedly designed to limit maladaptive inflammatory responses against endogenous microflora; however, it may also limit the ability of the host to respond quickly and effectively against enteric bacterial pathogens. We and others have hypothesized that, as a result, the innate immune system may have developed unique compensatory actions within the GI tract, and in fact, recent studies have found that commensal bacterial stimulation of intestinal TLRs helps maintain intestinal homeostasis and mucosal integrity, rather than trigger acute inflammation. This unique mutualism was recently found to be essential in protecting the intestine against chemical-induced injury suffered during DSS colitis (Rakoff-Nahoum et al., 2004).

To address how innate immune responses in the GI tract function in response to bacterial pathogens, we chose to study the minimally invasive Gram-negative murine pathogen C. rodentium. Like its clinically important relatives, EPEC and EHEC, C. rodentium resides primarily in the gut lumen. Despite this location, C. rodentium infection leads to a slowly developing colitic response involving neutrophil and macrophage recruitment into the infected intestine. Moreover, significant tissue pathology, including epithelial hyperplasia, accompanies this infection. We recently determined that much of the inflammation and pathology exhibited by infected mice is TLR4-dependent (Khan et al., 2006). Despite this role, loss of TLR4 had little impact on host defence. Similarly, we recently determined that TLR2 is also dispensable for controlling C. rodentium burdens (Gibson et al., 2007). These findings raised the question of whether the innate responses that are induced against C. rodentium actually protect the host or, alternatively, merely cause inflammation and tissue damage without providing significant protection against infection. Our current study demonstrates that MyD88-dependent signalling contributes to host defence within the GI tract, not only by controlling pathogen burdens and selective aspects of the host inflammatory response, but also by playing a critical role in promoting epithelial homeostasis during infection.
findings are supported by recent studies of Lebeis and colleagues (2007) demonstrating that TLR signalling via MyD88 is required for a protective immune response to C. rodentium. Although neither study has ruled out possible effects of IL-1R signalling, it is likely that MyD88 is activated through TLR signalling, considering that C. rodentium activates at least TLR2 (Gibson et al., 2007) and TLR4 (Khan et al., 2006). Additionally, C. rodentium did not induce IL-1β or IL-18 after 6 days of infection with C. rodentium, a time point prior to severe tissue damage (data not shown). However, we have not completely eliminated the possibility of IL-1R signalling, and further investigation is warranted.

Our findings confirm earlier studies that colonic epithelial cells undergo a significant increase in proliferation during C. rodentium infection. Exceeding a concurrent increase in epithelial cell turnover, the end result is crypt hyperplasia that is observed even in mice lacking an adaptive immune system (Vallance et al., 2002a). Cliffe et al. (2005) recently and elegantly outlined how accelerated colonic epithelial cell turnover aids in the expulsion of nematode parasites from infected hosts, and this process has also been proposed as a generalized means of shedding epithelial adherent or invasive pathogens. We hypothesize that during C. rodentium infection of immunocompetent mice, the rapid turnover of infected epithelial cells limits pathogen burdens while the increase in crypt heights keeps C. rodentium at the apex of infected crypts, distant from vulnerable crypt stem cells. Our current studies reveal that both the increases in cellular proliferation and the crypt hyperplasia are MyD88-dependent. As a result, C. rodentium were able to penetrate deeply into the crypts of MyD88–/– mice. This was accompanied by an increase in epithelial cell injury, impaired barrier function, exaggerated goblet cell depletion and, ultimately, crypt loss and extensive ulcerations throughout the distal colon and caecum.

Notably, MyD88–/– mice are the only mouse strain we have identified that suffers such a major defect in epithelial cell proliferation and in the induction of crypt hyperplasia following infection. In fact, both TLR2–/– and TLR4–/– mice exhibit normal epithelial proliferative responses during infection (data not shown) even though, at least, TLR2–/– mice were more susceptible to a lethal colitis induced by C. rodentium. This raises the question of whether the MyD88 dependency of these responses reflects the activation of other TLRs during infection, or whether TLR2 and TLR4 functionally compensate for each other in mediating this aspect of the host response. The involvement of these TLRs during C. rodentium infection differs from findings in DSS colitis, where both TLR2–/– and TLR4–/– mice exhibited increased susceptibility to DSS colitis, albeit to a lesser extent than MyD88–/– mice (Rakoff-Nahoum et al., 2004). These differences in susceptibility may reflect that DSS reduces, rather than promotes, epithelial cell proliferation, with the resulting impairment especially striking in colitic MyD88–/– mice, leading to dramatic reductions in the number of epithelial cells per crypt unit. Therefore, while the noxious stimulus may differ, the innate regulation of epithelial cell proliferation and homeostasis appears to play a broad and critical role in protecting the GI tract against mucosal injury. The cell types involved in MyD88-dependent signalling to C. rodentium still remain to be specifically identified; however, they appear to include both haemopoietic and non-haemopoietic cells (Lebeis et al., 2007). Importantly, studies by Brown and colleagues (2007) have found that mesenchymal stromal cells play a key role in maintaining MyD88-dependent intestinal homeostasis during DSS-induced colitis. These investigations found that MyD88 signalling repositions a subset of prostaglandin-endoperoxide synthase (Ptgs2)-expressing stromal cells from the mesenchyme surrounding the middle and upper crypts to an area surrounding the crypt base adjacent to colonic epithelial stem cells, thereby preserving epithelial cell proliferation during injury (Brown et al., 2007). We are currently investigating the role of MyD88-dependent Ptgs2 in C. rodentium-induced colitis.

Our studies also show that MyD88-dependent signalling plays a critical, yet selective role in the inflammatory response elicited by C. rodentium. Expression of the inflammatory cytokines IL-6 and TNF-α was impaired in the colons and BMDM of MyD88-deficient mice. This is similar to impaired responses by MyD88–/– macrophages to Gram-negative pathogens like Salmonella typhimurium (Weiss et al., 2004), and likely contributes to some of the pathology we observed in MyD88–/– C. rodentium-infected mice. Previous studies have implicated TNF-α in a number of pro-inflammatory roles, including acting as a chemoattractant for neutrophils (Ramos et al., 2005). Moreover, TNF signalling has been shown to limit both bacterial burdens and tissue pathology during C. rodentium infection (Goncalves et al., 2001). Similarly, IL-6 has been proposed to play both pro-inflammatory and cytoprotective roles, including acting to promote epithelial cell repair (Wang et al., 2004). In fact, IL-6 has been implicated in mucosa-protective actions in the C. rodentium colitis model (Eckmann, 2006), suggesting that the impaired expression of this cytokine may contribute to the loss of mucosal integrity and barrier function seen in infected MyD88–/– mice.

Aside from attenuated cytokine responses, we identified a delayed inflammatory response in the MyD88–/– mice, with fewer macrophages and neutrophils recruited to the colons of these mice at day 6 p.i. Because we identified no major defects in expression of the chemokines MIP-2 and MCP-1 in these mice, deficiencies in other
chemoattractants, such as TNF-α, may explain the reduced inflammation. These findings agree with recent studies showing that timely neutrophil infiltration is reliant on MyD88-dependent induction of KC chemoattractant (Lebeis et al., 2007). Notably, once ulcers had developed, inflammatory cell recruitment was observed, with neutrophils and macrophages identified in the mucosa and submucosa underlying ulcerated regions. This is similar to other studies which found a robust inflammatory cell recruitment to the intestines of MyD88−/− mice during S. Typhimurium-induced colitis (Hapfelmeier et al., 2005). Although inflammatory cell recruitment to the infected colon appears to be for the most part MyD88-independent, the function of these recruited cells is likely impaired, as evidenced by the abrogated cytokine expression by MyD88−/− BMDM exposed to C. rodentium.

While the loss of epithelial homeostasis undoubtedly played a major role in the susceptibility and pathology suffered by MyD88−/− mice, they also suffered bacterial overgrowth, carrying C. rodentium burdens 10- to 100-fold greater than those carried by wild-type mice. Moreover, mucosal injury in these mice was focused in regions of the lower bowel, where C. rodentium colonization was heaviest. While the basis for this bacterial overgrowth is probably multifactorial, including a reduced ability to slough infected epithelial cells as well as a delayed inflammatory cell infiltration, the impaired TNF-α and iNOS responses seen in these mice are also likely involved. Mice deficient in TNF-α signalling were previously found to carry C. rodentium burdens at levels 10- to 100-fold greater than wild-type mice (Goncalves et al., 2001). Furthermore, we have previously identified a modest bacteriostatic role for nitric oxide, with iNOS−/− mice carrying roughly 10-fold higher numbers of C. rodentium (Vallance et al., 2002b). It is of note that despite the increased C. rodentium burdens carried by TNFRp55−/− and iNOS−/− mice, they exhibited only modest increases in pathology and mortality compared with wild-type mice. These findings corroborate our hypothesis that the extreme susceptibility to C. rodentium infection exhibited by MyD88−/− mice primarily reflects their inability to maintain epithelial cell homeostasis; however, the bacterial overgrowth these mice suffer likely exacerbates the extent and severity of the resulting mucosal damage.

Clearly a delicate balance of TLR regulation is required for healthy GI function, and future studies will be needed to dissect out the actions of specific TLRs in the promotion of host defence in this and other enteric infections. However, our results make clear that the innate host response to enteric bacteria is a complex process that requires assessment not only of inflammatory mediators and pathogen burdens, but of the function and integrity of the resident cells that make up the GI tract. While it remains to be determined whether TLR signalling also promotes host defence against EPEC or EHEC infections through regulation of mucosal homeostasis, there is evidence that innate immunity is activated by these pathogens in vivo, because infection by EPEC and EHEC leads to significant GI inflammation, with fecal leucocytes more frequently recovered from patients infected with EHEC than from those infected with Salmonella and Shigella spp. (Klein et al., 2002). Similarly, studies examining colonic biopsies have revealed inflammatory cell infiltration into the intestinal lamina propria and crypts of infected tissues (Murray and Patterson, 2000). While a broad immunodeficiency in patients like that seen in MyD88−/− mice seems unlikely, selective defects in innate regulation of epithelial cell homeostasis may underlie the exaggerated pathology and susceptibility seen in some individuals exposed to enteric bacterial pathogens, and may well underlie host susceptibility not only to enteric infections, but also to the maladaptive host–microbial interactions that occur in inflammatory bowel disease.

**Experimental procedures**

**Mice**

MyD88−/− mice were raised in our animal facility at the Child and Family Research Institute (CFRI), while 6- to 8-week-old sex-matched C57BL/6 J mice were obtained from Charles River (Wilmington, MA, USA). Mice were maintained in sterilized, filter-topped cages, handled in tissue culture hoods, and fed autoclaved food and water under specific pathogen-free conditions in our animal facilities at the CFRI. Sentinel animals were routinely tested for common pathogens. The protocols used were approved by the University of British Columbia’s (UBC) Animal Care Committee, and in direct accordance with guidelines drafted by the Canadian Council on the Use of Laboratory Animals.

**Bacterial strains and infection of mice**

Mice were infected by oral gavage using 0.1 ml of Luria broth (LB) containing ~2.5 × 10^8 cfu of wild-type C. rodentium DBS100 or bioluminescent C. rodentium as previously described (Schauer and Falkow, 1993; Khan et al., 2006). A bioluminescent C. rodentium derivative was obtained by transposon mutagenesis with the mini-Tn5 vector carrying an unpromoted luxCDABE operon from the nematode symbiont Photobahbus luminescens and a kanamycin-resistance cassette (pUTmini-Tn5 luxKm2) (Winson et al., 1998a, b), inserted within the pCRP3p06 GeneID:3207191. pUTmini-Tn5 luxKm2 was maintained in E. coli S17/1 λpir, and transposon mutagenesis was carried out by mixing donor (E. coli with transposon cassette) and recipient (C. rodentium) cells from overnight broth cultures described previously (Winson et al., 1998b). Transposants were isolated on LB agar plates containing kanamycin, and bioluminescent transposants were selected by eye in a darkened room. Bioluminescent colonies were plated to purity as single colonies and lux phenotype confirmed by luminometry prior to storage in glycerol at ~80°C. The disruption of pCRP3p06 gene in C. rodentium was found to have no effect on growth in vitro or colonization
dynamics in vivo (data not shown). C57BL/6 and MyD88−/− mice were infected with the same bacterial preparation to minimize variability. Mice were euthanized at various time points p.i., and tissues were prepared for the analyses described below.

Survival and bodyweight measurement
Mice were monitored for mortality and morbidity throughout the infection, and any that showed signs of extreme distress were euthanized. Mice were weighed immediately prior to infection and at specified time intervals until day 8 p.i. The survival and bodyweight data presented are pooled from three independent experiments (n = 9). Bodyweight data are presented as the mean percentage of the starting weight of each mouse at each time point, whereas survival data are presented as the percentage of the initial mice still surviving at each time point.

In vivo imaging
At various times p.i., mice were anaesthetized with 2% isoflurane carried in 2% O2 and imaged using IVIS (Xenogen, Almeda, CA). Greyscale reference images taken under low illumination were collected and overlaid with images capturing the emission of photons from the lux-expressing bioluminescent C. rodentium using LIVING IMAGE software (Xenogen) and Igor (WaveMetrics, Seattle, WA). Live mice were returned to their cages or euthanized, after which their colon and caecum were removed, opened longitudinally, washed with phosphate-buffered saline (PBS) and imaged as above.

Tissue collection and bacterial counts
Tissue collection and bacterial counts were performed as described previously (Khan et al., 2006). Briefly, mice were euthanized over the course of infection, dissected, and their large intestines including the caecum were collected in 10% neutral buffered formalin (Fisher) for histological analyses, or processed for tissue pathology assays. For viable cell counts, colon tissues, also recognizes C. rodentium LPS, polyclonal rabbit antisera generated against E. coli LPS (Poly8; Biotec Laboratories, England) that also recognizes C. rodentium LPS, polyclonal rabbit antisera generated against the macrophage marker F4/80 (Serotec) for macrophages, polyclonal rabbit antisera generated against MPO (NeoMarker) for neutrophils, polyclonal rabbit antisera generated against BrDU (Serotec), and polyclonal rabbit antisera generated against Ki67 (Abcam) for cellular proliferation. This was followed by secondary Alexa568- or Alexa488-conjugated goat anti-rabbit or anti-rat IgG antibodies (Molecular Probes) and Prolong® Gold antifade reagent containing 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen). Tissues were visualized at 350 and 594 nm, with dilutions plated onto Lb agar plates.

Histopathological scoring
To assess tissue pathology, we used a scoring system adapted from previously described scoring systems (Barthel et al., 2003; Khan et al., 2006). In brief, paraffin-embedded colonic tissue sections (3 μm) that had been stained with haematoxylin and eosin were examined by two observers blinded to the experimental condition. Two tissue sections from five mice per group were assessed for submucosal oedema (0 = no change; 1 = mild; 2 = moderate; 3 = profound), goblet cell depletion (scored based on numbers of goblet cells per high-power field averaged from five fields at 400× magnification, where 0 = > 50; 1 = 25–50; 2 = 10–25; 3 = < 10), epithelial hyperplasia (scored based on percentage above the height of the control, where 0 = no change; 1 = 1–50%; 2 = 51–100%; 3 = > 100%), and epithelial integrity (0 = no change; 1 = < 10 epithelial cells shedding per lesion; 2 = 11–20 epithelial cells shedding per lesion; 3 = epithelial ulceration; 4 = epithelial ulceration with severe crypt destruction).

BrDU incorporation
Uninfected and day 6 p.i. mice were administered by i.p. injection with 1 mg ml−1 of BrDU in PBS. Intestines were excised 2 h p.i., and segments of the lower GI tracts were fixed as described above and stained as described below. The number of cells per crypt column was quantified by counting the number of cells in intact, well-oriented crypts.

FITC-dextran
Uninfected or day 6 p.i. mice were gavaged with 150 μl of 80 mg ml−1 FITC-dextran (Sigma; FD4) in PBS 4 h prior to sacrifice. Mice were anaesthetized and blood was collected by cardiac punctures, which was added immediately to a final concentration of 3% acid-citrate dextrose (20 mM citric acid, 100 mM sodium citrate, 5 mM dextrose) (Harald Schulze, Shvidasani Laboratory, DFCI). Mice were euthanized, and the lower GI tract were excised and fixed with 4% paraformaldehyde as previously described (Khan et al., 2006). Sections were cut and examined for fluorescence as described below. Plasma was collected and fluorescence was quantified using a Wallac Victor (Perkin-Elmer Life Sciences, Boston, MA) at excitation 485 nm, emission 530 nm for 0.1 s.

Immunofluorescence staining of colon tissues
Immunofluorescence staining of control and infected tissues was performed using previously described procedures (Khan et al., 2006). For the identification of C. rodentium, macrophages, neutrophils and proliferating (BrDU and Ki67-positive) cells, paraffin-embedded sections were deparaffinized and rehydrated, followed by antigen retrieval using 0.1 M citric acid monohydrate (Sigma) with 0.05% Tween 20 (pH 6.0) and steam for 45 min. Slides were blocked in PBS with 2% normal horse serum, 1% BSA, 0.1% Triton X-100 and 0.05% Tween 20 for 1 h at room temperature, followed by polyclonal rabbit antisera generated against E. coli LPS (Poly8; Biotec Laboratories, England) or anti-rat IgG antibodies (Molecular Probes) and Prolong® Gold antifade reagent containing 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen). Tissues were visualized at 350 and 594 nm using a Leica DM 4000B microscope (Leica Microsystems) equipped with a Retiga 1300i camera (QImaging, Burnaby, British Columbia, Canada) operating through Open Laboratory software 4.0.2.

RNA extraction and quantitative real-time polymerase chain reaction (PCR)
MyD88−/− pups were screened by collecting mouse ear notches to obtain genomic DNA. The primers used for genotyping were: MyD88 KO forward (5′-TGG CAT GCC TTC ATC ATA GTT AAC C-3′) and reverse (5′-ATC GGC TTC TAT CGC CTG GAC

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analysed. Supernatants were then collected and stored at -80°C. Total RNA was purified using Qiagen RNEasy kits (Qiagen) in the presence of DNase I (Qiagen) according to the manufacturer’s instructions. cDNA was synthesized with Superscript II Reverse Transcriptase (Invitrogen) and Oligo dT 12–16mer (Invitrogen), followed by quantitative real-time PCR techniques. Quantitative PCR was carried out on a Bio-Rad MJ Mini-Opticon Real-Time PCR System (Bio-Rad), using IQ SYBR Green Supermix (Bio-Rad) and MCP-1, MIP-2, INOS and GAPDH primers and conditions previously described (Khan et al., 2006). Quantification was carried out using Gene Ex Macro OM 3.0 software (Bio-Rad), where PCR efficiencies for each of the primer sets were incorporated into the final calculations.

**Generation and infection of BMDM**

Bone marrow was isolated from the femurs of C57BL/6 and MyD88−/− mice, cultured for 6 days in DMEM (HyClone) supplemented with 20% fetal bovine serum (Invitrogen), 1x Glutamax™ (Gibco), 10 000 units penicillin and 10 mg streptomycin per ml (P/S) (Gibco) and 100 ng ml−1 M-CSF (R and D Systems) at 37°C in 5% CO2. BMDM (5 ¥ 106 cells per well) were cultured overnight in the above media under conditions lacking P/S and M-CSF, and then exposed to media or an overnight culture of C. rodentium corresponding to a multiplicity of infection of 100:1.

**Colon organ culture**

A modification of the protocol of Siegmund et al. (2001) was used. Briefly, segments corresponding to the distal parts of the colon were washed in ice-cold PBS supplemented with P/S. These segments were then cultured in 24-well flat bottom culture plates (Falcon) in serum-free RPMI 1640 medium (Gibco) supplemented with P/S for 18–24 h at 37°C and 5% CO2. Supernatants were then collected and stored at -80°C until analysed.

**Cytokine analysis by ELISA**

Supernatants from cultured BMDM or from cultured whole colons were removed from wells and immediately frozen at -80°C in 96-well 1 ml Corning Assay Blocks (Costar®). Cytokines are represented as ng detected per ml of supernatant. Supernatants from cultured whole colons were standardized to the amount of total protein in the supernatant by quantification using the DC Protein Assay (Bio-Rad) and presented as pg cytokine per mg of protein in the supernatant. IL-6 and TNF-α were assayed using specific ELISA cytokine kits (Biosource) outlined by the manufacturer. Cytokines were quantified using an ELISA plate reader and software (Bio-Rad).

**Statistical analysis**

All the results are expressed as the mean value with standard error of the mean (SEM). Non-parametric Mann-Whitney T-tests were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA; http://www.graphpad.com).

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