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Pathogenic and protective roles of MyD88 in leukocytes and epithelial cells in mouse models of inflammatory bowel disease

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Abstract

Background & Aims—Toll-Like Receptors (TLR) are innate immune receptors involved in recognition of the intestinal micro-flora; they are expressed by numerous cell types in the intestine, including epithelial cells, myeloid cells and lymphocytes. Little is known about the relative contributions of TLR signaling in distinct cellular compartments to intestinal homeostasis. We aimed to define the roles of TLR signals in distinct cell types in the induction and regulation of chronic intestinal inflammation.

Methods—We assessed the roles of the shared TLR signaling adaptor protein, MyD88, in several complementary mouse models of inflammatory bowel disease (IBD), mediated by either innate or adaptive immune activation. MyD88-deficient mice and bone marrow chimeras were used to selectively disrupt TLR signals in distinct cellular compartments in the intestine.

Results—MyD88-dependent activation of myeloid cells was required for development of chronic intestinal inflammation. By contrast, although epithelial cell MyD88 signals were required for host survival, they were insufficient to induce intestinal inflammation in the absence of a MyD88-competent myeloid compartment. MyD88 expression by T cells was not required for their pathogenic and regulatory functions in the intestine.

Conclusions—Cellular compartmentalization of MyD88 signals in the intestine allow the maintenance of host defense and prevent deleterious inflammatory responses.

Keywords

Helicobacter hepaticus; colitis; PRR; Treg

INTRODUCTION

Human and murine studies have strongly implicated dysregulated immune responses to the intestinal bacterial micro-flora as a central component of inflammatory bowel disease (IBD) aetiology¹, yet the host-commensal relationship also promotes host fitness, as evidenced by

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the multiple immune and physiological defects observed in germ-free animals². Thus, the immune signaling events mediating this dynamic dialogue are of interest in both intestinal health and disease.

Toll-like receptors (TLR) represent the best-characterised family of innate immune receptors and recognize conserved microbial motifs including lipopeptides, lipopolysaccharide, flagellin and unmethylated DNA. Furthermore, TLR are expressed by multiple cell types present in the intestine, including intestinal epithelial cells (IEC), dendritic cells, monocyte/macrophages, granulocytes and lymphocytes³⁻⁵. Numerous TLR polymorphisms have been linked to IBD susceptibility in humans, including TLR1, TLR2, TLR4, TLR6 and TLR9^{5,6}, but the functional outcome of these polymorphisms is unclear. Both hyper- or hypo-morphic TLR mutations may conceivably drive inflammation; through enhanced innate immune activation in the case of the former, or due to diminished protective immune responses and enhanced bacterial translocation in the latter.

Studies using mice deficient in MyD88, the major signaling adaptor molecule of the TLR family, suggest a dominant role for TLR/MyD88 signal transduction may be the prevention of intestinal inflammation following acute epithelial injury, by promoting epithelial restitution and repair, and the production of cytoprotective factors^{7,8}. This concept is further supported by the spontaneous colitis observed in mice with selective depletion of downstream IKK family members in IEC⁹. However, recent studies suggest TLR/MyD88 signaling also promotes intestinal inflammation¹⁰⁻¹² and inhibition of NF- κ B, a canonical target of this pathway, can ameliorate commensal driven colitis in IL-10^{-/-} mice¹³. In order to resolve these conflicting findings, the cellular compartment required for this pro-inflammatory signal transduction demands clarification, as do micro-flora constituents leading to its activation.

We and others have shown that the murine intestinal bacterium, *Helicobacter hepaticus* (*Hh*), can induce intestinal immune pathology through the activation of innate immunity alone^{14,15}, or through induction of *Hh*-specific colitogenic T cell responses^{16,17}. In addition, we have identified many of the downstream pathogenic mediators of chronic intestinal pathology, such as the pro-inflammatory cytokines IL-23 and TNF- α , and found that colitis is associated with potent Th1 and Th17 responses^{14,16-18}. However, the role of TLR/MyD88 signaling in the induction of *Hh*-driven typhlocolitis remains uncharacterized. Indeed, recent studies suggest that exposure of IEC to *Hh* could render them refractory to TLR activation¹⁹. Despite extensive investigation, similar studies with the closely related human pathogen *Helicobacter pylori* have yet to provide a clear consensus as to a TLR-driven mechanism leading to chronic gastric inflammation²⁰.

TLR expression by effector T cells and especially by CD4⁺CD25⁺ regulatory T cells (Treg) has also been reported, as has their functional modulation by TLR agonists²¹⁻²³. This introduces another level at which TLR/MyD88 signaling may influence intestinal homeostasis through 'T cell intrinsic' mechanisms.

Here, we have used MyD88-deficient mice to assess the functional significance of MyD88 signaling in the induction of both T cell-dependent and innate immune mediated chronic intestinal pathology. We report that MyD88 signaling is strictly required for the induction of inflammation. Furthermore, through selective ablation of MyD88 from hematopoietic cells, we demonstrate that *Hh*-mediated activation of this cellular compartment is critical for the development of chronic intestinal inflammation. By contrast, MyD88 signaling by IEC was insufficient to propagate this inflammatory cascade, although such signals contributed to host survival. In contrast, T cell intrinsic MyD88 signals were dispensable for the induction

of chronic intestinal inflammation by pathogenic CD4⁺ T cells and for the regulation of colitis by Treg.

MATERIALS AND METHODS

Mice

C57BL/6, C57BL/6MyD88^{-/-}, C57BL/6RAG1^{-/-} (RAG1^{-/-}), 129SvEv, 129SvEvMyD88^{-/-}, 129SvEvRAG2^{-/-} (RAG2^{-/-}) and 129SvEvRAG2^{-/-}MyD88^{-/-} mice were bred and maintained under specific-pathogen free conditions. C57BL/6MyD88^{-/-} mice were backcrossed onto a 129SvEvRAG2^{-/-} background using a microsatellite marker-assisted speed congenic approach²⁴. 129SvEvRAG2^{-/-}MyD88^{+/-} mice from the F5 and F6 generations were intercrossed to produce 129SvEvRAG2^{-/-}MyD88^{-/-} and littermate controls. Experiments were performed in accordance with the UK Animal Procedures Act (1986).

Bone marrow chimeras

Bone marrow was isolated from either 129SvEvRAG2^{-/-} or 129SvEvRAG2^{-/-}MyD88^{-/-} mice, and 5×10^6 cells injected IV into gamma-irradiated (5.5Gy, 550 rad) 129SvEvRAG2^{-/-} mice, which were left to reconstitute for 12wk.

Induction and regulation of innate immune typhlocolitis

Helicobacter hepaticus—NCI-Frederick isolate 1A (strain 514449, American Type Culture Collection) was cultured as described^{14, 18}. Mice were orally gavaged three times on alternate days with $0.5\text{--}1.0 \times 10^8$ CFU of *Hh* and sacrificed 8–12 wks post-infection. For Treg suppression experiments, 129SvEvRAG2^{-/-} mice were adoptively transferred with 2×10^5 CD4⁺CD25⁺ T cells IP and infected with *Hh* 24h later.

Induction of T cell dependent colitis in C57BL/6 mice

C57BL/6 mice were infected with *Helicobacter hepaticus* as above and injected with 1 mg anti-IL-10R monoclonal antibody IP on days 0, 7, 14 and 21 post-infection¹⁷. Mice were sacrificed and assessed for disease on day 28 post-infection.

Induction of IBD by adoptive transfer of naive CD4⁺ T cells

CD4⁺ T cells were purified from spleens and labeled with a cocktail of fluorescent antibodies as described^{18, 25}. Naïve (CD4⁺CD45RB^{HI}) and regulatory (CD4⁺CD45RB^{LO}CD25⁺) T cell populations were isolated using a Mo-Flo cell sorter (DakoCytomation) to >99% purity. For colitis induction, C57BL/6RAG1^{-/-} mice were adoptively transferred with 4×10^5 CD4⁺CD45RB^{HI} T cells IP and sacrificed upon the onset of symptoms of severe colitis (>6 wks post-transfer).

Assessment of intestinal inflammation

Samples of cecum and colon were fixed in buffered 10% formalin solution. 4 µm paraffin-embedded sections were stained with H&E and inflammation evaluated in a blinded fashion using a scoring system described previously²⁵; where 0–3 = normal; 4–6 = mild inflammation; 6–9 = moderate inflammation; 9–12 = severe inflammation. Colon scores represent the mean score of the proximal, mid and distal sections.

Quantitation of *Helicobacter hepaticus* colonization

Upon sacrifice, cecal contents were immediately collected in PBS and stored at –20°C. DNA was purified from cecal contents with the DNA Stool Kit (QIAGEN) as per

manufacturer's instructions. *Hh* DNA was quantitated using a Q-PCR method based on the *cdtB* gene ¹⁴.

Immunofluorescent staining of colon sections

Colon sections were snap frozen in OCT compound (Tissue-Tek) and cryosectioned to 6µm. Slides were fixed in 2% paraformaldehyde solution, washed with PBS and blocked with PBS containing 3% goat serum, 1% BSA and 0.3% Triton X-100 (Sigma Aldrich)). Following washing, sections were stained with a polyclonal anti-*Hh* serum (rabbit) followed by Alexa 633 conjugated goat anti-rabbit IgG (Invitrogen). Sections were co-stained with FITC-conjugated mouse anti-E-Cadherin (BD Biosciences) and mounted in ProLong Gold Antifade Reagent with DAPI (Invitrogen). Sections were imaged using a 63× oil immersion lens on a Zeiss LSM510 confocal microscope. Data were analyzed using LSM 5 Image Software (Zeiss).

Quantitation of cytokines and antimicrobial peptides (AMP) in colonic tissue

Colonic protein and RNA was isolated from frozen colonic tissue as described ¹⁸. Protein concentrations were measured using cytometric bead assay (BD Biosciences) (TNFα and IFNγ) or with Luminex 100 assay (Bio-Rad Laboratories) (IL-1β) as described ¹⁸. Real-Time PCR using a Chromo4 detection system (MJ Research) was used to determine colonic cytokine mRNA levels as described ¹⁸. Taqman Gene Expression Assays (Applied Biosystems) were used for the quantification of antimicrobial peptides RegIIIβ, RegIIIγ and S1008a.

Flow cytometry

Preparations of spleen, mesenteric lymph node and lamina propria leukocytes were isolated as described previously ²⁶ and enumerated by hemocytometer. For analysis of innate leukocytes, cells were stained with fluorescent antibodies against CD11b, Gr1 (Ly6G) and CD11c ^{14, 18}. For phenotypic characterization of lymphocytes, cells were surface stained with fluorescent antibodies against CD4, TcRβ and CD25, followed by intracellular staining with fluorochrome-labeled anti-mouse FoxP3 or isotype control (eBioscience) as described ^{18, 25}. Data were analysed with FlowJo software (Tree Star, Inc.).

Statistics

Statistical analysis was performed with Prism 5.0 (GraphPad Software). The non-parametric Mann Whitney test was used for all statistical comparisons, with the exception of log-rank (Mantel-Cox) test used for Kaplan-Meier plots. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Helicobacter hepaticus (*Hh*)-induced innate immune intestinal inflammation is MyD88-dependent

Infection of lymphocyte-deficient 129SvEvRAG2^{-/-} (RAG2^{-/-}) mice with *Hh* triggers potent intestinal and systemic innate inflammation ¹⁴. To assess the role of MyD88 signaling in chronic innate inflammatory responses in the gut, we generated RAG2^{-/-}MyD88^{-/-} double-knock out mice. Even when maintained under strict SPF conditions, the RAG2^{-/-}MyD88^{-/-} mice exhibited poor fitness with a progressive mortality in excess of 70%, as opposed to the 100% survival of RAG2^{-/-}MyD88^{+/-} littermate controls (Figure 1A). RAG2^{-/-}MyD88^{-/-} mice died suddenly without signs of wasting disease and did not have overt signs of immune pathology. Despite this issue, we were able to infect a sufficient

number of RAG2^{-/-}MyD88^{-/-} mice to assess the role of MyD88 signaling in the development of innate immune IBD.

In contrast to MyD88-sufficient littermate controls, *Hh*-infected RAG2^{-/-}MyD88^{-/-} mice displayed no cecal or colonic inflammation (Figure 1B,C), demonstrating that *Hh*-driven innate inflammation is strictly MyD88-dependent. We also found *Hh*-triggered activation of systemic innate inflammation was completely MyD88-dependent, as shown by the absence of splenomegaly and granulocyte accumulation in RAG2^{-/-}MyD88^{-/-} mice (Figure 2A-D).

We hypothesized that in the absence of MyD88-driven pro-inflammatory signals, infected mice might exhibit outgrowth of *Hh*, as has been reported following infection of MyD88^{-/-} mice with intestinal pathogen *Citrobacter rodentium*⁸. However, we found that RAG2^{-/-}MyD88^{-/-} mice exhibited decreased *Hh* colonization (Figure 3). The increased *Hh* colonization observed in MyD88-competent mice suggests intestinal inflammation may confer a competitive advantage to *Hh*, as has been described for other enteric pathogens^{27, 28}.

Hematopoietic MyD88 signaling is required for innate intestinal inflammation

Intestinal epithelial cells and leukocytes of the lamina propria express TLRs and MyD88 and much current debate has focused on their relative contribution to the development of intestinal inflammation^{29, 30}. We therefore created bone marrow chimeras to selectively deplete MyD88 from the hematopoietic cellular compartment. Thus, we irradiated RAG2^{-/-} mice and reconstituted them with bone marrow from RAG2^{-/-}MyD88^{-/-} or RAG2^{-/-} control donor mice. Bone marrow chimeras were then infected with *Hh* and assessed for disease development.

We found a complete absence of intestinal inflammation in the chimeric mice with selective ablation of MyD88 in hematopoietic cells, as opposed to the severe typhlocolitis observed in MyD88-competent bone marrow chimeras (Figure 4A,B). Following *Hh* infection, hematopoietic MyD88^{-/-} chimeras were resistant to crypt hyperplasia, goblet cell depletion and leukocytic infiltrate of the lamina propria indicating that epithelial cell MyD88-signaling alone was insufficient to evoke the innate inflammatory response to *Hh*. Moreover, levels of pro-inflammatory cytokines including TNF α , IFN γ , IL-1 β , IL-23p19, IL-17A, IL-22, and IL-6 were significantly reduced in the colons of *Hh* infected chimeric mice lacking hematopoietic MyD88 signaling (Figure 4C). Concordant with the absence of intestinal inflammation, mice with selective depletion of MyD88 from hematopoietic cells were also refractory to *Hh*-driven systemic inflammation (Supplementary Figure 1). As with the complete RAG2^{-/-}MyD88^{-/-} mice, the absence of *Hh*-driven disease was not due to a lack of *Hh* colonization and lack of MyD88-signaling from the hematopoietic compartment did not lead to *Hh* outgrowth or to increased epithelial colonization (Figure 5A, Supplementary Figure 2). Together, these findings reveal an indispensable role for MyD88-signaling receptors upon hematopoietic cells for the induction of innate inflammatory responses to *Hh*.

Due to the poor fitness of RAG2^{-/-}MyD88^{-/-} mice (Figure 1A), we were unable to perform the inverse selective ablation of MyD88 in non-hematopoietic, radioresistant cells. Notably, in stark contrast to complete RAG2^{-/-}MyD88^{-/-} mice (Figure 1A) selective depletion of MyD88 from the hematopoietic compartment did not predispose to mortality, either prior to, or following, *Hh* infection (Figure 5B). Together, these findings suggest a critical role for epithelial MyD88 signals for host survival under specific pathogen-free conditions. We therefore examined expression of the antimicrobial peptides (AMP) RegIII β , RegIII γ and S100A8 in the colons of mice with or without intact epithelial MyD88 signaling following infection with *Hh*. Whereas *Hh*-infected RAG2^{-/-}MyD88^{-/-} mice exhibited a marked

reduction in AMP expression as compared to RAG2^{-/-} controls (Figure 5C), chimeric mice selectively lacking MyD88 in the hematopoietic compartment alone maintained normal AMP expression following *Hh* infection (Figure 5D). Thus, epithelial MyD88 signaling efficiently drives AMP expression in the colon and this correlates with survival under SPF conditions.

T cell-dependent intestinal inflammation is MyD88-dependent

Following infection with *Helicobacter hepaticus*, C57BL/6 mice mount a range of *Hh*-specific CD4⁺ T cell responses, comprising both regulatory and effector T cell populations³¹. Blockade of IL-10 signaling by treatment with anti-IL-10R blocking mAb disrupts the dominant effects of regulation, revealing colitogenic CD4⁺ T responses that mediate chronic intestinal inflammation¹⁷. We used this model to address whether MyD88 was required for the development of such pathogenic T cell responses in the gut. As expected, infection with *Hh* alone did not provoke any intestinal pathology in WT or MyD88^{-/-} mice (Figure 6A,B). However, wild type mice infected with *Hh* and concomitantly treated with anti-IL-10R mAb developed severe typhlocolitis that was characterized by epithelial hyperplasia, edema, crypt abscesses and dense leukocytic infiltrates (Figure 6A-C). By contrast, MyD88^{-/-} mice infected with *Hh* and treated with anti-IL-10R mAb exhibited no significant signs of cecal or colonic inflammation (Figure 6A-C).

Similarly, both splenomegaly and granulocyte accumulation induced by *Hh* + anti-IL-10R mAb treatment of immune competent mice were completely MyD88-dependent (Figure 6D,E). As uninfected wild type and MyD88^{-/-} mice harbour comparable numbers and proportions of splenic granulocytes (³²and data not shown) this resistance to systemic inflammation is due to failure to activate and/or recruit these inflammatory leukocytes rather than any intrinsic neutropenia. These findings demonstrated that both mucosal and systemic inflammatory responses to *Hh* were MyD88 dependent.

It was recently reported that MyD88 signaling within T cells was required for their pathogenic function in the intestine³³. However, a subsequent study found no such requirement for T cell intrinsic MyD88 signals for intestinal pathology³⁴. Therefore, using the classical 'T cell transfer' model of colitis, we compared the ability of naïve CD45RB^{HI} CD4⁺ T cells isolated from wild type or MyD88^{-/-} mice to induce colitis upon adoptive transfer to lymphopenic C57/BL6RAG1^{-/-} recipients. Consistent with the findings of Tomita *et al.*³⁴, we found MyD88 signaling by T cells was dispensable for the induction of T cell transfer colitis (Supplementary Fig 3), with equivalent levels of disease incidence and severity induced following adoptive transfer of wild type or MyD88^{-/-} CD45RB^{HI} CD4⁺ T cells. This suggests that the resistance of MyD88^{-/-} mice to *Hh*-driven T cell-dependent inflammation is due to a defect in innate leukocyte activation, rather than intrinsically hyporesponsive T cells.

Intrinsic MyD88 signals are dispensable for the suppression of immune pathology by regulatory T cells

A number of studies have reported that regulatory T (Treg) cells selectively express TLR and that TLR agonists may directly inhibit CD4⁺CD25⁺ Treg suppressive function²¹⁻²³. However, given that it was recently reported that MyD88^{-/-} Treg lacked regulatory function *in vivo*³³, whether TLR/MyD88 signaling positively or negatively modulates Treg remains contentious.

We have shown previously that CD4⁺CD25⁺ Treg cells suppress *Hh*-driven intestinal inflammation¹⁴. Therefore, we examined whether MyD88 signaling by Treg was required

for this regulation. Immediately following infection with *Hh*, cohorts of RAG2^{-/-} mice received either wild type or MyD88^{-/-} CD4⁺CD25⁺ Treg cells and were sacrificed ten weeks later. We found that mice transferred with either wild type or MyD88^{-/-} CD4⁺CD25⁺ Treg cells exhibited comparable attenuation of *Hh* driven typhlitis and colitis (Figure 7A-C) and also exhibited similar decreases in splenomegaly (Figure 7D). These findings demonstrated that MyD88^{-/-} Treg exhibited no defect in their ability to suppress either intestinal or systemic inflammation. Finally, we found no difference in the accumulation of either wild type or MyD88^{-/-} FoxP3 cells in the mesenteric lymph node (Figure 7E). These findings are consistent with the comparable frequency of FoxP3⁺ CD4⁺ T cells in WT and MyD88^{-/-} mice (Supplementary Figure 4). Together, these results indicate that T cell intrinsic MyD88 signals are not required for development of CD4⁺CD25⁺ Treg cells or for the expression of their suppressor function *in vivo*.

DISCUSSION

In the large intestine, the protective monolayer of epithelial cells and leukocytes in the lamina propria, such as DC, macrophages and lymphocytes, share the potential to be activated through TLR. However, their functional significance to intestinal immune homeostasis and disease remains unclear.

Recent murine studies yielded contrasting findings on the role of MyD88 in chronic intestinal inflammation. Whilst the spontaneous colitis in IL-10^{-/-} and NEMO^{IEC-KO} mice required MyD88 signaling^{9, 10}, intestinal pathology in IL-2^{-/-} mice was MyD88-independent¹⁰. In agreement with the studies in IL-10^{-/-} mice, we observed that chronic intestinal inflammation in C57BL/6 mice (induced by *Hh* infection plus IL-10R blockade) was strictly MyD88-dependent. Functionally, this MyD88 dependency could reflect a failure to activate innate immunity or a failure to prime a colitogenic T cell response, since IL-10 may regulate either mechanism^{14, 17}.

The use of a T cell independent IBD model allowed us to address the role of MyD88 signals in the development of bacterially-triggered innate immune typhlocolitis. Our data demonstrated that this inflammatory pathway required MyD88 signal transduction, as evidenced by the striking lack of pathology in *Hh*-infected 129SvEvRAG2^{-/-}MyD88^{-/-} mice. In contrast, the predominant view of intestinal MyD88 signaling is that it promotes barrier function, and hence acts to prevent intestinal inflammation. This anti-inflammatory role was supported by findings that MyD88^{-/-} mice exhibited diminished epithelial restitution and exacerbated acute intestinal inflammation following disruption of the intestinal epithelium by dextran sodium sulphate (DSS)⁷, or the invasive bacterial pathogen *Citrobacter rodentium*⁸. The ameliorating effects of TLR agonists on DSS colitis further support this hypothesis²⁹. However, this protective role is thought to be driven by activation of TLR in IEC and may be required for epithelial repair following insult, rather than being strictly required for maintenance of the epithelium in the steady state¹⁰. Moreover, in contrast to the studies on acute colitis, MyD88-deficiency instead conferred complete resistance to *Hh*-induced chronic intestinal pathology. Together, these findings suggest *Hh* itself does not directly mediate acute epithelial injury, but that disruption is a downstream effect of MyD88-driven host inflammatory responses. Consistent with this hypothesis, we found decreased colonic expression of several key pro-inflammatory cytokines, including IL-23, TNFα and IFNγ, in the absence of hematopoietic MyD88 signals. We speculate that dendritic cells may play an essential role in the elaboration of these cytokine responses, particularly given recent findings that transgenic mice harbouring a CD11c-specific ablation of MyD88 showed a strong reduction of systemic IL-12/IL-23p40 responses to TLR agonists³⁵. In agreement with these results, our previous work has shown that chronic

colitis depends on IL-23^{17, 18} and that CD11c+ cells are a major source of IL-23 in the inflamed intestine²⁶.

Although IEC may typically be refractory to stimulation by the commensal micro-flora, enteric pathogens may induce IEC to release numerous inflammatory mediators, including TNF α , IL-6, IL-1, IL-18 and IL-8³⁶. Through the selective ablation of MyD88 from hematopoietic cells, we demonstrated that autonomous activation of the epithelium was insufficient to evoke the chronic innate inflammatory response to *Hh*, despite the intimate association between *Hh* and epithelial cells¹⁹. Whilst we cannot completely exclude a role for epithelial MyD88-signal transduction in the immunopathogenesis of *Hh*, any such role is functionally dependent on concurrent MyD88-dependent activation of hematopoietic innate immune cells. These results are consistent with a recent report that myeloid rather than epithelial cell NF κ B activation drives chronic colitis in IL-10^{-/-} mice³⁷.

Although insufficient to drive inflammatory pathology, MyD88 signaling by non-hematopoietic cells was critical for host survival in the absence of adaptive immunity. Even under strict SPF conditions, RAG2^{-/-}MyD88^{-/-} mice exhibited extremely poor fitness, whereas RAG2^{-/-} mice with selective ablation of MyD88 from hematopoietic cells exhibited comparable survival to RAG2^{-/-} control mice. Furthermore, MyD88 signaling by non-hematopoietic cells was sufficient to drive efficient expression of AMPs in the colon, including RegIII β , RegIII γ and S100A8, in response to infection with *Hh*. Taken together, our findings implicate distinct roles for MyD88 signaling in leukocytes and epithelial cells in the development of chronic inflammation and host protective 'tonic' immune signaling respectively.

Like TLRs, IL-1R and IL-18R express TIR domains and both dimerize to signal via MyD88³⁸. Furthermore, MyD88 signaling augments IFN γ -activity via post-transcriptional modification of pro-inflammatory response genes including TNF and IP-10³⁹. Thus, our findings may reflect a requirement for TLR-mediated recognition of *Hh* and/or in the downstream effector responses driven by these potent inflammatory mediators. The partial resistance of IFN γ ^{-/-} mice to *Hh*-induced colitis¹⁷ suggests IFN γ R/MyD88 signaling may contribute to the immune pathology. Whilst the role of IL-1/IL-18R in *Hh* immunopathogenesis is to date undetermined, we have previously shown *Hh* induces a massive increase in colonic IL-1 β levels¹⁸. Moreover, ablation of IL-1 and IL-18 signaling can ameliorate disease in other models of IBD⁴⁰. Indeed, whilst IL-1 and IL-18 may be induced by classical TLR stimulation, activation of caspase-1 by cytosolic NLRs is required for efficient processing and secretion of the active form of these mediators³⁸. It will be important to assess the contribution of these additional innate inflammatory pathways to chronic intestinal pathology.

Although MyD88 signaling in leukocytes elicits protective immunity to intestinal pathogens⁴¹, we did not observe *Hh* outgrowth or increased epithelial colonization in MyD88-deficient animals. Thus, *Hh* may occupy an immunological niche, with the potential to persistently activate innate immune cells leading to chronic, non-resolving inflammation. Thus, the development of various *Hh*-reactive regulatory populations in infected WT mice³¹ is probably essential to temper these deleterious responses. One key regulatory component are CD4⁺CD25⁺ Treg cells, which we have shown can suppress *Hh*-mediated immune pathology¹⁴. CD4⁺CD25⁺ Treg cells express multiple TLR⁴ and TLR2 and TLR8 signals have been reported to modulate their expansion and suppressor activity²¹⁻²³. However, we observed that adoptive transfer of either WT or MyD88^{-/-} Treg cells resulted in equivalent suppression of *Hh*-mediated intestinal and systemic immune pathology. Indeed, given the deleterious consequences of the *Hh*-induced inflammatory response to the host, and the ability of *Hh* to persist in this inflammatory milieu, TLR-mediated constraints

on Treg activity would likely act to exacerbate chronic intestinal immune pathology. We also found MyD88 expression by conventional T cells was dispensable for their induction of colitis. Whilst initial evidence suggested that MyD88-deficient naïve T cells failed to become primed pathogenic effector cells³³, it is apparent from a recent study³⁴ and our data that MyD88-deficient T cells are indeed pathogenic in the intestine. Given the extended time course of these latter experiments, any T cell intrinsic effects of MyD88 may be diminished in the setting of chronic intestinal inflammation.

Our findings strongly support the notion that innate MyD88 signals are maintained at a critical threshold to maintain intestinal homeostasis. Above this threshold, innate inflammatory responses to resident bacteria such as *Hh* may be evoked from the hematopoietic compartment, resulting in chronic intestinal inflammation. However, insufficient MyD88 signaling by non-hematopoietic cells may impair functions required for host survival, such as antimicrobial peptide secretion, acute inflammatory responses or epithelial restitution and repair. Compartmentalization of this spectrum of MyD88-dependent functions may thus permit the maintenance of robust host defense in the epithelium, whilst avoiding deleterious inflammatory responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

AMP	Antimicrobial peptide
<i>Hh</i>	<i>Helicobacter hepaticus</i>
IEC	intestinal epithelial cells
TLR	Toll-like receptor
NLR	NOD-like receptor
Treg	regulatory T cells

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Fig 1. *Helicobacter hepaticus*-triggered innate immune typhlocolitis is MyD88-dependent
 (A) Survival curve of uninfected 129SvEvRAG2^{-/-}MyD88^{-/-} (n=12) and 129SvEvRAG2^{-/-}MyD88^{+/-} littermates (n=14) during post-weaning period. (B&C) 129SvEvRAG2^{-/-}MyD88^{-/-} and littermate control mice (MyD88^{+/+} and MyD88^{+/-}) were infected with *Helicobacter hepaticus* for >8 weeks and assessed for cecal (B) and colonic (C) inflammation. Each symbol represents a single animal, and the data were pooled from three independent experiments (n=7–12 per group). Horizontal lines represent group means. ** p < 0.01, *** p < 0.001.

Fig 2. *Helicobacter hepaticus*-triggered systemic innate immune activation is MyD88-dependent 129SvEvRAG2^{-/-}MyD88^{-/-} and littermate control mice (MyD88^{+/+} and MyD88^{+/-}) were infected with *Helicobacter hepaticus* for >8 weeks and assessed for (A) spleen weight, (B) total splenocyte counts, (C) number and (D) frequency of splenic granulocytes (FSC^{HI}SSC^{HI}CD11b^{HI}Gr1^{HI}CD11c⁻ cells). Each symbol represents a single animal, and the data were pooled from three independent experiments (n=7–12 per group). Horizontal lines represent group means (A & B). Graphs (C & D) represent group means $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig 3. Equivalent colonization by *Helicobacter hepaticus* in the absence of MyD88
 129SvEvRAG2^{-/-}MyD88^{-/-} and littermate control mice (MyD88^{+/+} and MyD88^{+/-}) were infected with *Helicobacter hepaticus* for >8 weeks. At sacrifice, DNA was purified from cecal contents and quantity of *Hh* DNA determined using Real-Time PCR. Graphs show pooled data from three independent experiments (n=7-12 per group). Group means $\pm$ SEM are shown. * p < 0.05.

Fig 4. MyD88 signaling by hematopoietic cells mediates *Helicobacter hepaticus*-driven intestinal pathology

(A) 129SvEvRAG2^{-/-} mice were γ -irradiated (5.5 Gy) and reconstituted with 5×10^6 bone marrow cells isolated from either 129SvEvRAG2^{-/-} MyD88^{-/-} or 129SvEvRAG2^{-/-}MyD88^{+/+} donors. Twelve weeks after reconstitution, chimeras were infected with *Helicobacter hepaticus* and assessed for cecal (A) and colonic (B) inflammation. Each symbol represents individual mouse scores pooled from two independent experiments (n=11-14 per group). Horizontal lines represent group means. *** p < 0.001. (C) Concentration of pro-inflammatory cytokines in colon tissue homogenate from the mice described above, normalized to the total amount of protein in each sample.

(D) Expression levels of cytokine mRNA in colon tissue homogenates from these mice, normalized to HPRT ($\times 10^4$). Bar graphs represent group means $\pm$ SEM ($n = 5-9$ mice per group). * $p < 0.05$.

Fig 5. MyD88 signals in non-hematopoietic cells facilitate host survival

Bone marrow chimeras lacking MyD88 in hematopoietic cells were generated and infected with *Helicobacter hepaticus* as described in Figure 4. (A) At sacrifice DNA was purified from cecal contents and quantity of *Hh* DNA determined using Real-Time Q-PCR. Bars represent group means $\pm$ SEM. (B) Survival curve of 129SvEvRAG2^{-/-} bone marrow chimeras following reconstitution with 5×10^6 bone marrow cells isolated from either 129SvEvRAG2^{-/-}MyD88^{-/-} or 129SvEvRAG2^{-/-}MyD88^{+/+} donors and during subsequent infection with *Hh*. Graphs show data pooled from two independent experiments (n=11-14 per group). (C,D) Antimicrobial peptide expression was assessed in colon homogenates isolated from (C) 129SvEvRAG2^{-/-}MyD88^{+/+} or 129SvEvRAG2^{-/-} mice infected with *Hh*

as described in Figure 1 or (D) from 129SvEvRAG2^{-/-} mice reconstituted with RAG2^{-/-} or RAG2^{-/-}MyD88^{-/-} bone marrow cells and infected with *Hh* as described above. Bar graphs represent group means $\pm$ SEM (n = 4 – 8 mice per group). * p < 0.05.

Fig 6. Intestinal and systemic pathology in lymphocyte replete mice is MyD88-dependent
 C57BL/6 wild type or MyD88^{-/-} mice were infected with *Hh* and treated with 1mg/week of anti-IL-10R mAb IP. On day 28, mice were sacrificed and (A) cecal and (B) colonic pathology evaluated. (C) Representative micrographs of cecum and proximal colon of *Hh* infected mice treated with anti-IL-10R mAb. (D) Spleen weight and (E) splenic granulocyte numbers were also quantified. Each symbol represents a single animal, and the data were pooled from two independent experiments (n=5-9 per group). Horizontal lines represent group means. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig 7. MyD88-deficient regulatory T cells suppress immune pathology in vivo
 129SvEvRAG2^{-/-} mice were infected with *Helicobacter hepaticus* and reconstituted with 2×10^5 CD4⁺CD25⁺ Treg cells from wild type or MyD88^{-/-} mice. Mice were sacrificed 8 wks post-transfer and (A) cecal and (B) colonic pathology evaluated. (C) Representative photomicrographs. (D) Spleen mass and (E) total numbers of mesenteric lymph node CD4⁺FoxP3⁺ cells were also quantified. Each data point represents individual mice from three pooled independent experiments (n=9-13 per group). Horizontal lines represent group means. * $p < 0.05$, ** $p < 0.01$.