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Effects of myeloid differentiation primary response gene 88 (MyD88) activation on *Helicobacter* infection in vivo and induction of a Th17 response

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Abstract

Background—*Helicobacter pylori* is a spiral-shaped Gram-negative microaerophilic bacterium associated with a number of gastrointestinal disorders, including gastritis, peptic ulcers, and gastric cancer. Several studies have implicated a Th17 response as key to protective immunity against *Helicobacter*.

Materials and Methods—Wild type (WT) and MyD88 deficient (MyD88^{-/-}) mice in the C57BL/6 background were infected with *H. felis* for 6 and 25 weeks and colonization density and host response evaluated. Real-time PCR was used to determine the expression of cytokines and antimicrobial peptides in the gastric tissue of mice.

Results—mRNA expression levels of the Th17 cytokines, interleukin-17A (IL-17A) and IL-22 were markedly up-regulated in WT compared to MyD88^{-/-} mice both at 6 and 25 weeks in response to infection with *H. felis*, indicating that induction of Th17 responses depends on MyD88 signaling. Furthermore, reduction of expression of the Th17-dependent intestinal antimicrobial peptide lipocalin-2 was linked with increased bacterial burden in the absence of MyD88 signaling.

Conclusion—We provide evidence showing that MyD88-dependent signaling is required for the host to induce a Th17 response for the control of *Helicobacter* infection.

Keywords

Helicobacter; MyD88; IL-17A; IL-22; lipocalin-2

Introduction

Helicobacter pylori (*H. pylori*) is the most common human bacterial pathogen in the world, infecting approximately 50% of the world's population (reviewed in [1]). *H. pylori* is responsible for most cases of inflammatory gastritis, peptic ulcer disease, and gastric cancer in the human population [1]. It has been widely reported that T lymphocytes play a central role in host immune response during *Helicobacter* infection [2]. We have previously described a predominantly Th1 response with increased IFN- γ production in infections with *Helicobacter* spp [3]. Recent studies have also shown a marked induction of a key Th17 cytokine, IL-17A during infection with *H. pylori* [4]. In mice infected with *Helicobacter*, IL-17A has been suggested to play a role in limiting the growth of *Helicobacter* following vaccination [5]. IL-17A is a potent inducer of CXCR2 cytokines [6, 7], which are implicated

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in neutrophil recruitment [8]. Antimicrobial peptide regulation at the mucosal surfaces is a potential additional yet important function of IL-17A. Indeed, IL-17A was reported to be essential for production of antimicrobial peptides in tissue during inflammation including induction of lipocalin-2 (Lcn2) [9]. Lcn2 is an antimicrobial peptide that inhibits bacterial growth through its interference with iron acquisition [10]. Up-regulation of Lcn2 was shown to improve host defense against bacterial pathogens such as *Klebsiella pneumoniae* [11], *Escherichia coli* [10], *Mycobacterium tuberculosis* [12], and *Salmonella typhimurium* [9]. Recently, Hornsby et al. [13] reported up-regulation of siderocalin in rhesus macaque monkeys infected with *H. pylori*, but the mechanism of this induction or the role of Lcn2 during infection with *Helicobacter* are not known. Additionally, the mechanism of *Helicobacter*-related induction of IL-17A remains unclear.

We recently demonstrated the importance of myeloid differentiation primary response gene 88 (MyD88) in host immune response to *Helicobacter* in vitro using bone marrow-derived macrophages [14]. MyD88 is a key intracellular adaptor molecule and mediates signals from most Toll-like receptors (TLRs) except for TLR3 [15]. We showed that MyD88 was required for *H. pylori* induction of many inflammatory cytokines including IL-6, IL-1 β , IL-12, and IL-10 [14]. In the present study, we investigated the role of MyD88 in induction of Th17 responses (IL-17A and IL-22) during infection with *H. felis*. In addition, the effect of IL-17A expression during infection with *H. felis* was determined by examining the expression of IL-17A target genes, particularly antimicrobial peptides in the gastric tissue of mice.

Methods

Mice

Six- to ten- week-old wild type (WT) and MyD88 deficient (MyD88^{-/-}) mice in the C57BL/6 background were used in this study. WT mice were purchased from The Jackson Laboratory (Bar Harbor, ME). MyD88^{-/-} mice were from our breeding colony originally provided by Dr. Akira (Osaka University, Japan). All animal procedures were approved by the Animal Care Committee at the University of California, San Diego.

Culture of *H. felis*

H. felis, strain CSI (ATCC 49179) was purchased from American Type Culture Collection (Manassas, VA). *H. felis* was routinely maintained on solid medium, Columbia agar (Becton Dickinson, MD) supplemented with 5% laked blood under microaerophilic conditions (5% O₂, 10% CO₂, 85% N₂) as previously described [14] in an incubator at 37°C. *H. felis* used to infect mice were subcultured into liquid medium, brain heart infusion broth (BHI, Becton Dickinson) supplemented with 5% fetal calf serum and cultured for 48 hours on a reciprocal shaker (100 rpm) at 37°C under microaerophilic conditions. Before infections, spiral bacteria were enumerated using a Petroff-Hausser chamber.

Infection of mice with *H. felis*

C57BL/6 WT and MyD88^{-/-} mice were inoculated by oral gavage three times at 2-day intervals as described elsewhere [16–18] with 10⁹ *H. felis* suspended in 0.3 ml BHI broth. At 6 and 25 weeks post-infection, mice were euthanized and the stomachs removed under aseptic conditions. The stomach was divided longitudinally into three parts and used for assessment of gene expression, histologic evaluation of inflammatory response, and recovery of *H. felis* to confirm colonization.

Histologic evaluation of colonization and inflammation

Mouse stomachs from C57BL/6 WT and MyD88^{-/-} mice were processed for histology as in our previous studies [3, 19]. Briefly, longitudinal sections of mouse stomach tissue from each mouse were fixed in neutral-buffered 10% formalin, embedded in paraffin, and 5 µm sections stained with May-Grunwald Giemsa [20] for microscopic evaluation of colonization. Two longitudinal stomach sections per mouse with an average of ten 400X high-powered fields were evaluated. *H. felis* colonization density was determined by grading both the corpus and antrum by a blinded veterinary pathologist (Dr. Rickman) using a semiquantitative scoring system of 0 to 4 as described by Wang et al. [21]. Scores were assigned as follows: 0, none; 0.5, rare; 1, occasional pits and/or glands with individual bacteria; 2, frequent pits and/or glands with individual bacteria; 3, infrequent pits and/or gland with dense bacterial colonies; and 4, frequent pits and/or glands with dense bacterial colonies.

For inflammation, 5 µm stomach sections tissues were stained with hematoxylin and eosin. Gastric histopathology was graded using the paradigm developed by Rogers et al. [22]. Briefly, inflammation was scored with a grade of 0 – 4, with "0" representing no inflammation and 1 thru 4 representing a range from mild to severe inflammation.

RNA isolation and reverse transcription (RT)

A section of a stomach tissue from each WT and MyD88^{-/-} mouse at sacrificing was immediately placed in RNA stabilizing solution (Ambion) and stored at -70°C. Total RNA from each mouse gastric tissue section was homogenized in 1 ml of Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The RNA preparations were further purified using the Turbo DNase treatment as directed by the manufacturer (Ambion). RNA was quantified by absorbance at 260 nm and integrity checked on a 1% agarose gel. 2 µg of RNA was reverse transcribed into complementary DNA (cDNA) using the High-capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA).

Gene expression in the mouse stomach by quantitative real-time PCR

Gene expression was determined by real-time PCR with SYBR Green dye (Eurogentec, San Diego, CA). Due to similar expression levels in the initial three genes (glyceraldehyde-3-phosphate dehydrogenase, GAPDH; gamma interferon, IFN-γ; and IL-17A) performed in individual mice from the same treatment group, cDNA samples from mice in each treatment group were pooled in subsequent experiments and reactions set in 10 µl reaction mix in 96-well plates. For each gene of interest, reactions were performed in triplicates using an amplification program consisting of initial 1 cycle at 95°C for 5 minutes, followed by 40 cycles of amplification with denaturation at 95°C for 15 seconds, annealing at 60°C for 20 seconds, and extension at 72°C for 40 seconds. For each sample a melting curve was generated at the end of the reaction to ensure specificity. Gene expression levels were normalized to GAPDH and the data analyzed using a comparative cycle threshold calculations ($\Delta\Delta C_T$, Applied Biosystems). Data were expressed as fold change relative to uninfected mice. Each real-PCR experiment was run three times. Samples that were undetected after 40 cycles were considered negative and 40 cycles used to calculate the fold change. A list of genes analyzed and their respective primers are provided in Table 1.

Statistics

Colonization scores were compared between WT and MyD88^{-/-} mice by a two-tailed nonparametric Mann-Whitney *U*-test. A *P* value of < 0.05 was considered statistically

significant. Student *t*-test (Microsoft excel) was used for statistical analysis on $\Delta\Delta C_T$ and SD real-time PCR results with significance at $P < 0.01$.

Results

H. felis colonization in WT and MyD88^{-/-} mice

To test whether MyD88 signaling influences *H. felis* colonization, WT and MyD88^{-/-} mice were infected with *H. felis* for 6 or 25 weeks. *H. felis* was detected in all infected WT and MyD88^{-/-} mice. *H. felis* infection scores were higher in MyD88^{-/-} than in WT mice (Fig. 1A). WT mice had significantly ($p < 0.05$) fewer bacteria at 6 weeks post-infection. At 25 weeks, WT mice still remained less heavily infected with *H. felis* when compared to MyD88^{-/-} mice (Fig. 1A) although the difference was not statistically significant. *H. felis* colonization of mice was also analyzed quantitatively by determining the relative mRNA levels for the *H. felis* flagellar filament B gene (FlaB) using real-time PCR [17, 18, 23]. As shown in Fig. 1B, *H. felis* colonization of MyD88^{-/-} mice was significantly ($p < 0.01$) greater than that of WT mice at both 6 and 25 weeks. Quantitative analysis of *H. felis* colonization by real-time PCR was more sensitive in detecting significant differences in colonization levels between WT and MyD88^{-/-} mice than the scoring system of bacterial density in histologic sections. Higher bacterial load has also been reported in MyD88^{-/-} mice infected with *H. pylori* [24]. However, we chose to analyze *H. felis* infection of C57BL/6 mice because this system more accurately recapitulates disease progression similar to that occurring in humans during infection with *H. pylori* [25]. We did not observe differences in inflammation between WT and MyD88^{-/-} mice (see supporting information). Rad *et al.* [24] reported marginal significant differences in inflammation between WT and MyD88^{-/-} mice infected with *H. pylori*. Although WT mice had a higher inflammatory score than MyD88^{-/-} mice the authors noted that some individual MyD88^{-/-} mice had severe inflammation.

Induction of gastric cytokines in response to infection with *H. felis*

IL-17A expression during infection with *Helicobacter* has been reported [4]. However, the mechanism of this up-regulation is unknown. Additionally, the effect of MyD88 signaling on the expression of Th17 effector cytokines, IL-17A and IL-22 during *Helicobacter* infection has not been investigated. Real-time PCR analysis showed that the mRNA levels of IL-22, IL-17A, and IFN- γ exhibited substantial increases in response to infection with *H. felis* (Fig. 2). Infection of mice with *H. felis* increased the gastric mRNA levels of IL-17A, IL-22, and IFN- γ more in WT than in MyD88^{-/-} mice, both at 6 and 25 weeks post-infection. There was a 53- and 26- fold change in expression of IFN- γ and IL-22, respectively in WT mice at 6 weeks (Fig. 2A). By 25 weeks post-infection the mRNA levels of IFN- γ and IL-22 had increased to 63- and 57- fold, respectively in WT mice (Fig. 2B). Among the cytokines we evaluated, IL-17A was the most prominently induced cytokine in response to infection with *H. felis* both at 6 and 25 weeks post-infection. Infection with *H. felis* resulted in a 332- and 12.9- fold change in IL-17A expression in WT and MyD88^{-/-} mice, respectively at 6 weeks post-infection (Fig. 2A). At 25 weeks post-infection, *H. felis* infection resulted in 154- and 59- fold change in IL-17A expression in WT and MyD88^{-/-} mice, respectively (Fig. 2B). Our study is the first to demonstrate that high level IL-17A/IL-22 expression during infection with *Helicobacter* requires activation of the MyD88 signaling pathway. Increased expression of IL-17A and IL-22, which was observed in WT mice, was associated with reduced *H. felis* colonization. Indeed, IL-17A is reported to be involved in protection against *Helicobacter* infection following vaccination [5]. IL-17A may contribute to limitation of *Helicobacter* growth in several ways, including induction of antimicrobial peptides.

Expression of antimicrobial peptides in mouse gastric tissue during infection with *H. felis*

Because of the prominence of IL-17A expression we observed in gastric tissue of WT mice infected with *H. felis*, we reasoned that IL-17A target genes would be highly expressed in the gastric tissues. We have previously demonstrated in vitro that *H. pylori* up-regulates antimicrobial peptides [26]. We predicted that induction of Th17-regulated antimicrobial peptides, such as Lcn2, would be markedly reduced in the MyD88^{-/-} mice compared to the WT. Real-time RT-PCR was performed on RNA isolated from gastric tissue of WT and MyD88^{-/-} mice at 6 and 25 weeks post-infection. As shown in Fig. 3, *H. felis* highly up-regulated expression of Lcn2 while the expression of other antimicrobial peptides including mouse β -defensin 1 (mBD1) and *Cnlp*, which encodes a cathelicidin-related antimicrobial peptide (CRAMP) was up-regulated only slightly ranging between 0.4- to 3- fold, both at 6 and 25 weeks post-infection. *H. felis* induced at least a 2-fold change in *Cnlp* in WT mice, both at 6 and 25 weeks post-infection. Expression of mBD1 was only detected at 25 weeks post-infection (Fig. 3B). The induction of Lcn2 was highest after six weeks of infection at 340-fold change in WT mice. The higher induction of Lcn2 expression in WT mice infected with *H. felis* was linked to higher IL-17A/IL-22 expression in these mice. In addition, *H. felis* induction of Lcn2 was dependent on MyD88 signaling, which was similar to what we observed for IL-17A and IL-22 expression. Our findings of lower Lcn2 expression in MyD88^{-/-} mice are similar to those reported in the pulmonary *K. pneumoniae* infection mouse model [11] in which lung homogenates from MyD88^{-/-} mice had reduced Lcn2. In the same study, it was shown that Lcn2 protein amounts were linked to bacterial burden. In our present study, higher expression of Lcn2 was related to reduced *H. felis* colonization.

Discussion

Despite a robust immune response during infection with *Helicobacter*, the bacteria persists causing disease conditions including gastritis, peptic ulcer, mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric cancer [1]. Understanding the host immune response during infection with this organism may lead to the definition of mechanisms involved in the clearance of these bacteria. We have previously demonstrated in vitro that MyD88 is critical in the *H. pylori*-induced inflammatory response [14]. However, the effects of MyD88 on Th17 effector cytokines had not been investigated. In addition, the role of IL-17A and/or IL-22 in induction of antimicrobial peptides during *Helicobacter* infection had not been previously described. We show here for the first time that *H. felis* induction of IL-17A and IL-22 is dependent on MyD88 signaling. Furthermore, we demonstrated an inverse relationship between IL-17A/IL-22 expression and *H. felis* colonization level in the mouse gastric mucosa. IL-17A has been shown to play a major role in host protection against bacterial and fungal infections especially at the mucosal surface [27]. In particular, IL-17A was reported to be involved in protection against *Helicobacter* infection following vaccination [5]. We observed a strong association between increased expression of IL-17A/IL-22 with reduced *H. felis* colonization. IL-17A may contribute to limitation of *Helicobacter* growth in several ways, including through induction of antimicrobial peptides. We identified antimicrobial peptides released during gastric inflammation in response to *H. felis* infection and report for the first time *Helicobacter* induction of the mouse *Cnlp* gene, which encodes the mouse cathelicidin, CRAMP. In addition, Lcn2, an antimicrobial peptide involved in binding microbial iron-siderophore complexes, was also markedly up-regulated in response to infection with *H. felis*. In particular, high expression of Lcn2 was linked to reduced *H. felis* colonization at both 6 and 25 weeks post-infection.

Elevated expression of Lcn2 has recently been reported in humans [28] and rhesus macaques [13] in response to *H. pylori* infection although the mechanism of this induction is unknown. Here we show that *H. felis* induction of Lcn2 expression was associated with up-regulation of IL-17A and IL-22 and the expression of all three was dependent on MyD88 signaling.

Additionally, it was reported in *H. pylori*-infected rhesus macaque monkeys that induction of Lcn2 was dependent on the presence of a cag pathogenicity island [13]. Our observation of high Lcn2 induction by *H. felis*, which lacks the cag pathogenicity island suggests involvement of a different system in this induction in mice. Mice that had high expression levels of IL-17A/IL-22, which coincided with high induction of Lcn2 had significantly reduced *H. felis* colonization both at 6 and 25 weeks post-infection. Our data suggesting that IL-17A may be involved in induction of Lcn2 during infection with *Helicobacter* is in agreement with a study showing that IL-17A induces Lcn2 in response to infection with *Klebsiella* [29]. In addition, it has been reported that the growth of *S. typhimurium* was inhibited by production of Lcn2 upon stimulation of intestinal epithelial cells with IL-17A and IL-22 in vitro [9]. Studies using Lcn2^{-/-} mice suggest that Lcn2 is pivotal in innate immune response to bacterial infections and its absence can lead to sepsis and death of these mice [10, 11]. These studies together with our data suggest that Lcn2 could have a role in the reduced *H. felis* colonization we observed in WT compared to MyD88^{-/-} mice. To the best of our knowledge, we are the first to show evidence connecting expression of Lcn2 and limitation of *H. felis* growth in mice.

In summary, we show for the first time that *Helicobacter* induction of IL-17A and IL-22 expression is dependent on MyD88 signaling. Increased IL-17A/IL-22 expression was accompanied with enhanced expression of Lcn2. We propose that higher expression of IL-17A/IL-22 in conjunction with up-regulation of Lcn2 may contribute to the reduced *H. felis* colonization levels we observed in WT mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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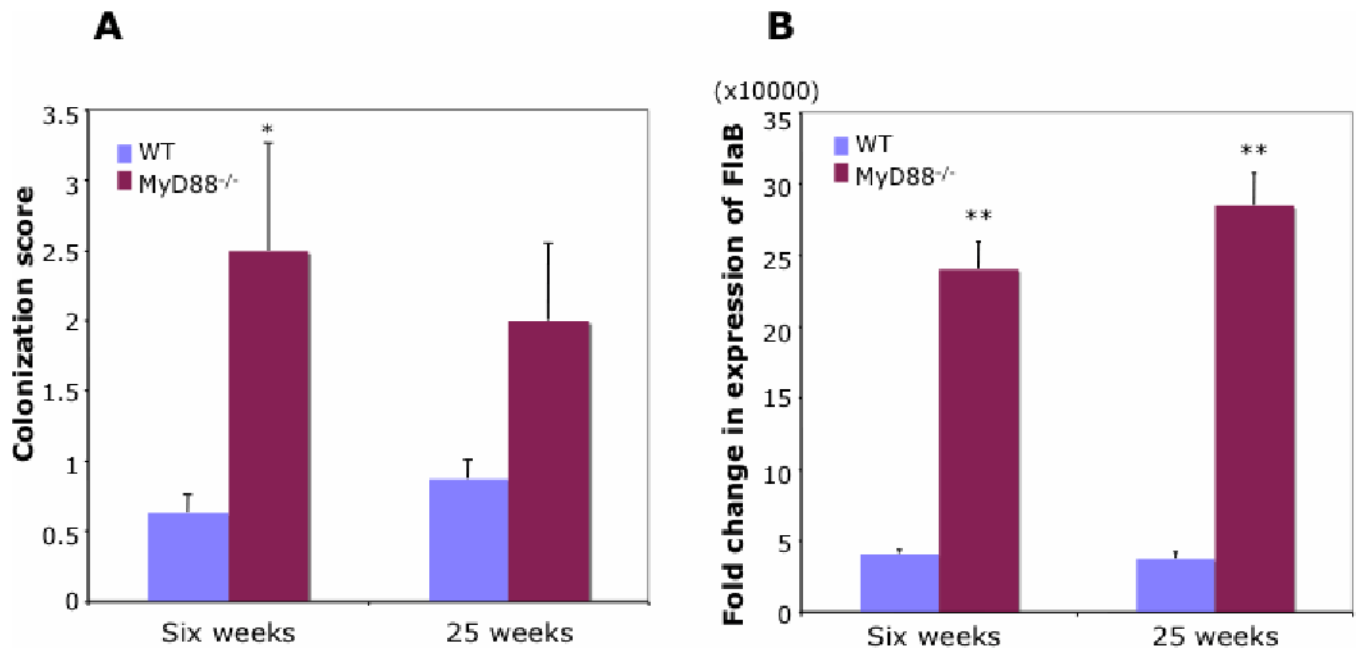


Figure 1.

Colonization of the mouse stomach tissue. WT and MyD88^{-/-} mice were infected with *H. felis* for 6 and 25 weeks. (A) Two longitudinal stomach sections per mouse with an average of ten 400X high-powered fields were evaluated to determine *H. felis* colonization density. Both the corpus and antrum were graded using a semiquantitative scoring system and the difference in bacterial load between WT and MyD88^{-/-} mice analyzed by a nonparametric Mann-Whitney *U*-test. (B) *H. felis* colonization of mice was also analyzed quantitatively by determining the relative gene expression of FlaB using real time PCR. Student *t*-test (Microsoft excel) was used to determine differences in expression of FlaB between WT and MyD88^{-/-} mice as a measure of *H. felis* colonization. All infected mice were colonized. Data are presented as means \pm SEM (WT: 6 weeks, n = 7; WT: 25 weeks, n = 7; MyD88^{-/-}: 6 weeks, n = 6; MyD88^{-/-}: 25 weeks, n = 9). **P* < 0.05, ***P* < 0.01.

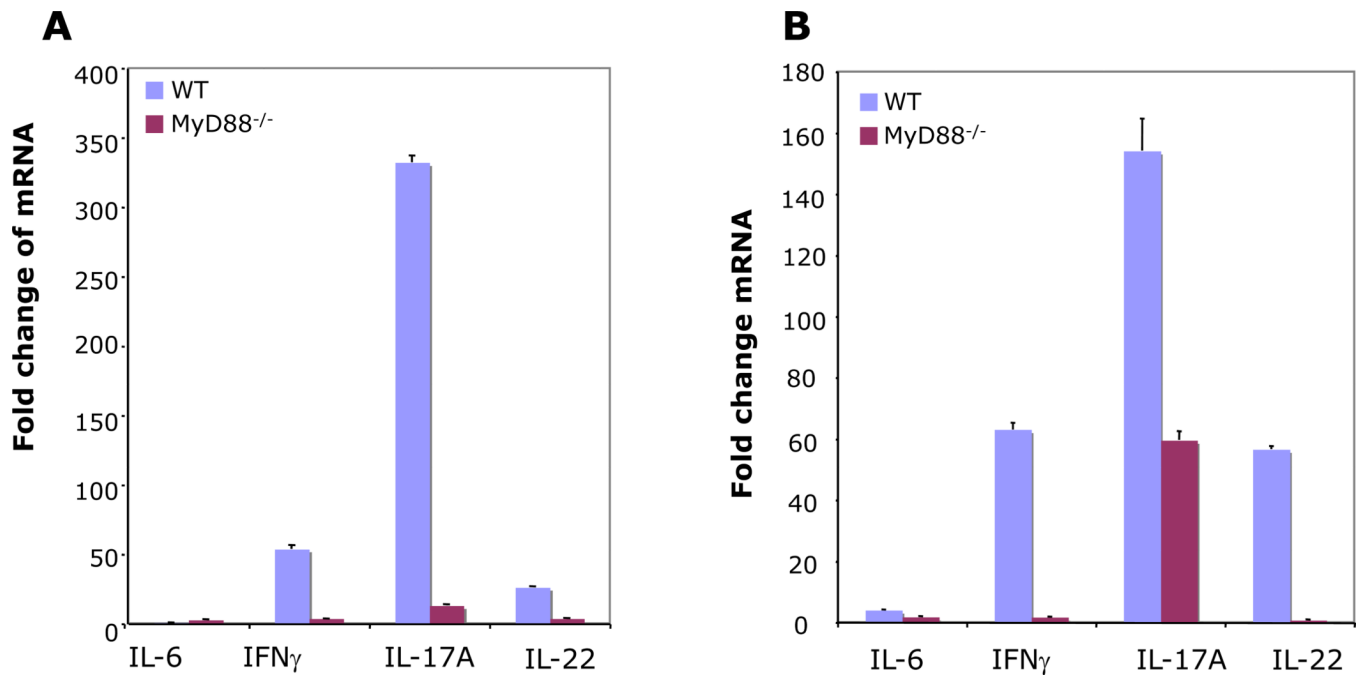


Figure 2. Cytokine expression in mouse gastric tissue. WT and MyD88^{-/-} mice were infected with *H. felis* for 6 (A) and 25 (B) weeks. Expression of IL-6, IFN- γ , IL-17A, and IL-22 was determined by real-time RT-PCR and each normalized to GAPDH. Data are expressed as means fold change \pm SEM compared to uninfected mice (WT: 6 weeks, n = 7; WT: 25 weeks, n = 7; MyD88^{-/-}: 6 weeks, n = 6; MyD88^{-/-}: 25 weeks, n = 9).

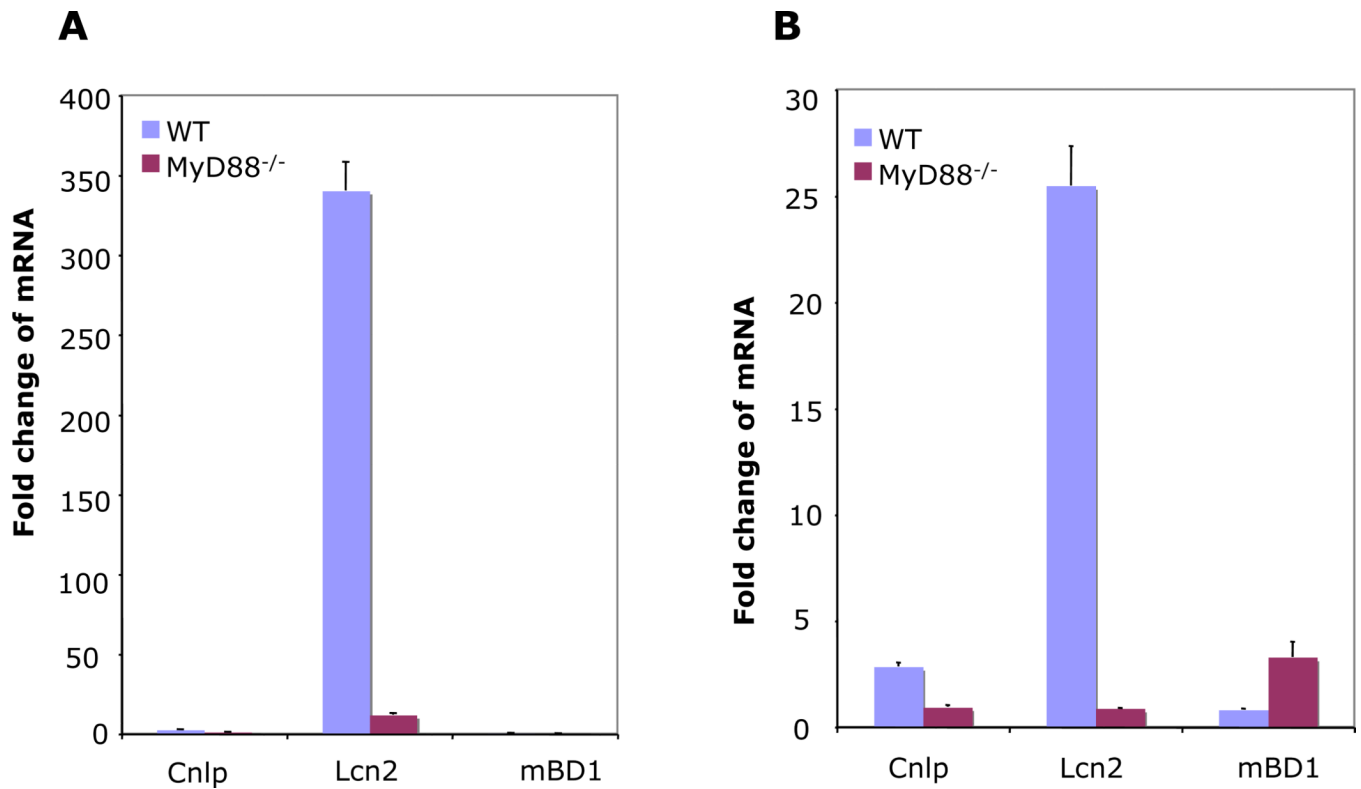


Figure 3.

Antimicrobial peptide expression in mouse gastric tissue. WT and MyD88^{-/-} mice were infected with *H. felis* for 6 (A) and 25 (B) weeks. Expression of *Cnlp*, mBD1, and *Lcn2* was determined by real-time PCR and normalized to GAPDH. Data are expressed as means fold change \pm SEM compared to uninfected mice (WT: 6 weeks, n = 7; WT: 25 weeks, n = 7; MyD88^{-/-}: 6 weeks, n = 6; MyD88^{-/-}: 25 weeks, n = 9).

Table 1

Primer sequences for real-time PCR

Gene	Direction	Sequence
IL-17A	Forward	AACATGAGTCCAGGGAGAGCTTCA
	Reverse	AGTGTTTGGACACGCTGAGCTTTG
IL-22	Forward	GACAGGTTCCAGCCCTACAT
	Reverse	ATCGCCTTGATCTCTCCACT
IL-6	Forward	ACCACTTCACAAGTCGGAGGCTTA
	Reverse	TGGTACTCCAGAAGACCAGAGGAA
IFN- γ	Forward	TCAAGTGGCATAGATGTGGAAGAA
	Reverse	TGGCTCTGCAGGATTTTCATG
Lcn ^{2a}	Forward	ACATTTGTTCCAAGCTCCAGGGC
	Reverse	CATGGCGAACTGGTTGTAGTCCG
Cnlp ^b	Forward	TCAACCAGCAGTCCCTAGAC
	Reverse	AAGGCACATTGCTCAGGTAG
mBD1	Forward	GGCATTCTACAAGTCTTGGACGAAG
	Reverse	AGCTCTTACAACAGTTGGGCTTATCTGG
GAPDH	Forward	TCAACAGCAACTCCCACTCTTCCA
	Reverse	ACCCTGTTGCTGTAGCCGTATTCA
FlaB ^c (<i>H. felis</i>)	Forward	TTCGATTGGTCTACAGGCTCAGA
	Reverse	TTCTTGTTGATGACATTGACCAACGCA

^aPrimer sequences previously described by Raffatellu et al. 2008 (ref # 9)

^bCnlp, gene encoding cathelicidin-related antimicrobial peptide (CRAMP)

^cPrimer sequences previously published (refs #s 17, 18, and 22).