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TOPIC HIGHLIGHT

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Role of Toll-like receptors in *Helicobacter pylori* infection and immunity

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Abstract

The gram-negative bacterium *Helicobacter pylori* (*H. pylori*) infects the stomachs of approximately half of the world's population. Although infection induces an immune response that contributes to chronic gastric inflammation, the response is not sufficient to eliminate the bacterium. *H. pylori* infection causes peptic ulcers, gastric cancer and mucosa-associated lymphoid tissue lymphoma. Disease outcome is linked to the severity of the host inflammatory response. Gastric epithelial cells represent the first line of innate immune defence against *H. pylori*, and respond to infection by initiating numerous cell signalling cascades, resulting in cytokine induction and the subsequent recruitment of inflammatory cells to the gastric mucosa. Pathogen recognition receptors of the Toll-like receptor (TLR) family mediate many of these cell signalling events. This review discusses recent findings on the role of various TLRs in the recognition of *H. pylori* in distinct cell types, describes the TLRs responsible for the recognition of individual *H. pylori* components and outlines the influence of innate immune activation on the subsequent development of the adaptive immune response. The mechanistic identification of host mediators of *H. pylori*-induced pathogenesis has the potential to reveal drug targets and opportunities for therapeutic intervention or prevention of *H. pylori*-associated disease by means of vaccines or immunomodulatory therapy.

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Key words: *Helicobacter pylori*; Toll-like receptor; Gastric epithelium; Monocyte; Macrophage; Dendritic cell; Cytokine; Lipopolysaccharide

Core tip: Eradication rates for *Helicobacter pylori* (*H. pylori*) infection have fallen. The development of therapeutic alternatives to antibiotics, such as immunomodulatory therapy and vaccines requires a clearer understanding of host-pathogen interactions. As Toll-like receptors are intimately involved in the regulation of inflammation in response to *H. pylori* and represent key activators of adaptive immunity, they represent a target for therapeutic manipulation. Elucidating innate immune signals triggered by *H. pylori* will provide an understanding of how the balance between pro-inflammatory and anti-inflammatory signals fine-tunes the response to infection and insight into how the immune response may be manipulated therapeutically to successfully eradicate the bacterium.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a gram-negative microaerophilic flagellated bacterium that specifically infects

the stomachs of approximately 50% of the world's population. Infection is thought to be acquired in early childhood and persists for life if left untreated, despite triggering vigorous innate and adaptive immune responses[1-4]. Prevalence of *H. pylori* infection varies throughout the world and is associated with lower socioeconomic conditions[5]. Most infected individuals are asymptomatic. However, infection may cause chronic gastritis and confers a 1%-10% risk of developing gastric or duodenal ulcers, a 0.1%-3% risk of developing gastric adenocarcinoma, and < 0.01% of developing mucosa-associated lymphoid tissue (MALT) lymphoma[2]. Disease risk varies in different populations and is associated with host genotype, strain-specific bacterial components and environmental factors. *H. pylori* colonization of the gastric mucosa is followed by infiltration of polymorphonuclear leukocytes, monocytes and lymphocytes[6]. Mucosal levels of pro-inflammatory cytokines and chemokines such as interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β) are significantly higher in *H. pylori*-positive compared to *H. pylori*-negative gastric specimens[7-9]. Although, *H. pylori* infection induces an immune response that contributes to chronic gastric inflammation, the response is not sufficient to eliminate the bacterium[6,10]. Progression of disease from superficial gastritis to gastric cancer is linked to the severity of the host inflammatory response[11-13].

All consensus guidelines recommend eradication of *H. pylori* in symptomatic individuals using a standard first-line triple therapy consisting of a proton pump inhibitor together with the antibiotics clarithromycin and amoxicillin or metronidazole[4]. However, eradication rates have fallen in recent years in line with a rapid increase in antimicrobial resistance[14]. The most recent multicentre European assessment on *H. pylori* antimicrobial susceptibility has indicated that resistance rates for metronidazole and clarithromycin are 34.9% and 17.5% respectively[15]. Clarithromycin resistance has almost doubled in Europe in the last 10 years[15]. Furthermore, a high resistance rate of 14.1% has emerged for levofloxacin, which is used in rescue therapy for *H. pylori* infection[15]. This rapid emergence of antibiotic resistant strains of *H. pylori* is a cause for concern. The development of therapeutic alternatives to antibiotics, such as immunomodulatory therapy and vaccines requires a more lucid understanding of host-pathogen interactions. The mechanistic identification of host mediators of *H. pylori*-induced pathogenesis has the potential to reveal drug targets and opportunities for therapeutic intervention or prevention of *H. pylori*-associated disease.

The immune system consists of innate and adaptive immunity, that cooperate to efficiently control infections. The evolutionary conserved innate immune system provides the first line of defence against invading microbes, whereas the adaptive immune system is developed in later phases of infection and is highly specific, long lasting and possesses immunological memory[16]. Innate immune recognition of microbes is mediated by families of pathogen recognition receptors (PRRs), which recognize pathogen-associated molecular patterns (PAMPs) that are broadly shared by pathogens[17]. Upon PAMP recognition by a particular PRR, cell signalling cascades are triggered that are necessary for initiation of the host response. Additionally, PRR signalling induces the maturation of the major antigen presenting cells, dendritic cells (DCs), and the subsequent induction of adaptive immunity[17].

Gastric epithelial cells of the stomach mucosa represent the first line of innate immune defence against *H. pylori*, and respond to infection by initiating numerous cell signalling cascades[11]. PRRs of the Toll-like receptor (TLR) family have been shown to mediate many of these cell signalling events. In particular, a key role for TLR2 has been described in the response to *Helicobacter* in multiple cell contexts[11,18-21]. Recent data also suggest associations between TLR2 polymorphisms and the severity of intestinal metaplasia in *H. pylori*-positive patients[22] and with gastric cancer risk[23]. Additionally, polymorphisms in the TLR1 gene, which encodes a TLR2 co-receptor, are associated with *H. pylori* prevalence[24].

TLRS AND PATHOGEN RECOGNITION

TLRs are the most widely studied of the PRRs. Members of the TLR family are type I transmembrane proteins, consisting of a leucine-rich repeat-containing ectodomain involved in PAMP recognition, a transmembrane region and an intracellular portion that harbours a Toll-IL-1 receptor (TIR) domain involved in the activation of downstream signalling pathways. There are 10 TLR genes in humans[25]. TLRs are expressed on the cell surface or associated with intracellular vesicles, such as endosomes[16,17] (Figure 1). TLR1, TLR2, TLR4, TLR5 and TLR6 bind their respective ligands on the cell surface and recognize microbial membrane components such as lipids, lipoproteins and proteins[16,17]. TLR3, TLR7, TLR8, TLR9 are found in intracellular vesicles such as the endosome or lysosome and the endoplasmic reticulum, and are mainly involved in the recognition of microbial nucleic acids[16,17].

TLR4 was the first human TLR to be identified and recognizes bacterial lipopolysaccharide (LPS), which is a major constituent of the outer membrane of gram-negative bacteria[26]. LPS is a surface exposed glycolipid that consists of a hydrophobic membrane anchor portion, known as lipid A, and a non-repeating core oligosaccharide coupled to a distal polysaccharide (O-antigen) that extends from the bacterial surface[27,28]. The lipid A domain is responsible for the endotoxic properties associated with LPS. There is considerable LPS structural variability, due to diversity in both the chemical composition of the polysaccharide O-antigen and in lipid A variations, which contribute to the ability of some gram-negative bacteria to evade immune detection[27,28]. Smooth LPS is composed of a polysaccharide O-antigen side chain and has complete core oligosaccharides,

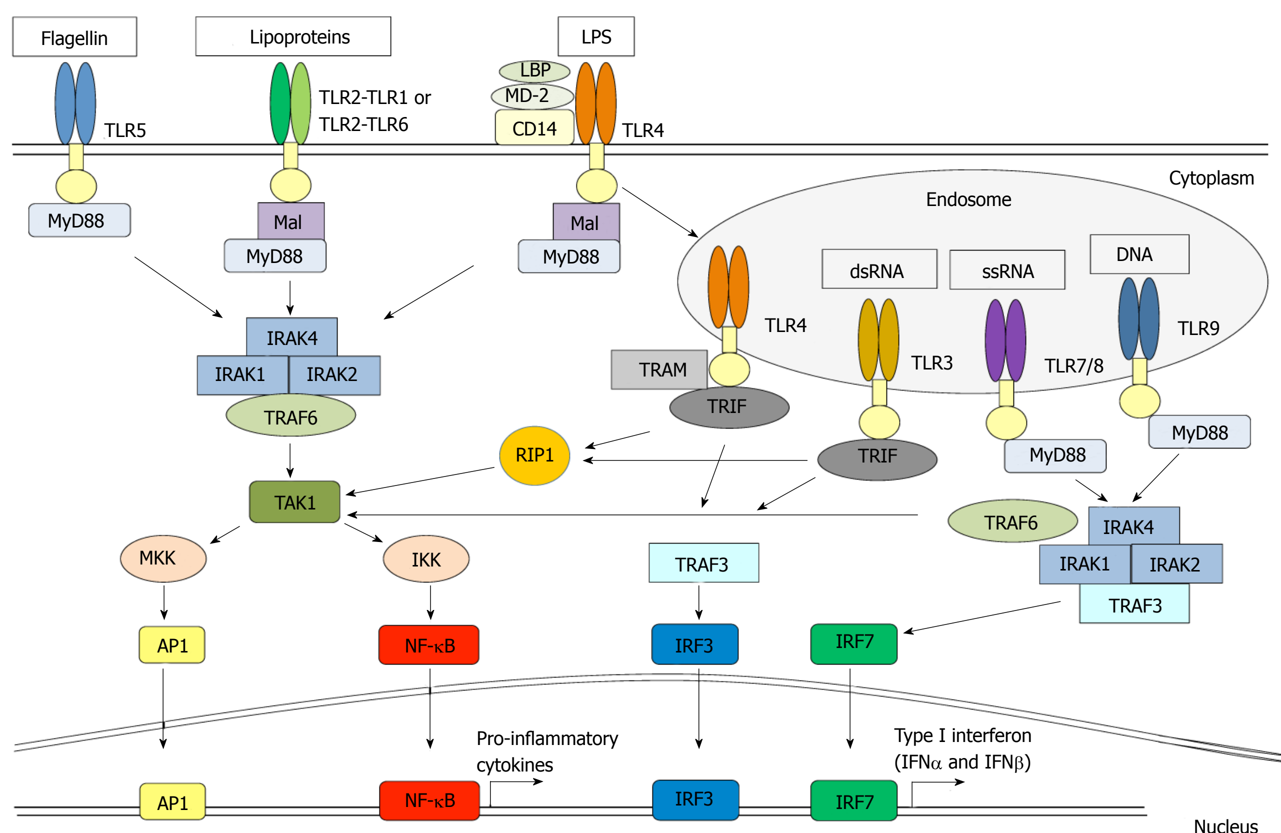

Figure 1 Toll-like receptor signalling. Toll-like receptors (TLRs) are type Ⅰ transmembrane proteins, consisting of a leucine-rich repeat-containing ectodomain involved in pathogen-associated molecular pattern (PAMP) recognition, a transmembrane region and an intracellular portion that harbours a Toll-IL-1 receptor (TIR) domain involved in adapter protein recruitment and the activation of downstream signalling pathways. TLR1, TLR2, TLR4, TLR5 and TLR6 bind to their ligands on the cell surface and recognize microbial membrane components. TLR3, TLR7, TLR8, TLR9 are found in intracellular vesicles and are mainly involved in the recognition of microbial nucleic acids. TLR signalling is initiated by ligand-induced receptor dimerization and TIR engagement with the adapter proteins MyD88 or TRIF. TLR4 localises from the cell membrane to endosomes to change signalling through MyD88 to TRIF. MyD88 is a central TLR adapter protein utilized by all TLRs, with the exception of TLR3, and transmits signals that result in the induction of inflammatory cytokines. The association between a TLR and MyD88 recruits members of the IRAK family. IRAK1 and IRAK4 are sequentially phosphorylated and dissociated from MyD88. This results in the activation of TRAF6, which in turn activates TAK1. TAK1 activates the IKK complex. In most resting cells, NF-κB is bound to the inhibitory IκB proteins (IκBα and IκBβ) in the cytoplasm. Upon activation of the IKK complex, IκB becomes phosphorylated and degraded, thus releasing NF-κB for translocation to the nucleus, where it interacts with promoters harboring κB binding elements. In addition, TAK1 stimulation results in the induction of MAP kinases kinases (MKKs) that activate p38, JNK and ERK, resulting in the subsequent activation of AP-1. In the case of TLR4, and to a lesser extent TLR2, the activation of this pathway involves the bridging adapter protein MAL, which links MyD88 to the TLR. The adapter protein TRIF is involved in the MyD88-independent TLR4 pathway, as well as the TLR3 signalling pathway. TRAM links TRIF to TLR4. Endosomal TLR-mediated signalling leads to the induction of type I interferon through the activation of the transcription factors IRF3 and IRF7.

whereas rough LPS lacks O-antigen and has shorter core oligosaccharides[16]. MD-2 is closely associated with TLR4 on the cell surface and is required for strong inflammatory cytokine induction in response to LPS. LPS-binding protein (LBP) and CD14 are also involved in the TLR4-mediated response to LPS[16]. Cells lacking CD14 are not responsive to smooth LPS but still respond to rough LPS or lipid A[16].

TLR2 recognizes a number of PAMPs on a variety of microorganisms, including zymosan from fungi, triacyl lipopeptides from bacteria and mycobacteria, diacyl lipopeptides from mycoplasma, and peptidoglycan and lipoteichoic acid from gram-positive bacteria[16,17]. TLR2 distinguishes between PRRs by hetero-dimerization with TLR1, TLR6, dectin-1 or CD14. TLR2 heterodimerizes with TLR1 to recognize triacylated lipopeptides from gram-positive bacteria[29,30] or with TLR6 to recognize diacylated lipopeptides, lipoteichoic acid and zymosan[31,32]. CD14 is involved in the recognition of diacylated lipopeptide, whereas the C-type lectin receptor dectin-1 collaborates with TLR2 in the recognition of β-glucan[16] found in the cell walls of fungi and yeasts. TLR2 has also been shown to recognize atypical forms of LPS[33-37]. TLR5 recognizes flagellin[38], a protein component of bacterial flagella. A role for TLR10 has not yet been shown, but the TLR10 sequence is most similar to TLR1 so TLR10 may heterodimerize with TLR2[25]. TLR3 recognizes double stranded RNA[39], which is a major component of many viruses. TLR9 is the receptor for CpG-rich hypomethylated DNA motifs[40], frequently found in bacterial DNA. TLR9 also responds to herpes virus DNA[41]. TLR7 and TLR8 sense single-stranded viral RNA[42-44].

TLR SIGNALLING

Upon PAMP recognition, TLRs trigger cell signalling pathways resulting in (1) the activation of the transcription factors nuclear factor-κB (NF-κB), activating protein-1 (AP-1) and interferon regulatory factors (IRFs); (2) expression of inflammatory cytokines, antimicrobial peptides and type I interferon (IFN); and (3) the subsequent recruitment of neutrophils, activation of macrophages and dendritic cells and the induction of IFN-stimulated genes. The specific response triggered by an individual TLR depends on the recruitment of a single or combination of TIR-domain containing adapter proteins[17]. MyD88 (myeloid differentiation primary response protein 88) is a key TLR adapter protein utilized by all TLRs, with the exception of TLR3, and transmits signals that result in the induction of inflammatory cytokines (Figure 1). The association between a TLR and MyD88 recruits members of the interleukin-1 receptor-associated kinase (IRAK) family. IRAK1 and IRAK4 are sequentially phosphorylated and dissociated from MyD88. This results in the activation of tumor necrosis factor receptor-associated factor 6 (TRAF6), which in turn activates transforming growth factor B-activated protein kinase 1 (TAK1). TAK1 activates the IKK [inhibitor of NF-κB (IκB) kinase] complex. In most resting cells, NF-κB is bound to the inhibitory IκB proteins (IκBα and IκBβ) in the cytoplasm. Upon activation of the IKK complex, IκB becomes phosphorylated and degraded, thus releasing NF-κB for translocation to the nucleus, where it interacts with promoters harboring κB binding elements to regulate gene transcription[45]. In addition, TAK1 stimulation results in activation of MAP kinase kinases (MKK) leading to the induction of the MAP kinases p38, JNK and ERK, resulting in the subsequent activation of AP-1[45] (Figure 1). In the case of TLR4, and to a lesser extent TLR2, the activation of this pathway involves the bridging adapter protein, MAL (MyD88 adapter-like, also known as TIR-domain-containing adapter protein, TIRAP)[46-49] , which links MyD88 to the TLR. The TIR-domain-containing adapter protein inducing IFNβ (TRIF, also known as TICAM1) is involved in the MyD88-independent TLR4 pathway, as well as the TLR3 signalling pathway[50-53] (Figure 1). TRIF-related adapter molecule (TRAM, also known as TICAM2) links TRIF to TLR4[54-56]. Endosomal TLR-mediated signalling leads to the induction of type I interferon through the activation of the transcription factors IRF3 and IRF7[25] (Figure 1).

THE ROLE OF TLRS IN *H. PYLORI* INFECTION

Epithelial cells

As gastric epithelial cells represent the first point of contact between *H. pylori* and the host, there has been a focus on the individual TLRs involved in the response to *H. pylori* infection in this cell context (Table 1). Expression of numerous TLRs has been confirmed in many gastric epithelial cells lines, including AGS, MKN28, MKN45, NUGC3 and KATOIII[19,57-60]. In addition TLR2 has been detected in epithelial cells from human gastric biopsy samples, with increased TLR2 expression reported in samples from *H. pylori*-infected patients[61,62]. Increased TLR4 expression has also been reported in the gastric mucosa of *H. pylori*-infected patients[58]. The Goldberg laboratory have reported a role for both TLR2 and TLR5 during *H. pylori* infection of MKN45 cells; inhibition of TLR2 or TLR5 (but not TLR4) function using dominant-negative mutant constructs decreased *H. pylori*-driven NF-κB activation[19]. In order to assess the contribution of individual TLRs during *H. pylori* infection, many investigators have utilised human embryonic kidney 293 (HEK293) cells stably expressing specific TLRs. HEK293 cells act as a suitable negative control as they do not express TLR2 or TLR4 endogenously[19,63,64]. Indeed additional studies from the Goldberg group have supported a role for TLRs during *H. pylori* infection by demonstrating that over-expression of TLR2 or TLR5 in HEK293 cells enhanced NF-κB activation and IL-8, macrophage inflammatory protein 3α (MIP-3α) and growth regulated protein α (GROα) mRNA expression in response to *H. pylori*[19]. Others have also confirmed that TLR2 expression in HEK293 cells results in enhanced IL-8 expression following *Helicobacter* infection[7,11]. Using HEK-TLR2 cells, the Goldberg group subsequently utilised microarray analysis to identify 28 TLR2-dependent genes whose expression was altered in response to *H. pylori* infection[20]. A number of these genes demonstrated distinct expression patterns between AGS cells (which do not express TLR2 endogenously[20,62]) and MKN45 cells (which express TLR2[19,57])[20].

Monocytes/macrophages

Following *H. pylori* infection, epithelial cells release a variety of cytokines and chemokines leading to the recruitment of monocytes/macrophages to the gastric mucosa. Mononuclear cell infiltration in the lamina propria is characteristic of *H. pylori*-induced chronic infection[65]. Human monocytes and macrophages express a wide repertoire of PRRs. *H. pylori* has been shown to induce secretion of inflammatory cytokines (IL-1β, IL-6, IL-8) from peripheral blood mononuclear cells and IL-8 from purified human monocytes and monocyte-derived macrophages[11]. Different studies have implicated alternative TLRs in the *H. pylori*-mediated response in monoctyes/macrophages (Table 1). Maeda *et al*[57] (2001) demonstrated that peritoneal macrophages from C3H/HeJ mice carrying a point mutation in the TLR4 gene showed decreased NF-κB activation and TNFα secretion compared with C3H/HeN macrophages in response to *H. pylori* infection. On the other hand, Gobert *et al*[66] (2004) found no significant difference in terms of IL-6 mRNA induction between peritoneal macrophages isolated from wild-type mice, TLR2-, TLR4- and MyD88-deficient mice in response to *H. pylori* infection. Using bone-

Table 1 Toll-like receptor involvement in the response to *Helicobacter pylori* infection in different cell types

Cell type	Cell type	TLR involvement	Readout of TLR activation	Ref.
Epithelial	MKN45 HEK-TLR2 HEK-TLR5	TLR2 TLR5	NF-κB-dependent reporter gene activity	Smith *et al*[19]
	HEK-TLR2	TLR2	MIP-3α mRNA expression IL-8 and GROα mRNA expression	Smith *et al*[19]
	HEK-TLR2	TLR2	IL-8 production	Mandell *et al*[11]
	HEK-TLR2 AGS MKN45	TLR2	mRNA expression of multiple genes	Ding *et al*[20]
	HEK-TLR2	TLR2	IL-8 mRNA expression	Smith *et al*[7]
Monocytes and macrophages	Mouse peritoneal macrophages	TLR4	NF-κB activation by electro mobility shift assay TNFα production	Maeda *et al*[57]
	Mouse peritoneal macrophages	TLR2-, TLR4- and MyD88-independent	IL-6 mRNA expression	Gobert *et al*[66]
	Mouse BMDMs	TLR2	IL-6 production	Mandell *et al*[11]
	Mouse BMDMs	TLR2 TLR4	IL-6 and IL-1β production IL-10 and IL-12 production	Obonyo *et al*[67]
	Mouse BMDMs	MyD88	IL-6 and IL-12 production	Rad *et al*[69]
Dendritic cells	Mouse BMDCs	MyD88	MHC II and co-stimulatory molecule induction IL-6, IL-12 and TNFα production mRNA expression of multiple genes	Rad *et al*[69]
	Mouse BMDCs	TLR2 TLR4 TLR9	mRNA expression of multiple genes IL-6 and IL-12 production	Rad *et al*[18]
	Mouse BMDCs	TLR2	IL-1β production	Kim *et al*[70]
	Mouse BMDCs	TLR2	IL-12, TNFα, IL-6 and IL-23 production	Sun *et al*[73]
B cells	Mouse B cells	MyD88	IL-6 and IL-12 production	Rad *et al*[69]
	Mouse B cells	MyD88 TLR2	IL-10, IL-6 and TNFα production CD80 and CD86 expression Secretion of antibodies	Sayi *et al*[21]

TLR: Toll-like receptor.

marrow derived macrophages (BMDMs) from knockout mice, Mandell *et al*[11] (2004) reported that the cytokine (IL-6) response to *H. pylori* was mediated by TLR2. *H. pylori*-infected BMDMs from wild-type or TLR4-deficient mice produced a robust cytokine response, whereas macrophages form TLR2-deficient mice were unresponsive. It is possible that alternative TLRs are involved in the *H. pylori*-mediated induction of individual cytokines within a particular cell context. Indeed, Obonyo *et al*[67] (2007) demonstrated that *H. pylori* induced IL-12 and IL-10 through TLR4/MyD88 signalling and IL-6 and IL-1β through TLR2/MyD88 signalling using BMDMs from knockout mice. As such, this study would suggest that *H. pylori* infection activates both TLR2 and TLR4 signalling in BMDMs leading to the secretion of distinct cytokines. This hypothesis is possible, given that individual *H. pylori* components have been suggested to trigger TLR2 or TLR4 signalling (Table 2).

Dendritic cells

In recent years, there has been an increasing interest in the mechanisms by which *H. pylori* initiates adaptive immunity and instructs the phenotype of the T cell response. During the activation of adaptive immunity, different T-helper (Th) cell subsets arise that exhibit characteristic patterns of cytokine secretion. As the major antigen presenting cells, DCs play a key role in the induction of the adaptive immune response. DCs express a wide range of PRRs[68] and possess the unique ability to capture antigen from the periphery and activate naïve T cells to direct T cell differentiation by producing three types of signals; antigen presentation, co-stimulation and cytokine secretion[18,69]. Rad *et al*[69] have shown that *H. pylori* activates DCs in a MyD88-dependent manner (Table 1). Production of pro-inflammatory cytokines (IL-6, IL-12 and TNFα), and induction of major histocompatibility complex class Ⅱ (MHC Ⅱ) and co-stimulatory molecules in MyD88-deficient DCs was impaired compared to wild-type cells following *H. pylori* stimulation. Further analysis of the *H. pylori*-controlled DC transcriptome by microarray analysis indicated that MyD88 was involved in the regulation of numerous genes involved in DC maturation, antigen uptake and presentation, as well as effector cell recruitment and activation[69]. *H. pylori*-mediated cytokine stimulation was also impaired in B cells and macrophages from the MyD88-deficient mice (Table 1). The *in vitro* findings were reflected *in vivo* in the form of reduced gastric inflammation and increased bacterial colonization following 4 mo *H. pylori* infection in MyD88-deficient mice, suggesting that the impaired immune response in MyD88-deficient mice enables better bacterial survival[69]. *Helicobacter*-specific

IgG2c/IgG1 ratios were reduced in MyD88-deficient mice, implying the involvement of the MyD88 pathway in the instruction of a Th1 phenotype[69]. Subsequent research by Rad *et al*[18] (2009) further characterized TLR-mediated signalling in DCs during *H. pylori* infection. They identified a MyD88-dependent component of the DC activation program that was induced by TLR2 and to a minor extent TLR4. Microarray analysis of *H. pylori*-stimulated DCs showed complementary, redundant and synergistic interactions between TLRs. Using TLR2-deficient cells the anti-inflammatory cytokine IL-10 was identified as a TLR2-dependent *H. pylori* responsive gene in DCs[18]. In addition, they demonstrated that IL-6 and IL-12 production was inhibited by approximately 50% in TLR2/TLR4/TLR9-deficient BMDCs compared to TLR2/TLR4-deficient cells in response to *H. pylori* infection, implying that TLR9-dependent recognition of *H. pylori* in DCs contributes to the cytokine response[18]. More recently Kim *et al*[70] (2013) have also implicated TLR signalling in response to *H. pylori* by demonstrating a role for TLR2 in *H. pylori*-induced IL-1β production in mouse BMDCs.

Although *H. pylori*-infected individuals generate a strong immune response, they fail to eradicate the bacterium. Emerging evidence suggests that failure to eliminate *H. pylori* may be due to its ability to induce a regulatory T cell (Treg) response, as expression of the Treg marker Foxp3 is increased in *H. pylori*-infected gastric tissue compared to that of uninfected individuals[71,72]. Sun *et al*[73] (2013) have recently investigated the functional role of TLR2 signalling in BMDCs in response to *H. pylori* and the subsequent effects on T cell responses. Firstly, they demonstrated that *H. pylori*-infected BMDCs from TLR2-deficient mice exhibited impaired production of the pro-inflammatory cytokines that promote both Th1 responses (IL-12 and TNFα) and Th17 responses (IL-6 and IL-23) compared to wild-type cells. Additionally, this report suggests that *H. pylori* may skew differentiation of naïve T cells towards Th17 and Treg responses as opposed to Th1 responses, as *H. pylori*-stimulated BMDCs from TLR2 knock-out mice induced a higher splenocyte production of IFNγ (Th1 response) and lower production of IL-17 (Th17 response) and IL-10 (Treg response)[73]. *In vivo* analyses following *H. pylori* infection for 2 mo showed a lower degree of gastric *H. pylori* colonization in TLR2 knock-out mice and more severe gastritis, implying that the TLR2-mediated response to *H. pylori* promotes a bacterial survival advantage. Sun *et al*[73] also demonstrated that the gastric mucosa of the infected TLR2 knock-out mice had lower Foxp3, IL-10 and IL-17A expression, but higher expression of IFNγ compared to wild-type mice. The *H. pylori*-specific Th1 response was higher and the Treg and Th17 responses were lower in the spleens of infected TLR2 knock-out mice, suggesting that *H. pylori* mediates immune tolerance through TLR2-derived signals and inhibits Th1 immunity, thus evading the host defence[73]. It is noteworthy that the Rad *et al*[69] (2007) study suggested MyD88-dependent TLR signalling promotes a Th1 response in *H. pylori*-infected mice and is protective against *H. pylori* colonization, while the Sun *et al*[73] (2013) study implies that TLR2 signalling inhibits Th1 immunity, supports a Treg/Th17 response and promotes *H. pylori* colonization. It is possible that different TLR ligands from the same pathogen induce distinct but opposing signals. Furthermore, it is likely that the complementary, redundant and synergistic interactions between TLRs in DCs subsequently reported by Rad *et al*[18] (2009) contribute to the observations from studies involving MyD88-deficient mice.

B cells

Evidence suggests that B cells contribute to the immunepathogenesis of *H. pylori* infection[74]. Sayi *et al*[21] (2011) have demonstrated that B cells play a role in regulating T cell responses and gastric immunopathology in response to *Helicobacter*. Building on the finding by Rad *et al*[69] (2007) that TLR2 is required for *Helicobacter*- mediated IL-6 and IL-12 induction in B cells, Sayi *et al*[21] showed that cytokine production (IL-10, IL-6 and TNFα), surface expression of the activation markers CD80 and CD86 and induction of antibody secretion was impaired in *Helicobacter*-stimulated B cells from both MyD88- and TLR2-deficient mice compared to wild type control cells (Table 1). The *Helicobacter*-stimulated B cells induced IL-10-producing $CD4^+CD25^+$ T regulatory-1 (Tr-1)-like cells in a TLR2- and MyD88-dependent manner[21]. The Tr-1 cells acquired suppressive activity *in vitro* and suppressed excessive gastric *Helicobacter*-associated immunopathology *in vivo*, suggesting that TLR2-mediated signalling in B cells plays a role in regulating the balance of *Helicobacter*-specific T cell responses to prevent excessive Th1-driven immunopathology and promote mucosal homeostasis, but enabling bacterial persistence[21].

RECOGNITION OF DISTINCT *H. PYLORI* COMPONENTS BY SPECIFIC TLRS

LPS

Based on the involvement of TLRs in regulating immunopathology in the context of *H. pylori* infection, many investigators have set out to elucidate the contribution of individual *H. pylori* components to the control of TLR-driven innate immune responses. *H. pylori* LPS has a lower endotoxicity than other gram-negative bacteria such as *Escherichia coli* or *Salmonella enterica*[75-78]. Although there has been substantial investigation into the innate immune response to *H. pylori* LPS, there have been conflicting findings with regard to the TLR responsible for its recognition (Table 2). Some studies have implicated the classic gram-negative bacterial LPS receptor TLR4[11,28,58,60,79,80], while others have suggested a role for TLR2[7,12,19,37,63]. Initial evidence for TLR4-mediated recognition of *H. pylori* LPS was provided by Kawahara *et al*[79] (2001) who demonstrated that LPS from clinical isolates of *H. pylori* induced increased superoxide anion (O_2^-) production in guinea pig gastric pit cells that ex-

Table 2 Toll-like receptors involved in the response to distinct *Helicobacter pylori* components

H. pylori component	TLR	Ref.
LPS	TLR4	Kawahara *et al*[79]
		Su *et al*[80]
		Ishihara *et al*[58]
		Mandell *et al*[11]
		Chochi *et al*[60]
		Cullen *et al*[28]
	TLR2	Smith *et al*[19]
		Lepper *et al*[63]
		Yokota *et al*[12]
		Triantafilou *et al*[37]
		Smith *et al*[7]
Flagellin	TLR5	Smith *et al*[19]
	TLR5 evasion	Lee *et al*[84]
		Gewirtz *et al*[83]
HSP60	TLR2	Takenaka *et al*[59]
		Zhao *et al*[85]
	TLR2-independent	Gobert *et al*[66]
HP0175	TLR4	Basak *et al*[87]
		Pathak *et al*[65]
		Basu *et al*[86]
NAP	TLR2	Amedei *et al*[6]
H. pylori DNA	TLR9	Rad *et al*[18]
H. pylori RNA	TLR7/TLR8	Rad *et al*[18]

TLR: Toll-like receptor; *H. pylori*: *Helicobacter pylori*.

press endogenous TLR4, but not TLR2. Subsequently, TLR4 antibodies were shown to inhibit LPS-mediated IL-8 secretion from phorbol myristate acetate (PMA)-stimulated THP1 macrophages and *H. pylori* demonstrated increased adherence to Chinese Hamster Ovary (CHO) cells transfected with TLR4 compared with that of CHO-TLR2 or untransfected CHOs[80]. Using reporter gene assays, Ishihara *et al*[58] (2004) described *H. pylori* LPS-mediated NF-κB activation and transcription from the IL-8 promoter in AGS gastric epithelial cells over-expressing TLR4 and MD2[58]. In addition, Mandell *et al*[11] (2004) demonstrated that although TLR2 plays a key role in the response to intact *H. pylori*, TLR4-deficient murine BMDMs were unresponsive to LPS isolated from clinical strains of *H. pylori* with regard to cytokine (IL-6) production. More recently, Chochi *et al*[60] (2008) demonstrated that a clinical isolate of *H. pylori* LPS augmented proliferation using a panel of gastric cancer cell lines (MKN28, MKN45, NUGC3 and KATOIII) in a TLR4-dependent manner. Lastly, while investigating the role of lipid A modifications in *H. pylori* pathogenesis, Cullen *et al*[28] (2011) reported that modification of *H. pylori* LPS in terms of lipid A dephosphorylation leads to decreased LPS-mediated NF-κB activation in HEK-TLR4 cells, providing a mechanism whereby *H. pylori* evades innate immune recognition.

In support of TLR2 as the *H. pylori* LPS receptor, Smith *et al*[19] (2003) demonstrated that LPS isolated from *H. pylori* NCTC 26695 induced NF-κB-dependent reporter gene activity in HEK293 cells transfected with TLR2, but not with TLR4. In addition, LPS from *H. pylori* strain LC11 and two clinical isolates activated NF-κB in HEK-TLR2 cells but not HEK-TLR4 cells. Also using HEK cell lines transfected with TLRs, studies from the Triantafilou laboratory indicated that *H. pylori* LPS induced TNFα production in TLR2-expressing cells, but not TLR4-expressing cells[37,63]. TLR2 was responsible for *H. pylori* LPS-mediated NF-κB-driven reporter gene activity in CHO fibroblasts and HEK cells over-expressing TLR2[37,63]. Inhibition of endogenous TLR2 expression in vascular endothelial cells by RNA interference resulted in a reduction of TNFα production[37]. Using fluorescence resonance energy transfer analysis, they also demonstrated that TLR2 is recruited to lipid rafts and associates with TLR1 in cells following LPS stimulation in vascular endothelial cells[37]. Further, Yokota *et al*[12] demonstrated that *H. pylori* LPS-mediated induction of IL-8 secretion from T24 uroepithelial cells was suppressed by expression of a dominant negative TLR2 mutant, but not with a TLR4 mutant. NF-κB-dependent luciferase reporter assays indicated that over-expression of TLR2 and TLR1 or TLR2 and TLR6 conferred LPS responsiveness in HEK293 cells. The combination of TLR2 and TLR1 expression resulted in higher responsiveness to *H. pylori* LPS than TLR2 and TLR6 expression[12].

Studies by Smith *et al*[7] (2011) have also supported a role for TLR2 in the innate immune recognition of *H. pylori* LPS. LPS prepared from 3 reference strains (NCTC 11637, NCTC 26695 and CCUG 17874) and 4 clinical isolates of *H. pylori* induced IL-8 mRNA expression in HEK293 cells over-expressing TLR2 but not TLR4. IL-8 induction in HEK-TLR2 cells was found to be dose-dependent with a significant level of induction observed at the lowest LPS concentration tested (250 ng/mL). The effect was shown to be LPS specific, as pre-incubation of the *H. pylori* LPS preparations with the antibiotic polymyxin B, a well-known inhibitor of the activating properties of LPS, resulted in a dose-dependent decrease in IL-8 induction in HEK-TLR2 cells[7]. It was also found that *H. pylori* LPS did not induce IL-8 expression in AGS cells, which do not express TLR2 endogenously[20,62], whereas IL-8 was induced in MKN45 cells and T84 colorectal carcinoma cells which have been shown to express endogenous TLR2[19,57,81]. In order to delineate LPS-mediated signalling downstream of TLR engagement, co-transfection using dominant negative constructs and small-interfering RNA demonstrated that *H. pylori* LPS functioned as a classic TLR2 ligand by signalling through pathways involving MyD88, MAL, IRAK1, IRAK4, TRAF6, IKKβ and IκBα to activate NF-κB and transcription form the IL-8 promoter[7]. Through a combination of microarrays, quantitative PCR and ELISAs, it was demonstrated that *H. pylori* LPS induced expression of ICAM1 and the chemokines CXCL1, CXCL2, CXCL3 and CCL20 in TLR2-expressing HEK cells and MKN45 gastric epithelial cells but not HEK293, HEK-TLR4 or AGS cells. Increased expression of these genes was confirmed in gastric tissue biopsy samples from *H. pylori*-infected patients when

compared to uninfected controls[7].

The reasons for the conflicting results between the studies are unclear. Possible explanations include differences in experimental systems involving alternative read-outs for TLR activation and various cell lines from different species. In addition, contamination of the LPS preparation with other components, such as protein, nucleic acids or other bacterial LPS molecules could account for conflicting findings. However, results from Smith *et al*[7] (2011) demonstrating that polymyxin B inhibited TLR2-mediated IL-8 induction in HEK-TLR2 cells would imply that the TLR2-mediated response observed was LPS-specific and not due to the presence of other contaminating TLR ligands, at least in this cell context. Contrasting findings may also have arisen due to heterogeneity of the structures of *H. pylori* LPS molecules resulting from strain differences and/or culturing conditions. Tran *et al*[75] (2005) reported that the lipid A portion of *H. pylori* LPS undergoes several structural modifications through the action of specific modifying enzymes. There is also considerable LPS structural variability due to diversity in the chemical composition of the polysaccharide O-antigen[27,28]. The study by Yokota *et al*[12] (2007) reported similar TLR2-dependent activities using LPS isolated from 6 different clinical isolates of *H. pylori* that demonstrated various characteristics, such as smooth/rough phenotypes and antigenicity of the polysaccharide portion[12]. Additionally, it has been shown that LPS isolated from other gram-negative bacteria that produce a mixture of lipid A species with modified forms of lipid A, such as *Porphyromonas gingivalis* and *Leptospira interrogans*, elicit immune responses through TLR2[33-37].

Flagellin

TLR5 has been identified as the receptor for bacterial flagellin[38], the protein subunit of the polymeric flagellar filament of different gram-positive and gram-negative bacteria. *H. pylori* flagella (5-7 per cell) confer motility and are composed of polymers of two protein subunits, the major flagellin FlaA and the minor flagellin FlaB[82,83], both of which are essential for the bacteria to survive in the stomach musica[84]. TLR5 is expressed on primary gastric epithelial cells and gastric epithelial cell lines, including AGS, HM02, MKN28 and MKN45[19,62,84]. Initial investigations into the innate immune recognition of *H. pylori* flagellin indicated that TLR5 expression in HEK293 cells conferred responsiveness to partially purified flagellin from *H. pylori* in terms of NF-κB-dependent reporter gene activity[19] (Table 2). In addition, transfection of MKN45 cells with a dominant negative TLR5 construct inhibited NF-κB activity in response to *H. pylori* flagellin[19]. However, other studies have since demonstrated that *H. pylori* flagellin is a significantly less potent stimulator of TLR5 signalling than flagellin from other gram-negative bacteria, such as *Salmonella typhimurium*[83,84]. Lee *et al*[84] (2003) demonstrated that although IL-8 release induced by *H. pylori* with mutations in one or both flagellins was delayed compared to wild type *H. pylori*, purified native or recombinant flagellins did not significantly stimulate IL-8 secretion from gastric epithelial cells despite the presence of TLR5, suggesting that the delayed effect with the mutant strains may have been a result of decreased bacterial motility or adherence. Gewirtz *et al*[83] (2004) found no impairment in the IL-8 inducing ability of *H. pylori* in AGS cells as a result of FlaA mutations compared to the wild type strain. In keeping with the findings of Lee *et al*[84] (2003), purified *H. pylori* flagellin failed to induce significant innate immune responses in gastric epithelial cells as assessed by p38 MAPK induction and IL-8 secretion. The low innate immune response to *H. pylori* flagellin in the stomach *in vivo* may provide another mechanism that contributes to the ability of *H. pylori* to evade host responses and to promote long term bacterial persistence.

Heat shock protein 60

The 60 kDa heat-shock protein (HSP60) of *H. pylori* plays a role in the adherence and attachment of *H. pylori* to the gastric epithelium and is a potent immune antigen that stimulates IL-8 induction in gastric epithelial cells[59]. HSP60-induced immune responses are associated with gastric inflammation and the pathogenesis of MALT[59]. Takenaka *et al*[59] (2004) have suggested that *H. pylori* HSP60 is a TLR2 ligand as HSP60-mediated NF-κB activation and IL-8 production in KATO Ⅲ human gastric epithelial cells was inhibited using a TLR2 blocking antibody (Table 2). *H. pylori* HSP60 has also been shown to induce IL-8 production in human monocytes. In support of TLR2 in the recognition of *H. pylori* HSP60, Zhao *et al*[85] (2007) reported that treatment of NOMO1 human monocytes with an anti-TLR2 blocking antibody or small interfering RNA for TLR2 inhibited NF-κB, ERK and p38 MAPK activation as well as IL-8 secretion in response to recombinant *H. pylori* HSP60 stimulation. In contrast to the findings in human cells, peritoneal macrophages from mice deficient in TLR2, TLR4, MyD88 or both TLR2 and TLR4 produced the same amount of IL-6 in response to *H. pylori* HSP60 as wild type macrophages, indicating TLR-independent IL-6 induction in this cell context[66].

H. pylori peptidyl prolyl cis-,trans-isomerase HP0175

H. pylori secretes the peptidyl prolyl *cis*-, *trans*-isomerase HP0175, which can induce apoptosis in gastric epithelial cells and is one of the highly and consistently reactive *H. pylori* antigens recognized in the sera of *H. pylori*-infected patients[65,86]. Studies from the Kundu laboratory have described a role for TLR4 in the recognition of HP0175 (Table 2). Initially, Basak *et al*[87] (2005) demonstrated interaction between TLR4 and HP0175 in AGS cells using pull-down immunoassays. Inhibition of TLR4 using a neutralizing antibody or a dominant negative construct inhibited HP0175-induced apoptosis in AGS cells[87]. Pathak *et al*[65] (2006) subsequently reported that HP0175 induced the release of IL-6 from PMA-differentiated

THP1 macrophages, whereas isogenic mutants of *H. pylori* 26695, in which the *Hp0175* gene was disrupted, elicited decreased IL-6 production. A role for TLR4 in this process was suggested because pre-treatment of cells with a TLR4 (but not TLR2) neutralising antibody or transfection with a dominant-negative TLR4 construct blocked HP0175-mediated IL-6 release. In addition, TLR4 expression (but not TLR2) in HEK293 cells conferred responsiveness to HP0175. Using ELISA-based binding assays, Pathak *et al*[65] also showed that HP0175 interacts with the extracellular domain of TLR4 in the absence of any accessory molecules. Finally, Basu *et al*[86] (2008) showed that HP0175 transactivates the epidermal growth factor receptor (EGFR) and stimulates EGFR-dependent vascular endothelial growth factor (VEGF) production in AGS cells in a TLR4-dependent manner.

NapA

The *H. pylori* neutrophil-activating protein (NAP) is a 150 kDa oligomeric virulence factor that is chemotactic for neutrophils, stimulates high production of oxygen radicals in neutrophils and their adhesion to endothelial cells. Amedei *et al*[6] (2006) have reported that the *H. pylori* NAP is a TLR2 agonist, because over-expression of TLR2 in HEK293 cells resulted in NAP-mediated NF-κB-dependent reporter gene activity (Table 2). NAP stimulation had no effect on untransfected HEK293 cells, or HEK293 cells over-expressing TLR3, TLR4, TLR5, TLR7, TLR8 or TLR9. In human monocytes and neutrophils, NAP stimulation induced the expression of IL-12[6], which is an important cytokine for the differentiation of naïve Th cells into the Th1 phenotype. NAP also induced monocytes to produce IL-23 and differentiate towards mature DCs[6]. Stimulation of antigen-induced T-cells with NAP resulted in increased numbers of IFN-γ-producing T cells and decreased numbers of IL-4-secreting cells, thus promoting a Th1 phenotype. In addition, using T cell clones generated from *in vivo*-activated T cells derived from the gastric mucosa of *H. pylori*-infected patients, NAP was shown to elicit Th1-polarizing capacity[6], implying that the TLR2: *H. pylori* NAP interactions promote the activation of innate immunity to drive IL-12 and IL-23 production and the subsequent promotion of Th1 immune responses.

Nucleic acids

TLR9 recognises unmethylated CpG DNA in bacteria[40] and also detects herpes virus DNA[41]. TLR7 and TLR8 have been shown to sense single-stranded viral RNA[42-44]. Rad *et al*[18] (2009) have demonstrated TLR9-mediated recognition of *H. pylori* DNA in DCs and the subsequent induction of pro-inflammatory cytokine secretion. They showed that IL-6 and IL-12 production was completely abrogated in TLR2/TLR4/TLR9-deficient BMDCs compared to TLR2/TLR4-deficient cells in response to purified *H. pylori* DNA following pre-treatment with ribonuclease. Expression of TLR9 is increased in mouse gastric tissue following *H. pylori* infection and is mainly localised to macrophages, DCs and $CD3^+$ cells in the gastric mucosa[88]. Although purified *H. pylori* DNA was reported to induce a TLR9-mediated increase in IL-6 and IL-12 expression in BMDCs[18], in a mouse model of *H. pylori* infection TLR9 signalling was shown to have an anti-inflammatory effect on the early phase of *H. pylori*-induced gastritis as genetic disruption of TLR9 resulted in an increase in *H. pylori*-induced gastric mucosal inflammation characterized by neutrophil infiltration and increased expression of TNFα and IFNγ[88]. In relation to TLR7 and TLR8-mediated recognition of *H. pylori*, Rad *et al*[18] (2009) showed that purified *H. pylori* RNA (pre-treated with deoxyribonuclease) induced pro-inflammatory cytokines in BMDCs in a MyD88-dependent manner involving the endosomal TLR8, possibly in collaboration with TLR7.

TARGETING TLR SIGNALLING THERAPEUTICALLY

As TLRs are intimately involved in the regulation of inflammation during innate immunity and represent key activators of adaptive immunity, they represent an attractive therapeutic target for treatment of inflammatory diseases. Indeed, oligonucleotide inhibitors of TLR7 and/or TLR9 have been shown to have therapeutic potential in animal models of systemic lupus erythematosus[89]. Additionally, an inhibitory TLR2 antibody was demonstrated to limit ischemia-reperfusion injury in the hearts of pigs[90] and kidneys of mice[91]. In the clinic, therapies involving the synthetic small molecule inhibitor of TLR4, Eritoran (also known as E5564), were used in trials for patients with sepsis, but only had marginal effects possibly as treatment was administered too late following disease onset[92,93]. TLR activation is also important for adjuvancy in vaccines and several TLR ligands have been shown to be efficacious as vaccine adjuvants[94,95]. For example, the vaccine adjuvant monophosphoryl lipid A, which is a less toxic version of LPS, promotes antibody responses via TLR4 activation[96]. Efficient preventative or therapeutic vaccination for *H. pylori* has not been achieved in humans to date[97]. Early signs of promise in animal models of *H. pylori* infection have been unsuccessful in humans. In a recent study to investigate the vaccine potential of *H. pylori* LPS, Altman *et al*[98] (2012) demonstrated enhanced antibody responses to a chemically modified LPS in mice and rabbits and partial protection against *H. pylori* challenge, warranting further investigation in this area. In terms of adjuvancy, immunization against *Helicobacter* using CpGDNA and cholera toxin demonstrated synergism leading to sterile immunity in mice[99]. More recently, Mori *et al*[100] (2012) constructed a chimeric flagellin by replacing the N- and T-terminal segments of *H. pylori* flagellin with a TLR5-stimulating adjuvant component of *E. coli* flagellin in order to enhance innate and adaptive immunity. The resulting chimeric flagellin activated TLR5 signalling and elicited a strong antibody response in mice. Together

with alum, vaccination with the chimeric flagellin protected mice from *H. pylori* infection[100]. In other disease settings, local administration of the TLR2 ligand *H. pylori* NAP was demonstrated to decrease tumour growth by activating a cytotoxic Th1 response in a mouse model of bladder cancer[101]. Taken together, these studies indicate that defining *H. pylori*-derived molecules responsible for TLR activation and elucidating innate immune signals triggered by *H. pylori*, may provide insight into the design and development of novel human vaccine adjuvants and therapeutics.

H. PYLORI RECOGNITION BY OTHER PRRS

Microbial pathogens activate multiple PRRs and different PRRs may recognize the same PAMP within an organism[17]. Insight into the co-operation between TLRs and other PRRs during infection is necessary for a complete understanding of the innate immune response during infection. These PRRs include nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) and C-type lectin receptors (CLRs). *H. pylori* peptidoglycan, delivered either by the type IV secretion system or through outer membrane vesicles secreted from the bacterium, is recognised by NOD1 in epithelial cells[102-107]. Moreover, studies by Kim *et al*[70] (2013) have described the cooperative interaction of TLR2 and NOD2 in the regulation of IL-1β in *H. pylori*-infected DCs. In addition to a role for TLR8 in the response to *H. pylori* RNA, findings by Rad *et al* (2009) indicated that *H. pylori* induces expression of type I interferon and interferon-stimulated genes in a MyD88- and TRIF-independent manner and demonstrated that the MyD88-independent type I IFN induction by *H. pylori* RNA was mediated by RIG-I[18]. In relation to *H. pylori*-mediated CLR signalling, Gringhuis *et al*[108] (2009) have reported that the fucose residues of *H. pylori* DC-SIGN ligands actively disrupt signalling down-stream of DC-SIGN to suppress pro-inflammatory cytokine induction.

CONCLUSION

Although, *H. pylori* induces a strong immune response, elimination of infection is not achieved. The pathogenesis of *H. pylori*-associated disease is linked to the severity of the host inflammatory response**.** Emerging evidence suggests that failure to eliminate *H. pylori* may be due to the ability of the bacterium to control T-cell responses. As TLRs are intimately involved in the regulation of inflammation during the innate immune response to *H. pylori* and represent key activators of adaptive immunity, a significant effort has been made to elucidate their role in the recognition of *H. pylori* and its components in multiple cell types. Much of the literature has focussed on the involvement of individual TLRs in the induction of pro-inflammatory cytokines in various *in vitro* cell culture models. There is substantial evidence to support the role of TLR2 in activating NF-κB or inducing cytokine expression in response to *H. pylori* infection in epithelial cells[7,11,19,20], monocytes/macrophages[11,67], dendritic cells[18,70,73] and B cells[21]. Numerous *H. pylori* ligands have been suggested to date that may contribute to these TLR2-dependent responses, including LPS[7,12,19,37,63], HSP60[59,85] and NAP[6]. TLR4 has also been implicated in the response to *H. pylori*[18,57,67], which may be mediated by LPS[11,28,58,60,79,80] and/or HP0175[65,86,87]. TLR9 has been identified as the receptor for *H. pylori* DNA[18]. Although *H. pylori* flagellin has been suggested as a TLR5 ligand[19], its activity as a TLR5 activator is low[83,84], providing a possible mechanism that contributes *H. pylori* persistence.

Recent studies using mouse models of infection have provided insight into the role of TLR signalling in regulating *H. pylori*-mediated T cell responses, gastric immunopathology and colonization *in vivo*. Interestingly, although a demonstrated role for TLR2 in the induction of pro-inflammatory cytokines has been described in distinct cell populations, the net effect of TLR2 signalling has been reported to mediate tolerance and promote bacterial persistence in mouse models of infection by skewing T cell responses[21,73]. Further *in vivo* studies elucidating innate immune signals triggered by *H. pylori*-mediated activation of TLRs, especially in cooperation with other PRRs, are necessary for a complete understanding of how the balance between pro-inflammatory and anti-inflammatory signals fine-tunes the immune response to *H. pylori* infection, and may provide insight into how the immune response may be manipulated therapeutically to successfully eradicate the bacterium.

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