The role of the pathogen recognition molecule, NOD1, in the induction of gastric epithelial cell signalling during *Helicobacter pylori* infection.

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General Declaration

PART A: General Declaration

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Declaration for thesis based or partially based on conjointly published or unpublished work

General Declaration

In accordance with Monash University Doctorate Regulation 17/ Doctor of Philosophy and Master of Philosophy (MPhil) regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 2 original papers published in peer reviewed journals and 0 unpublished publications. The core theme of the thesis is characterisation of host cell signalling responses to the gastric pathogen, *Helicobater pylori*. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Microbiology Department under the supervision of John Keith Davies.

[The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.]

In the case of chapter 2, my contribution to the work involved the following:

[If this is a laboratory-based discipline, a paragraph outlining the assistance given during the experiments, the nature of the experiments and an attribution to the contributors could follow.]

Thesis chapter	Publication title	Publication status	Nature and extent of candidate's contribution
2	*Helicobacter pylori* induces MAPK phosphorylation and AP-1 activation via a NOD1-dependent mechanism.	Published	75%
2	The beta1 integrin activates JNK independent of CagA, and JNK activation is required for *Helicobacter pylori* CagA+-induced motility of gastric cancer cells.	Published	10%

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed: .....[redacted].....

Date: 30-9-10

Abstract

Helicobacter pylori colonises a large proportion of the world's population, with infection invariably leading to chronic, life-long gastritis. While the infection often persists undiagnosed and without causing severe pathology, there are a number of host, bacterial and environmental factors that can influence disease outcome. In particular, the *H. pylori cag* pathogenicity island (*cag*PAI) encodes a Type IV secretion system (T4SS), which facilitates the translocation of bacterial effectors, such as peptidoglycan, into the host cell. The cytosolic pathogen recognition molecule, Nucleotide Oligomerisation Domain 1 (NOD1), senses the peptidoglycan of *H. pylori* and other Gram-negative organisms, resulting in the rapid initiation pro-inflammatory responses that are critical for the induction of innate and eventually adaptive immune responses. *H. pylori* is generally considered to be an extracellular pathogen and gastric epithelial cells are the pathogen's primary point of contact with the host. Therefore, complete characterisation of *H. pylori*-induced signalling in these cells, particularly with respect to the role of NOD1 and the *cag*PAI, will offer further insights into the mechanisms involved in the activation and recruitment of host immune effectors. Furthermore, these studies were designed to investigate the existence of a positive feedback mechanism between inflammatory responses and gastric epithelial cell signalling once infection is established.

In order to ascertain the involvement of NOD1 in host signalling responses to *H. pylori*, this study employed an *in vitro* co-infection model using various isogenic *H. pylori* mutants and a gastric epithelial cell line stably expressing siRNA to knock-down NOD1 expression. In addition, gastric biopsies from patients with differing degrees of gastritis or gastric cancer were analysed to assess the expression of various immune-related genes involved in inflammation and disease.

Initially, we addressed the respective contributions of NOD1 and *H. pylori* virulence factors to transcription factor activation and the expression of signalling molecules during infection. Accordingly, this study confirmed the role of NOD1 in the activation and nuclear translocation of "Nuclear Factor-kappa B" (NF-κB). Furthermore, we found that *H. pylori* induced the rapid phosphorylation of p38 and Extracellular-signal Regulated Kinase (ERK) Mitogen Activated Protein Kinases (MAPKs) in a NOD1-dependent manner and that this was essential for the downstream activation of "Activating Protein-1" (AP-1), an important

pro-inflammatory transcription factor involved in host responses to infection. Intriguingly, while NOD1 was essential for "c-Jun N-terminal Kinase" (JNK) MAPK phosphorylation in *Shigella flexneri*-infected cells, it was not required for the *cag*PAI-dependent phosphorylation of JNK, nor was it involved in the induction of a characteristic cell scattering and elongation phenotype observed in *H. pylori*-stimulated epithelial cells.

These findings were extended to investigate potential cross-talk between NOD1 and the chronic inflammatory responses induced during *H. pylori* infection. We show that *H. pylori* is capable of activating and enhancing certain components of the IFN-γ signalling pathway in gastric epithelial cells, specifically through NOD1 and the *cag*PAI. Many of these responses are known to be involved in the recruitment of immune cells to the site of infection, which would further exacerbate inflammation. In agreement with this hypothesis, the expression of several key factors involved in NOD1 or IFN-γ signalling pathways were found to be significantly upregulated in gastric biopsies from patients infected with *H. pylori*, particularly in those exhibiting either severe gastritis or tumour formation.

Taken together, the findings demonstrate a mechanism through which virulent strains of *H. pylori* are able to exacerbate inflammatory responses via activation of NOD1-dependent signalling pathways other than those previously reported. Furthermore, the recruitment of immune cells to the gastric mucosa may enhance epithelial responses in a *cag*PAI-dependent manner, offering insight into how virulent *H. pylori* isolates perpetuate the cycle of chronic inflammation. This work has identified a number of novel signalling and inflammatory responses not previously reported for *H. pylori* or associated gastric pathologies, which will form the basis for exciting future research.

List of Figures

List of Tables

TABLE OF CONTENTS

CHAPTER 1. Introduction

1.1 Colonisation

Helicobacter pylori is a spiral-shaped Gram-negative pathogen that resides in the harsh environment of the human stomach. Although there is limited knowledge available describing how individuals are initially colonised, the infection is generally acquired during childhood (1). Transmission of *H. pylori* is thought to occur primarily through person to person contact via the oral-oral, gastric–oral and/or possibly faecal–oral routes, with sanitation and hygiene practices influencing the incidence of infection (2). Developing countries tend to be associated with an elevated prevalence of *H. pylori* infections than developed countries, where the incidence of infection is steadily declining (3). There is, however, substantial variation in the carriage rates of *H. pylori* within developed nations (3), which is partially attributed to differences in hygiene practices, exposure to antimicrobials and ethnic/genetic predisposition to infection (2).

Once in the stomach, *H. pylori* produces large quantities of urease, which hydrolyses urea to produce carbon dioxide and ammonia, thus neutralising the surrounding pH and protecting the bacteria from the harsh acidic environment (4). This is a crucial step in the initial stages of colonisation that protects *H. pylori* whilst it traverses from the highly acidic lumen to the more neutral gastric mucosa, where the bacteria interact with the host epithelium. Motility is facilitated by unipolar flagella, which enable *H. pylori* to penetrate the mucus layer and persist in the dynamic gastric mucosa. Both urease- (5-7) and flagella-deficient (8-9) *H. pylori* mutants are unable to colonise the stomach, highlighting how well this pathogen has adapted to such a specialised niche.

1.2 Adherence

H. pylori express a number of proteins belonging to the "*H. pylori* outer membrane porins" (Hop) family. Many of these proteins act as adhesins that enable bacterial attachment to the gastric epithelium (10-11) and in some cases, promote inflammatory responses and influence

disease outcome. There is inter-strain variation in the distribution of certain adhesins and a remarkable capacity to regulate expression in response to environmental cues (12-16). In particular, sialic acid-binding adhesin (SabA) expression is highly variable and correlates inversely with low pH (16). Furthermore, SabA expression is associated with gastric cancer (16) and given its ability to bind and activate neutrophils (17), SabA may perpetuate the chronic inflammation that precedes such malignancies. Another highly variable adhesin is blood group antigen-binding adhesin A (BabA), which has been shown to undergo continual genetic shuffling with Hop paralogues (18), thus creating substantial variation amongst *babA* orthologues between different strains. Interestingly, BabA expression was found to be lost in *H. pylori* isolates recovered following long-term infection in a number of animal models (14, 19). This may be due to a reduction in the expression of the host BabA receptor as disease progresses (20), highlighting how *H. pylori* can evolve with the dynamic gastric environment during chronic infection.

1.3 Virulence Factors

H. pylori possesses a number of virulence factors that enhance disease by either directly causing localised cell damage or by exacerbating the host immune response (Table 1.1). While many factors may also serve to attenuate host immune responses, the presence of certain bacterial factors is known to be associated with the development of severe disease.

1.3.1 *cag* Pathogenicity Island (*cag*PAI)

The "*cag* PAthogenicity Island" (*cag*PAI) is found in some *H. pylori* strains and encodes a ~40 kb region of DNA comprising 27-31 genes (21-23). Many of these genes are required for the assembly of a Type IV secretion system (T4SS) that secretes the bacterial effectors, CagA and peptidoglycan, into the host cytoplasm (24-26). It is estimated that approximately 60% of *H. pylori* isolates from Western countries and virtually all East Asian isolates possess the *cag*PAI (27). The presence of the *cag*PAI is the most reliable bacterial indicator of disease outcome, strongly correlating with increased bacterial density (28-29), inflammation, and as a result, severe disease such as peptic/gastric ulcer, atrophic gastritis and gastric cancer (30-38).

Virulence Factor	Colonisation/Cell damage	Inflammation/Immunity	Disease Outcome
*cag*PAI	- increased bacterial density	- increased production of cytokines and antimicrobials - infiltration of immune cells	- gastritis - peptic ulcer - gastric cancer
CagA	- disruption of epithelial junctions - epithelial motility and scattering	- cytokine production? - upregulation of oncogenic proteins	- atrophic gastritis - gastric cancer
Urease	- colonisation - cellular injury (ammonia) - disruption of tight junctions	- activation and recruitment of monocytes and neutrophils - sequestration of IgA antibodies - inhibition of phagocytosis	- gastric damage
VacA	- colonisation? - epithelial permeability - epithelial erosion and necrosis - vacuolation - apoptosis	- disruption of antigen processing - inhibition of T-cell proliferation	- ulceration
OipA	- adherence to epithelial cells - colonisation?	- cytokine production?	- dysplasia - gastric cancer - duodenal ulcer
Hp-NAP	- adherence to host proteins - resistance to iron oxidation	- activation and recruitment of monocytes, DCs and neutrophils - ↑ monocyte and neutrophil survival - cytokine production - polarization of Th1 immune responses	- gastric cancer? - peptic ulcer?

Table 1.1 Association of *H. pylori* virulence factors with host responses and disease outcome.

1.3.2 Cytotoxin-associated gene A (CagA)

The *cagA* gene is located within the *cag*PAI (21) and encodes a 120-140 kDa protein (39-40) that is translocated into host cells by the T4SS. Upon entry, CagA multimerises (41) and is subsequently tyrosine phosphorylated by host Src and Abl kinases at multiple residues known as "EPIYA" motifs (25-26, 42). CagA phosphorylation facilitates its interaction with a number of cytoplasmic host proteins, thus inducing aberrant signalling events and influencing pathways that affect proliferation, chemokine production, cytoskeletal rearrangement and epithelial integrity (43). The EPIYA motifs are encoded within the 3´ region of the *cagA* gene, which is highly variable and enables the classification of Eastern and Western isolates based on the flanking DNA sequences. There are four distinct EPIYA variants: EPIYA-A, -B, -C and –D. Representative Western isolates generally possess a copy of EPIYA-A, –B and –C, whilst Eastern isolates possess EPIYA-A, -B, and –D (44). EPIYA-A and –B bind and activate the host "C-terminal Src kinase" (Csk), which inhibits phosphorylation of the CagA protein and thus negatively regulates CagA-induced signalling (45-47). Phosphorylated EPIYA-C from Western and EPIYA-D from East Asian isolates bind and activate a host cytoplasmic phosphatase, "Src homology 2-containing tyrosine phosphatase" (SHP-2) (44, 48), which is largely responsible for the disruption of host signalling pathways. The flanking sequence of EPIYA-D binds SHP-2 with greater affinity than that of EPIYA-C (44-45) and as such, East Asian *cagA*$^{+}$ isolates correlate more strongly with severe disease than Western *cagA*$^{+}$ isolates (49-53). Certain Western strains, however, may produce a CagA with multiple copies of the EPIYA-C segment and these isolates are generally more virulent than those with only one copy (44, 49, 54). Regardless, CagA containing a EPIYA-D motif is considered to possess the greatest potential for inducing aberrant epithelial cell signalling (44).

CagA has been consistently shown to induce cytoskeletal rearrangements *in vitro* (55-57), interfering with epithelial tight-junction proteins, such as Zonular Occludin-1 (ZO-1) and the Junction Adhesion Molecule (JAM), thus resulting in a loss of barrier function (58). Epithelial cells transiently expressing CagA were shown to lose polarity, acquire the ability to degrade the basement membrane and to invade the extracellular matrix (59). In addition, *cag*PAI^{+} *H. pylori* strains upregulate host expression of matrix metalloproteinases (MMPs) (60-63), which can further degrade the extracellular matrix and are often associated with malignant metastasis of a number of cancers (64).While *cag*PAI^{-} *H. pylori* strains are

capable of inducing host cell motility (55), this occurs without concomitant MMP production by epithelial cells. Furthermore, CagA has been reported to promote proliferative and anti-apoptotic gene regulation via activation of serum response elements (65), growth factor receptors and signalling molecules (66-68). Indeed, studies have found elevated proliferation scores in the stomachs of patients infected with *cagA*$^{+}$ *H. pylori*, as compared to those individuals infected with *cagA*$^{-}$ strains (69-70). Interestingly, CagA^{-} isolates were found to induce higher levels of apoptosis, whereas CagA^{+} strains did not (69-70). Therefore, the uncontrolled cellular growth induced by CagA^{+} *H. pylori*, in combination with a disruption in epithelial integrity and elevated expression of MMPs, may potentiate pre-cancerous changes in the stomach and partly explain the elevated incidence of gastric adenocarcinoma in individuals infected with CagA^{+} *H. pylori*.

1.3.3 Vacuolating cytotoxin A (VacA)

The cytotoxic effects of "vacuolating cytotoxin A" (VacA) were initially thought to be induced by CagA, until disruption of the *cagA* gene was found to have no effect on the vacuolating activity of *H. pylori* on host cells (71). Subsequently, the *vacA* gene was identified and its disruption was shown to ablate these cytotoxic effects (72). Early confusion regarding the function of CagA may be attributed to the strong correlation between the presence of the most active forms of VacA and the presence of the *cag*PAI, which is associated with an increased risk of severe disease (28, 53, 73-75).

The *vacA* gene encodes a 140 kDa autotransporter pre-protoxin that is proteolytically cleaved to produce the secreted ~88 kDa mature VacA toxin (76-77), which is activated in the extracellular milieu by an acidic or alkaline pH (78-79). Although all *H. pylori* isolates possess a copy of the *vacA* gene, not all strains secrete cytolytically active toxin (80) and allelic variation within *vacA* amongst different strains markedly affects toxin potency (72, 74). Polymorphic variations in the *vacA* gene enable classification based on three distinct variable regions: signal (s1a, s1b and s2), intermediate (i1 and i2) and middle (m1 and m2). Allelic variants tend to cluster into specific subsets, such as s1/i1/m1 and s2/i2/m2, with a *vacA* s1/i1/m1 type being associated with more severe disease (81). The s2 alleles encode an altered signal peptide that prevents toxin secretion and therefore renders VacA nontoxic (74). Compared to VacA m1, m2 variants are able to act on fewer types of epithelial cell lines, due to altered receptor-binding capabilities (82). VacA m2 variants, however, retain

their ability to bind and induce cytotoxicity in primary gastric epithelial cells (82), indicating that there may not be a difference in clinical outcome between infections with VacA m1 or a VacA m2 strains. The intermediate region was described only recently and shown to be responsible for the cytotoxic activity of VacA (81). Therefore, the i1 allele is thought to be the best indicator of disease outcome, correlating strongly with the development of peptic ulcer, duodenal ulcer and gastric cancer (81, 83).

VacA has been reported to bind a number of different host cell receptors including receptor protein tyrosine phosphatase (RPTP) α and β (79, 84-85), the epidermal growth factor receptor (EGFR) (86) and lipid raft-associated receptors (87-88). Interestingly, mice deficient in RPTPβ were found to be resistant to the formation of gastric ulcers following oral administration of purified VacA (89). It appears that VacA may bind a number of receptors within lipid rafts, which results in endocytosis of the toxin into the host cell (87-88, 90). Intoxicated cells typically contain characteristic vacuoles, which are fusions of late endosomal and early lysosomal compartments (91). Due to its ability to bind a number of cell types, VacA not only inflicts direct cell damage, but can also interfere with many processes that are involved in inflammatory and immune responses. Specifically, VacA induces apoptosis (92-93) and disrupts essential cellular functions such as protein degradation (94) and the processing of extracellular ligands in antigen presenting cells (95). Furthermore, several reports have demonstrated that VacA directly inhibits the activation and proliferation of human B cells, as well as both $CD4^+$ and $CD8^+$ T cells (96-99). Finally, VacA has been shown to increase the permeability of polarised epithelial cell monolayers to low molecular weight molecules and ions (100), which may be a mechanism employed by *H. pylori* to acquire nutrients in the harsh gastric environment.

1.3.4 Outer-membrane Inflammatory Protein (OipA)

OipA acts as an adhesin that facilitates bacterial attachment to gastric epithelial cells *in vitro* (101) and was reported to be important for *H. pylori* colonisation in the Mongolian gerbil model (102). In contrast, another study reported that an *H. pylori ΔoipA* mutant was able to colonise guinea pigs to the same extent as the parental strain (103). While the *oipA* gene is found in all strains, isolates may possess variants that have either been switched "on" or "off" via slip-strand mispairing (16), thereby implying that *oipA* expression is not required by all isolates to efficiently colonise the host. There is a strong correlation, however,

between the presence of an “on” *oipA* gene and other virulence factors, such as CagA and the potent VacA variant (101). This may explain why the presence of a functional *oipA* gene also correlates with high bacterial density, neutrophil infiltration, severe gastritis and gastric cancer (16, 104). Although Yamaoka and colleagues reported a role for OipA in pro-inflammatory cytokine production (15-16, 104-107), other groups have been unable to replicate this finding (101-102, 108-110). Elucidation of the cognate host receptor for OipA may provide insights into the mechanism of action of this protein.

1.3.5 *H. pylori* neutrophil-activating protein (Hp-NAP)

"*H. pylori*-Neutrophil Activating Protein" (Hp-NAP) is a highly immunogenic ~150 kDa protein that acts as a potent chemoattractant for monocytes and neutrophils (111-112), and promotes their adhesion to endothelial cells (112). The adhesive properties of Hp-NAP can also facilitate bacterial binding to mucin and other host proteins (113-114), which may assist the persistence of *H. pylori* in the stomach. In addition, Hp-NAP stimulates the release of "reactive oxygen species" (ROS) from neutrophils (115), and induces the production of pro-inflammatory cytokines by immune cells (111-112). These reports highlight how *H. pylori* can directly recruit immune cells to the site of infection and facilitate their persistence, thereby exacerbating inflammation.

1.3.6 Outer Membrane Vesicles (OMVs)

"Outer Membrane Vesicles" (OMVs) are released during growth *in vitro* and *in vivo* by all Gram-negative organisms (116), including *H. pylori* (117). Given that *cag*PAI^{-} *H. pylori* strains are capable of causing gastritis and severe disease, albeit less often than *cag*PAI^{+} strains (34), OMVs may be another mechanism through which strains lacking a T4SS can induce immune responses. Indeed, *H. pylori* OMVs contain cell wall components and biologically active VacA that is capable of inducing vacuolation (117-119). These vesicles were also shown to inhibit cellular proliferation at low doses and to induce growth cycle arrest and apoptosis at higher doses, an effect found to be independent of VacA (118, 120). Considering that approximately only 2% of *H. pylori* are in contact with the gastric epithelium at any one time (121), OMVs may serve to sustain inflammatory responses without the need for intimate association between bacteria and host cells.

1.4 Innate immune recognition of H. pylori

1.4.1 Pathogen Recognition Molecules (PRMs)

Innate immune responses to pathogenic organisms are largely dependent on the recognition of invariant microbial structures known as "pathogen-associated molecular patterns" (PAMPs) (122). A number of "pathogen recognition molecules" (PRMs) have been identified and shown to respond to a wide variety of PAMPs, resulting in the initiation of potent signalling cascades that culminates in pro-inflammatory cytokine production and the recruitment and activation of immune cells (122). In this way, PRMs are crucial for the development of specific adaptive immune responses that result in pathogen clearance. Epithelial cells are often the first point of contact for pathogenic organisms and both "Toll-like Receptors" (TLRs) (123) and "Nucleotide Oligomerisation Domain" (NOD) receptors (124-125) have been shown to play important roles in the initiation of host responses by these cells and a number of other cell types (122) (Table 1.2; Figure 1.1).

1.4.2 Toll-like Receptors (TLRs)

Ten TLR molecules have been identified in humans whereas 12 have been found in mice. Nevertheless with highly conserved pattern recognition and associated responses between both species (126). With respect to bacterial recognition, TLR2 senses a range of molecules, including lipoproteins from Gram-negative organisms (127-128), whereas TLR4 generally recognises Gram-negative lipopolysaccharide (LPS) (129-130). In addition, TLR5 responds to flagella (131) and TLR9 recognises un-methylated DNA motifs that are frequently present in bacteria (132). TLR2, 4, 5 and 9 all utilise the adaptor molecule "Myeloid Differentiation primary response protein 88" (MyD88), which recruits "Interleukin-1 Receptor-associated kinases" (IRAKs) (133-136). IRAK interacts with "Tumour Necrosis Factor (TNF) Receptor-Associated Factor" 6 (TRAF6) to induce the activation of "transforming growth factor-β (TGFβ)-associated kinase" (TAK1), which in turn can activate "Mitogen-Activated Protein Kinases" (MAPKs), "Activating Protein 1" (AP-1) and "Nuclear Factor-kappa B" (NF-κB) (122, 137-139). These responses, however, are dependent on both the type and cellular context of stimulation.

1.4.3 Nucleotide Oligomerization Domain (NOD) Receptors

Bacterial peptidoglycan is sensed by host cytosolic NOD proteins, of which NOD1 recognises a distinct diaminopimelate-containg tripeptide motif of Gram-negative peptidoglcyan (140), whereas NOD2 recognises muramyl dipeptide motifs common to both Gram-positive and -negative peptidoglycan (141-142). While NOD1 is broadly expressed in a wide range of tissues, NOD2 expression is generally restricted to monocytic cells (Ogura, JBC, 2001). Upon recognition of peptidoglycan, NOD1 or NOD2 associate with "Receptor Interacting Caspase-like Kinase" (RICK) (124, 143-144), which leads to the activation of IκB kinase (IKK) and subsequent IκB degradation (124-125, 143), thus allowing the translocation of NF-κB from the cytoplasm to the nucleus. The precise pathway and intermediate signalling molecules, however, have yet to be elucidated.

1.4.4 Transcription factor activation by *H. pylori*

Colonisation of the gastric mucosa by *H. pylori* results in the initiation of a robust innate immune response and the production of pro-inflammatory molecules that are involved in the direct killing of *H. pylori* and/or the recruitment of immune cells to the site of infection (145). In particular, *cag*PAI$^+$ *H. pylori* are potent activators of the transcription factors, NF-κB (146-147) and AP-1 (148-149), which are crucial links between pathogen recognition and cytokine production. In particular, *cag*PAI$^+$ *H. pylori* are able to induce IκBα degradation, which releases sequestered NF-κB and allows its nuclear translocation (150-151). AP-1 is induced via the activation of host MAPKs, p38, c-Jun N-terminal kinase (JNK) and extracellular signal-related kinase (ERK)(152). Interestingly, aside from ERK MAPK, p38 and JNK are activated only by *cag*PAI$^+$ *H. pylori* (149, 153-154), though the precise pathway(s) is(are) unclear. Asides from the importance of the T4SS for transcription factor activation, there are conflicting reports regarding the ability of other *H. pylori* virulence factors to induce pro-inflammatory signalling cascades in epithelial cells. For example, while some groups have reported that CagA can induce NF-κB (109, 155) and AP-1 activation (148, 156), CagA is generally considered to be dispensable for cytokine responses induced by these transcription factors (24, 65, 157-160). Discrepancies in the reported functions of CagA may highlight strain-specific variations in the virulence and function(s) of this protein amongst different *H. pylori* isolates, or even differences in the expression levels of host molecules within epithelial cell lines.

Host Receptor	Ligand	*H. pylori* factor	Cell type	Cell-specific response
NOD1	Gram negative bacterial peptidoglycan	Peptidoglycan	Epithelial cells	NF-κB and AP-1 activation Cytokine production Antimicrobials Polarization of Th1 immune responses
			Dendritic cells	IL-17 production
TLR2	Multiple bacterial lipoproteins and glycolipids	*H. pylori* LPS?	Epithelial cells	NF-κB activation Cytokine production
		Hp-NAP	Monocytes	NF-κB activation Cytokine production
			Dendritic cells	Cytokine production Th1 polarization
TLR4	Bacterial LPS	*H. pylori* LPS?	Macrophages ?	Cytokine production
TLR5	Bacterial flagella	Non-responsive to *H. pylori* flagella	Non-responsive	Non-responsive
TLR8 / TLR13	Single-stranded RNA?	*H. pylori* RNA?	Dendritic cells?	No Data
TLR9	Bacterial DNA	*H. pylori* DNA	Dendritic cells	Cytokine production
RIG-I	Viral RNA	*H. pylori* RNA	Epithelial cells	Interferon-β others?

Table 1.2 Role of pathogen recognition molecules (PRMs) in detecting *H. pylori*.

1.4.5 NOD1-dependent recognition of *H. pylori*

Viala *et al.* found that NOD1 was required for NF-κB activation by *cag*PAI$^+$ *H. pylori*. Specifically, they demonstrated that NOD1 recognised *H. pylori* peptidoglycan, which was translocated into host epithelial cells via the T4SS. (24). Furthermore, NOD1 knock-out mice had increased bacterial loads and diminished pro-inflammatory cytokine and Th1 immune responses upon *H. pylori* challenge (24, 161). These data indicated that NOD1 may play a critical role in driving innate and adaptive immune responses to infection by *cag*PAI$^+$ *H. pylori* strains. In contrast, Hirata *et al*. reported that in epithelial cells, *H. pylori* activated NF-κB independently of either NOD1 or RICK and instead induced signalling via the critical TLR adaptor molecule, MyD88 (162). The reason for this discrepancy is unclear, as epithelial cell lines are relatively non-responsive to TLR-2 and -4 activation by live *H. pylori* bacteria, due to defective receptor signalling (163).

1.4.6 TLR-dependent recognition of *H. pylori*

The ability of TLRs to recognise *H. pylori* and activate inflammatory pathways in epithelial cells is controversial. *H. pylori* LPS is substantially less endotoxic and immunogenic than the LPS molecules of other Gram-negative pathogens (164-167) and studies are conflicted over whether it is recognised in gastric epithelial cells by TLR4 (168-172) or unconventionally, by TLR2 (173-176). Regardless, *H. pylori* LPS is a weak activator of epithelial signalling and live bacteria are still capable of inducing robust pro-inflammatory responses, even in the absence of TLR2 and TLR4 signalling in these cells (24, 140, 151, 177).

Despite having minimal effects on gastric epithelial cells, some studies have reported that *H. pylori* LPS can be recognised via TLR4 in monocytes and macrophages (169, 172), whereas TLR2 is thought to be responsible for macrophage recognition of live *H. pylori* bacteria (172). Indeed, Hp-NAP, which is likely released upon bacterial lysis (113), was shown to stimulate TLR2-transfected HEK293 cells (111). Furthermore, the treatment of murine splenocyte preparations with Hp-NAP induced robust cytokine responses (178). In contrast, Obonyo *et al,*. reported that in addition to TLR2 and MyD88, TLR4 was in fact essential for cytokine responses to *H. pylori*-infected macrophages (179). Despite the element of cell-specificity regarding TLR-dependent responses to *H. pylori*, MyD88 is

undoubtedly a critical mediator of host responses, as knock-out mice had an elevated bacterial burden and impaired adaptive immune responses during *H. pylori* infection (180).

Another key TLR molecule involved in host defence at mucosal surfaces is TLR5, which responds to bacterial flagella (131) and is expressed in many gastric epithelial cell lines (163, 174, 181). *H. pylori* flagellin, however, fails to induce gastric epithelial responses (174, 181-182), as it lacks the consensus amino acid motifs that are critical for recognition by TLR5 (183). Indeed, flagellin-responsive epithelial cell lines are not responsive to purified *H. pylori* flagella (181-182). Furthermore, isogenic *H. pylori* mutants, lacking FlaA and/or FlaB proteins, retained their ability to induce "Interleukin-8" (IL-8) production by gastric epithelial cells (181), whereas transfection of a dominant-negative TLR5 receptor into epithelial cells only partially diminished epithelial responses to *H. pylori* (174). As mentioned previously, *H. pylori* flagella mutants are unable to colonise the stomach, suggesting that *H. pylori* may have evolved to express immunologically inert flagella in order to efficiently colonise the host without inducing excessive inflammation. Interestingly, this characteristic appears to have been conserved across other *Helicobacter* species and members of the *Campylobacterales* (184) (185) (182-183).

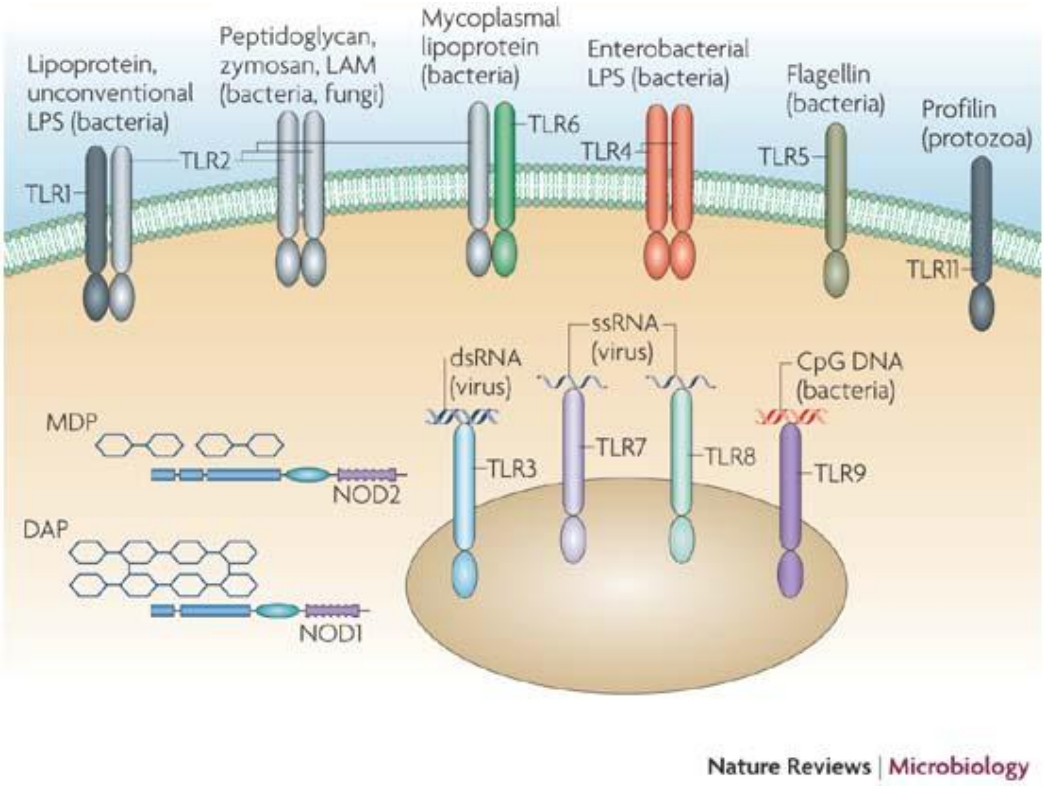

Figure 1.1 TLR and NOD receptor family

1.4.7 Signal Transducers and Activators of Transcription (STATs)

Signal Transducers and Activators of Transcription (STATs) are a family of 7 transcription factors that exist latently in the cytoplasm, which can associate with self or other STAT proteins upon phosphorylation via reciprocal phosphotyrosine-SRC homology 2 (SH2) domain interactions (186). These homo- or hetero-dimers translocate to the nucleus to regulate the expression of a wide variety of genes (187). STATs 2, 4 and 6 have limited activation stimuli, whereas STATs 1, 3 5a and 5b are responsive to a wider array of signals (188). Paradoxically, whilst certain STAT members can exert opposing effects on each other, in some instances they can unite as hetero-dimers, imparting an elevated degree of complexity and specificity on signalling responses (189-190).

Until now, few studies have investigated the role of STAT proteins during *H. pylori* infection and as such, the specific role of these transcription factors in epithelial responses to the pathogen remain unclear. With respect to STAT1, one group reported that *H. pylori* induces STAT1 tyrosine phosphorylation in gastric epithelial cells via OipA (191), whilst another study found that *H. pylori* actually inhibits IFN-γ-induced STAT1 activation and subsequent gene expression in a VacA-, CagA- and *cag*PAI-independent manner (192). A similar *H. pylori*-dependent inhibitory effect on IL-4-induced STAT6 activation in epithelial cells was also reported (193). Although no studies to date have investigated the role of STAT3 responses during *H. pylori* infection, a recent report found that transgenic mice with hyperactivated STAT3 spontaneously developed gastric tumours (194). The anti-apoptotic and pro-proliferative effects of STAT3 and the anti-tumourigenic properties of STAT1 (190), warrant further investigation into the role of these transcription factors in host responses to *H. pylori* and the development of associated malignancies.

1.5 Innate immune responses to H. pylori

1.5.1 Secretion of pro-inflammatory factors

Studies have consistently shown elevated levels of Interleukin-1β (IL−1β), Interleukin-2 (IL-2), Interleukin-6 (IL-6), IL-8, Growth-related oncogene-α (Gro-α) and tumour necrosis

factor-α (TNF-α) in the gastric mucosa of *H. pylori*-infected individuals (195-199). IL-1, IL-8 and Gro-α expression in particular, are generally higher in patients infected with *cag*PAI$^+$ *H. pylori* (199-200). Furthermore, *cag*PAI$^+$ *H. pylori* bacteria have been shown to induce epithelial cell secretion of chemokines such as IL-8 (160, 201), Gro-α (202), "monocyte chemotactic protein-1" (MCP-1) (203) as well as the chemokine, "Regulated upon Activation, Normal T cell Expressed and Secreted" (RANTES) (204). Many of these chemokines/cytokines have overlapping functions during *H. pylori* infection, which include apoptosis, proliferation, recruitment, activation and differentiation of lymphocytes, neutrophils, macrophages and DCs (145).

1.5.2 Antimicrobial molecules

The expression of a number of host antimicrobial compounds is upregulated in the gastric mucosa of *H. pylori* infected individuals. These include the neutrophil-derived alpha-defensins 1, 2 and 3 (205-206) and the epithelial-derived peptides, LL37/hCAP18 (207) and human β defensins (hβDs; (208)). hβDs are potent antimicrobial peptides and both hβD2 and hβD3 have been demonstrated to exert potent microbicidal action against *H. pylori* (209-211). *H. pylori* upregulates LL37 (207), hβD2 and hβD4 expression in a *cag*PAI-dependent manner in gastric epithelial cells (212), whereas, hβD3 expression is upregulated independently of the *cag*PAI (212-213). The production of these potent anti-microbial peptides may assist the host in controlling *H. pylori* infection by attacking extracellular bacteria that are not easily reached by immune cells.

1.5.3 Infiltration of immune cells

The interaction of *H. pylori* with the epithelium results in the activation and rapid recruitment of monocytes and neutrophils to the site of infection (164, 196, 214-219). This is partially attributed to the direct chemotactic activity of virulence factors, such as urease (216) and Hp-NAP (112, 115), which are expressed by all *H. pylori* isolates. As mentioned previously, T4SS effector translocation into gastric epithelial cells by *cag*PAI$^+$ *H. pylori* results in the secretion of chemokines (24, 160, 201) and the further recruitment of immune cells. As a result, *cag*PAI$^+$ *H. pylori* strains are generally associated with more severe gastritis than infection with *cag*PAI$^-$ isolates (220). Neutrophils, macrophages and DCs that

are recruited to the gastric mucosa perpetuate the inflammatory cycle by sampling antigens and secreting additional pro-inflammatory cytokines. It is important to note that inflammatory cell infiltration and corresponding gastritis is minimal in *H. pylori*-infected SCID mice (221) and the adoptive transfer of $CD4^+$ T-cells is capable of restoring the phenotype in these animals (222). These data indicate that the recruitment of inflammatory mediators to the site of infection is a largely T-cell mediated event.

1.5.4 Disease-associated polymorphisms

A number of studies have investigated the contribution of host genetic polymorphisms to the development of *H. pylori*-induced morbidity. Certain pro-inflammatory polymorphisms in the Interleukin-1 (IL-1) gene cluster, encoding IL-1α, IL-1β and their receptor, IL-1Ra, have been demonstrated to influence disease outcome (223). IL-1β polymorphisms were shown to be associated with a significantly increased risk of developing precancerous abnormalities (224) and intestinal and diffuse-type (non-cardia) gastric cancer in individuals with *H. pylori* infection (224-225). An even stronger correlation was noted if the patient was infected with a *cagA*$^+$ or VacA s1/m1 *H. pylori* strain (226). IL-1β is a potent inhibitor of gastric acid secretion (227) and hypochlorhydria is a hallmark of non-cardia gastric cancer (223). Indeed, transgenic mice expressing human IL-1β in the stomach were shown to spontaneously develop gastritis and carcinomas, an effect that was exacerbated by concomitant *Helicobacter felis* infection (228). Pro-inflammatory polymorphisms in the genes encoding TNF-α, Interleukin-10 (IL-10) and TLR4 have also all been linked to an elevated risk of non-cardia gastric cancer (225, 229-230). In addition, a common polymorphism in the *CXCL8* (IL-8) gene promoter, which results in enhanced mucosal IL-8 production, has been linked to an increased risk of severe gastritis and pre-malignant changes (231). The analysis of genetic polymorphisms in inflammatory molecules, which are associated with an elevated risk of developing severe disease, will continue to provide novel insights into the roles of different factors in host responses to *H. pylori* infection.

1.6 Adaptive Immunity

Infection with *H. pylori* results in the development of vigorous humoral and cell-mediated adaptive immune responses. Nevertheless, in most cases these responses appear to be relatively ineffective in eliminating the pathogen from the gastric mucosa (232). Results from immunisation studies in mice have revealed that the immune response generated during chronic infection differs markedly from responses induced by vaccination (232). This indicates that early events in the course of an *H. pylori* infection may actively skew the development of an effective immune response.

As mentioned previously, certain *H. pylori* virulence factors can either directly or indirectly, via the stimulation of epithelial cells, recruit neutrophils, macrophages and "Dendritic Cells" (DCs) to the gastric mucosa. Neutrophils and macrophages perpetuate non-specific inflammatory responses, whilst DC sampling and process antigens to present to pathogen-specific lymphocytes. The cytokines secreted by antigen presenting cells during lymphocyte priming polarise T-helper (Th) cells into specific functional classes, such as Th1, Th2 and Th17, which secrete particular cytokine profiles and have different roles in *H. pylori* infection (232-233).

1.6.1 T-helper (Th)-1, Th2 and Th17 responses to *H. pylori*

"T-helper" (Th)-1 $CD4^+$ lymphocytes produce a cytokine profile that includes Interferon-gamma (IFN-γ), Interleukin-2 (IL-2) and Interleukin-12 (IL-12) and is associated with cell-mediated immunity, which is important in the protection against intracellular parasites and viral infections (234). Th2 cells produce the cytokines, Interleukin-4 (IL-4), Interleukin-5 (IL-5) and Interleukin-13 (IL-13) and are associated with antibody-driven humoral immunity (234). Early studies investigating the immune responses in *H. pylori*-infected individuals reported a predominately Th1 phenotype, characterised by the gastric infiltration of $CD4^+$ T-helper cells (235-236) and the production of large quantities of IFN-γ and IL-12 (237-240). While there are conflicting reports linking the number of IFN-γ-secreting cells with the severity of gastritis (238, 241), infusion of IFN-γ into mice has been shown to induce pre-cancerous lesions, even without concomitant *H. pylori* infection (242). Likewise, pre-treatment of mice with IL-4 prevented the development of gastritis in mice infected with *Helicobacter felis* (243). Paradoxically, *in vivo* neutralization of IFN-γ was shown to

alleviate *H. felis*-associated gastritis in mice, without affecting vaccine-induced protection (244) and studies employing IFN-γ knock-out mice demonstrated that immunised animals were capable of inducing a protective immune response to *H. pylori* infection (240, 245). In contrast, however, others have reported that IFN-γ is indispensable for vaccine-induced protection (246). Discrepancies between these findings highlights the complexity of adaptive immune responses and the limitations of the Th1/Th2 model, particularly given the identification of additional T-helper subsets. Th17 $CD4^+$ T-cells are one such example and have emerged as important mediators of autoimmune inflammatory disorders (247) and immune responses to extracellular bacteria (248). Th17 $CD4^+$ cells secrete large amounts of IL-17 that can act on a variety of target cells to upregulate the production of pro-inflammatory molecules (249). In this regard, studies of mycobacterial infections in IFN-γ knock-out mice have demonstrated a significant increase in the amount Th17 $CD4^+$ T-cells and associated IL-17 production (250). Likewise, *in vivo* administration of IFN-γ to IFN-γ knock-out mice completely abrogated the artificially enhanced IL-17 and Th17 $CD4^+$ T-cell responses in a model of arthritis (251). While both T-helper subsets are capable of inducing potent inflammatory responses, their antagonistic effects offer some insight into the perplexing results of numerous *H. pylori* immunisation studies. While the precise role of Th17 $CD4^+$ T-cells in host responses to *H. pylori* is currently unclear, IL-17 cytokine levels are elevated during infection (252-253), suggesting that Th1/IFN-γ-driven immunity may not be solely responsible for the gastritis and inflammation associated with *H. pylori* infection.

1.6.3 Regulatory T-cell (T-reg) control of inflammation during *H. pylori* infection

Another confounding factor influencing disease progression is the failure to successfully clear *H. pylori* infection due to ineffective immune responses. While *H. pylori*-treated Dendritic cells (DCs) stimulate IFN-γ production by naïve T-cells, prolonged exposure of DCs to the bacterium was shown to result in impaired cytokine production and an inability to induce sufficient T-cell responses (254). Furthermore, memory T-cells isolated from *H. pylori*-infected patients had impaired proliferation and IFN-γ-secreting potential when re-challenged with *H. pylori* (255-256), despite retaining the ability to respond to stimulation with other antigens. This impaired T cell function was shown to be mediated by the presence of "Regulatory T-cells" (T-regs), the numbers of which are elevated in the gastric mucosa of

H. pylori-infected individuals (257-258). These cells indirectly facilitate the persistence of *H. pylori* in the stomach, by suppressing excessive inflammatory responses in an attempt to protect the host (256, 258). Indeed, there is a negative correlation between gastritis and gastric expression of the T-reg marker, Foxp3 (258-259). Levels of IL-10 and TGF-β are also elevated in the gastric mucosa during *H. pylori* infection (260-261) and these cytokines are known to be important mediators of T-reg function (262-264). T-regs may be a means by which *H. pylori* establishes an inflammatory equilibrium during persistent infection, thereby preventing pathogen clearance and minimising excessive damage to the host. This strategy is probably successful, considering that the proportion of infections that result in severe disease, such as peptic ulcer and gastric adenocarcinoma, are 15 % and 0.5 %-2 % respectively (265).

1.6.4 Antibody responses to *H. pylori*

From acute infection studies, it has been shown that *H. pylori* infection elicits a strong antibody response in the host, with detectable serum Immunoglobulin (Ig)A and IgM antibodies present 2 weeks after infection (266). IgA- and IgM-secreting cells can be found in the gastric mucosa (267) and IgA antibodies are detectable in both the gastric mucosa and gastric juice (32, 266, 268). The role of *H. pylori*-specific antibodies in host immunity or clearance is questionable, however, as IgA-deficient patients do not experience enhanced gastritis or an increased susceptibility to disease (269). Akhiani *et al*. even found that IgA knock-out mice had reduced *H. pylori* colonisation (270), suggesting that IgA antibodies may actually facilitate bacterial persistence, possibly through immune evasion (271-272).

Results from the numerous *Helicobacter* immunisation studies suggest that cell-mediated, rather than humoral immune responses are the most effective in controlling *H. pylori* infection (273). Indeed, both IgA- and antibody- deficient mice achieved the same levels of vaccine-induced protective immunity as wild-type animals (274-277). There are, however, differences in antibody responses induced by live *Helicobacter* and those induced by immunisation. Interesting comparisons were made in a study analysing the antibody profiles of mice infected with *H. felis* and mice immunised with bacterial sonicate. *H. felis* infection induced the proliferation of large numbers of IgA-secreting cells and associated IgA production, which were not seen in immunised animals (278). Instead, immunisation induced

the production of IgG production in both the salivary glands and the gastric mucosa, indicating a specific localised response that was absent in unimmunised mice (278). These results highlight the inability of the host to mount an effective immune response during *Helicbacter* infection and may be one mechanism that enables pathogen persistence.

It is noteworthy that no vaccine to date has achieved sterilising immunity against *H. pylori* and as such, no specific polarised immune response or component can be discounted when considering how infection is best controlled. It is also important to note that almost all of the vaccination studies to date have been performed in mice of the C57BL/6 background, which are more prone to develop a default Th1 response (279) and thus are more likely to rely on cell-mediated immunity to fight infection.

1.7 Aims of this study

H. pylori rapidly activates host signalling pathways upon interaction with gastric epithelial cells. In particular, NOD1-dependent recognition of *cag*PAI^{+} bacteria is responsible for the induction of robust pro-inflammatory responses and profoundly affects the establishment and maintenance of adaptive immune responses. Characterisation of how epithelial cells respond to *H. pylori* early in the acute phase of infection and also to the ongoing stimuli of immune response during chronic infection may provide insight into how the pathogen is able to persist.

In order to ascertain the role of NOD1 and the *cag*PAI in the activation of different inflammatory signalling pathways in epithelial cells, this study employed a number of isogenic *H. pylori* mutants and a specialised gastric epithelial cell line stably expressing siRNA to knock-down NOD1. An *in vitro* time-course co-culture assay was established to assess the kinetics of MAPK activation in these cells, focussing on the involvement of NOD1 and the *cag*PAI. Furthermore, we investigated the role of these factors in the activation and nuclear translocation of the transcription factors, NF-κB and AP-1, in addition to cytokine production.

Although many studies have evaluated the type of adaptive immune response generated during *H. pylori* infection, there is limited information available on how this organism influences epithelial cell responses to ongoing immune stimulation. Therefore, the second major aim of this study was to determine the ability of *H. pylori* to directly activate molecules involved in IFN-γ signalling and to assess the effects of *H. pylori* on IFN-γ-stimulated responses in epithelial cells. *In vitro* co-culture assays were utilised to assess whether NOD1-dependent recognition of *cag*PAI^{+} bacteria could activate such pathways. In addition, this study investigated a potential positive feedback mechanism between IFN-γ- and NOD1-dependent responses during *H. pylori* infection. In this way, the expression patterns of various molecules involved in host responses to *H. pylori* were analysed in gastric biopsies from *H. pylori*-infected and -uninfected patients with different stages of gastritis or gastric cancer.

This work describes for the first time, the downstream signalling events initiated by *cag*PAI^{+} *H. pylori* activation of the NOD1 pathway. Furthermore, the work identifies a molecular link between NOD1 and IFN-γ signalling pathways, thereby providing a novel insight into the mechanisms that facilitate the persistence of chronic and destructive inflammation during *H. pylori* infection.

CHAPTER 2. NOD1 is essential for *cag*PAI-dependent MAPK phosphorylation and AP-1 activation in gastric epithelial cells.

2.1 Summary

Infection with *cag*PAI$^+$ *H. pylori* bacteria results in the rapid initiation of pro-inflammatory cascades in gastric epithelial cells and the secretion of a number of cytokines involved in the recruitment and modulation of immune cells (145). These potent inflammatory responses are known to be induced via the *cag*PAI-encoded T4SS, which secretes the effectors, CagA and peptidoglycan, into the host cell (24-26). The cytosolic pathogen recognition molecule, NOD1, was shown to recognise peptidoglycan delivered into the cell via the T4SS, which in turn led to the activation of NF-κB (24). In that study, however, NF-κB activity was reported using an artificial reporter assay (162). It was thus unclear whether other signalling molecules were involved.

Although the transcription factor, AP-1, is also known to be activated in a *cag*PAI-dependent manner (148-149), no specific mechanism has yet been reported. It was previously reported that MAPKs are essential for *H. pylori*-induced AP-1 activation. Furthermore, both p38 and JNK MAPKs were shown to be phosphorylated only by *cag*PAI$^+$ *H. pylori* bacteria (149, 153). In contrast, ERK MAPK can be phosphorylated by *H. pylori* in a number of different ways, one of which is via CagA (68, 148, 153, 280) (154). Although the role of CagA in NF-κB and AP-1 activation is contentious, it is generally considered to be dispensable for transcription factor activation and cytokine expression in epithelial cells (24, 65, 157-160). CagA is, however, known to induce the formation of characteristic host cell hummingbird-like cytoskeletal rearrangements during *in vitro* stimulation (56, 281). It was reported that phosphorylated ERK (68, 280), in addition to a number of other, as yet unidentified host proteins, are required for CagA effects on the cytoskeleton.

The following series of experiments were thus designed to, firstly, examine the role of NOD1 in NF-κB activation and nuclear translocation. Secondly, we wished to determine

whether NOD1 is required for *cag*PAI-dependent p38 and JNK MAPK, and ultimately, AP-1, activation. Finally, the role of NOD1 and MAPKs in the formation of the CagA-dependent hummingbird phenotype was also assessed.

Declaration for Thesis Chapter 2

Declaration by candidate

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
My contribution entailed the project conceptualisation, experimental design. In addition I performed all experiments, interpreted data and the undertook the majority of writing.	75%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Thomas Kufer	Reagents and experimental assistance	
Elisabeth Kremmer	Reagents	
Maria Kaparakis-Liaskos	Project conceptualisation and data interpretation	
Richard Ferrero	Project conceptualisation, experimental design, data interpretation and manuscript editing	

Candidate's Signature	[redacted]	**Date** 30-9-10

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	

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Signature 1	[redacted]	**Date** 28/9/2010
Signature 2		28/9/2010
Signature 3		29/9/10
Signature 4		29/9/2010

2.2 *H. pylori* activates MAPKs and AP-1 in a NOD1-dependent manner

y

Helicobacter pylori Induces MAPK Phosphorylation and AP-1 Activation via a NOD1-Dependent Mechanism[1]

Cody C. Allison,*† Thomas A. Kufer,‡ Elisabeth Kremmer,§ Maria Kaparakis,*† and Richard L. Ferrero[2]*†

Helicobacter pylori **rapidly activates MAPKs and transcription factors, NF-κB and AP-1, in gastric epithelial cells following host attachment. Activation of these signal transducers is largely dependent on the *cag* pathogenicity island (*cag*PAI)-encoded Type IV Secretion System. *H. pylori* was shown to translocate peptidoglycan through the Type IV Secretion System, which is recognized by the pathogen recognition molecule, NOD1, thus resulting in NF-κB activation. The mechanisms of *H. pylori*-induced MAPK and AP-1 activation, however, are less well defined and therefore, we assessed the contribution of NOD1 to their activation. For this, we used gastric epithelial cell lines, stably expressing siRNA to either *NOD1* or a control gene. In si*NOD1*-expressing cells stimulated with *cag*PAI$^+$ *H. pylori*, we observed significant reductions in p38 and ERK phosphorylation ($p < 0.05$), whereas the levels of Jnk phosphorylation remained unchanged. Consistent with a previous report, however, we were able to demonstrate NOD1-dependent Jnk phosphorylation by the invasive pathogen *Shigella flexneri*, highlighting pathogen-specific host responses to infection. We also show that NOD1 was essential for *H. pylori* induction of not only NF-κB, but also AP-1 activation, implying that NOD1 induces robust proinflammatory responses, in an attempt to rapidly control infection. Pharmacological inhibition of p38 and ERK activity significantly reduced IL-8 production in response to *H. pylori*, further emphasizing the importance of MAPKs in innate immune responses to the pathogen. Thus, for the first time we have shown the important role for NOD1 in MAPK and AP-1 activation in response to *cag*PAI$^+$ *H. pylori*.** ***The Journal of Immunology,*** **2009, 183: 8099–8109.**

Infection with *Helicobacter pylori* is associated with chronic gastritis, which is characterized by infiltration of neutrophils, lymphocytes, and other inflammatory mediators into the gastric mucosa (1–3). This immune cell migration is largely regulated by the production of IL-8 and other proinflammatory chemokines by gastric epithelial cells in response to bacterial attachment (1–4). Strains of *H. pylori* that possess a *cag* pathogenicity island (*cag*PAI)[3] are associated with the production of higher levels of IL-8 and more severe disease than *cag*PAI$^-$ strains (5–8). The *cag*PAI encodes a type IV secretion system (T4SS), through which effectors, such as CagA (9) and peptidoglycan (10), are translocated into the host cell.

The secretion of effectors through the T4SS activates a signaling cascade culminating in the migration of the transcription factor NF-κB to the nucleus and transcription of inflammatory mediators, such as IL-8 and MCP-1 (5, 8, 10–14). *H. pylori* was also reported to rapidly activate another transcription factor, AP-1, in a *cag*PAI-dependent manner (15, 16). AP-1 is activated by MAPKs and is capable of inducing a strong proinflammatory response, often in concert with NF-κB (11). Indeed, early studies demonstrated that chemokine production could be diminished in *H. pylori* stimulated cells that had been pretreated with MAPK inhibitors (13, 17–20).

The signaling events leading to rapid MAPK phosphorylation during *H. pylori* infection are not well understood, although the T4SS is known to be required for complete p38, SAPK/Jnk, and ERK phosphorylation (19, 21). Additional *H. pylori* factors are believed to be involved in MAPK activation. The vacuolating cytotoxin produced by many isolates, VacA, was found to activate p38 and ERK (22, 23), however, these experiments were performed using purified VacA and not with live bacteria. Subsequent studies identified that *H. pylori* Δ*vacA* mutants were able to induce AP-1 transactivation to the same extent as wild-type bacteria (16, 20), implying that VacA is dispensable for AP-1 activity. A second *H. pylori* factor, CagA, encoded by the *cag*PAI, induces ERK phosphorylation via the Ras→Raf→Mek→ERK→NF-κB pathway, which can further augment IL-8 production (24). However, the effects of CagA on IL-8 production are not seen until 24 h after infection (24). Also, ERK can still be induced by *cag*PAI-independent mechanisms (19, 21), though CagA is required for complete AP-1 activation (15, 16), suggesting that CagA plays an additive role in transcription factor activation. Jnk activation during *H. pylori* infection has also been reported to require a functional T4SS (19, 21).

The cytosolic pathogen recognition molecule, nucleotide binding and oligomerization domain 1 (NOD1), recognizes specific conserved motifs found almost exclusively in the cell wall peptidoglycan of Gram-negative bacteria (25, 26). *H. pylori* peptidoglycan is delivered to the host cell via the T4SS, where it is recognized by cytosolic NOD1 (10). Upon stimulation with purified

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[3] Abbreviations used in this paper: *cag*PAI, *cag* pathogenicity island; T4SS, type IV secretion system; NOD1, nucleotide binding and oligomerization domain 1; RICK, receptor-interacting protein serine-threonine kinase 2; CARD, caspase-activation and recruitment domain; EGFP, enhanced GFP; OMV, outer membrane vesicle; DAF, decay-accelerating factor.

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agonist, NOD1 associates with the receptor-interacting protein serine-threonine kinase 2, (RICK), triggering a proinflammatory response, characterized by NF-κB activation and IL-8 production (27, 28). The involvement of RICK in this signaling cascade, however, has not yet been demonstrated during *H. pylori* infection.

In addition to activation of the classical NF-κB pathway, NOD1 was reported to be required for MAPK activation in response to bacterial pathogens. Infection of HEK293 cells with *Shigella flexneri* activates Jnk in a NOD1-dependent manner (29) and NOD1 was shown to be essential for p38 phosphorylation in endothelial cells during *Listeria monocytogenes* infection (30). This NOD1-dependent p38 activation was also found to induce IL-8 production (30). Furthermore, purified NOD1 agonist rapidly induced activation of p38, ERK, and Jnk in murine macrophages (31). Despite the knowledge that NOD1 is clearly capable of activating MAPKs, however, the role of NOD1 in MAPK-dependent inflammatory responses during *H. pylori* infection is unknown.

The mechanisms of *H. pylori*-induced MAPK activation are unclear and though many factors are thought to play a role, most are expendable. To better understand the mechanisms of *H. pylori*-dependent MAPK activation, we examined the requirement for NOD1 in MAPK and AP-1 activation during *H. pylori* infection. We observed that NOD1 was necessary for complete MAPK activation in the early stages of infection and that NOD1 was essential for the activation of both NF-κB and AP-1, as well as the release of proinflammatory cytokines in response to *H. pylori*. Thus, NOD1-dependent MAPK activation may represent an additional signaling pathway through which this pathogen controls the transcription of a subset of novel downstream target genes during infection.

Materials and Methods

Cell culture and reagents

Human adenocarcinoma gastric epithelial cells (AGS) as well as AGS cells stably expressing siRNA to either the caspase-activation and recruitment domain (CARD) of *NOD1* (AGS si*NOD1*) or an irrelevant gene, enhanced GFP, *EGFP* (AGS si*EGFP*), were cultured in RPMI 1640 (Life Technologies) containing 10% FBS (Thermo Electron). The detailed characterization of this cell line will be reported elsewhere (A. Grubman, M. Kaparakis, J. Viala, C. Allison, L. Badea, A. Karrar, I. Boneca, L. Le Bourhis, S. Reeve, I. Smith, et al., manuscript in preparation). The human embryonic kidney cell line, HEK293, was cultured in DMEM (Life Technologies) containing 10% (v/v) FBS. All cell lines were supplemented with 1% (v/v) penicillin-streptomycin (Life Technologies) and 1% (v/v) Glutamax (Life Technologies) and grown at 37°C, with 5% CO_2. Additionally, AGS *siNOD1* and si*EGFP* were supplemented with 400 μg/ml Geneticin (Life Technologies).

For MAPK inhibition experiments, cells were pretreated with pharmacological MAPK inhibitors, SB203580 (p38 inhibitor) and U0126 (ERK inhibitor) (10 μM) (all from Calbiochem) for 1 h before the addition of bacteria. As a positive control, TNF-α (10 ng/ml) (Chemicon) was added to cells for indicated periods of time.

The NOD1-specific mAb was prepared as described previously (32). In brief, Abs were raised in rats immunized with the GST-tagged CARD domain (aa1–126) of NOD1 expressed in bacteria.

Expression vectors and transient transfections

For NF-κB and AP-1 reporter assays, cells were transfected with 300 ng/well of either Ig-κ (29, 33) or AP-1 luciferase (34) and 250 ng/well of dTK renilla plasmid vector (Promega). The total amount of DNA to be transfected was standardized to 1150 ng/well by the addition of pCDNA3 (10). AGS and HEK293 cells were seeded in triplicate in 24-well plates at a concentration of 1×10^5 cells/ml and incubated for 18–24 h. Transfection was achieved using 4 μl of polyethyleneimine (Polysciences) to 1 μg of total DNA. Cells were cultured in a final volume of 1 ml of complete culture medium per well and incubated for 16–24 h at 37°C in 5% CO_2. Following stimulation, cells were lysed by adding 100 μl of Reporter Lysis Buffer (Promega). Sample aliquots (20 μl) were incubated with either coelenterazine renilla (50 μl) (Synchem) or Luciferin substrate (30 μl) (Promega) solutions. Luminescence was measured using a TECAN infinite M200 luminometer (TECAN) and Magellan version 6.0 software.

Vector constructs expressing RICK (29), NOD1 (35), and NOD1 ΔCARD (35) have been described previously. In brief, AGS cells were seeded in 24-well tissue culture plates at a concentration of 1×10^5 cells/ml and incubated for 18–24 h. Cells were transfected with 100 ng/well of NOD1, NOD1 ΔCARD, or RICK. Cells were incubated for 16 h before stimulation with bacteria.

Bacterial strains and isogenic mutants

H. pylori strains 251 (36) and G27 (37) are clinical isolates and isogenic 251 Δ*cag*PAI was constructed by natural transformation (38, 90) and G27 Δ*cagA* and Δ*cagM* mutants were constructed as described previously (10, 39). Bacteria were routinely cultured on blood agar medium, supplemented or not with 10 μg/ml kanamycin, under microaerophilic conditions (40). Liquid broth cultures were incubated overnight at 37°C with shaking at 125 rpm in 25-cm^3 tissue culture flasks (Iwaki) containing 10 ml of brain heart infusion broth (Oxoid) with 10% (v/v) newborn calf serum (Life Technologies).

Wild-type *Shigella flexneri* (M90T serotype 5A) and its noninvasive derivative (BS176) cured of the 220kB virulence plasmid, pWR100, have been described previously (29, 41, 42). *S. flexneri* strains were routinely cultured on Trypticase Soy Agar (BD Biosciences), supplemented with 0.01% (v/v) Congo Red (British Drug Houses), to differentiate between colonies with and without a virulence plasmid. Liquid broth cultures were prepared by inoculating 8 ml of trypticase soy broth with a single colony. Cultures were grown shaking at 160 rpm overnight at 37°C.

Infection of AGS and HEK293 cells with bacteria

Overnight *H. pylori* broths were washed twice in PBS and pelleted at 1250 × *g* for 10 min. Bacterial pellets were resuspended in RPMI 1640 or DMEM and added to AGS or HEK293 cells, respectively, at a multiplicity of infection of 1:10. *H. pylori* was centrifuged onto cells at 200 × *g* for 5 min before incubation for the appropriate times. For IL-8 assays, bacteria were washed off in PBS after 1 h of stimulation and the wells replaced with fresh tissue culture medium followed by further incubation.

For *S. flexneri* coculture experiments, overnight cultures were used to inoculate trypticase soy broth at a 1/100 dilution. Bacteria were grown at 37°C to mid-exponential phase, washed twice in PBS, and then pelleted at 3220 × *g* for 10 min. Pellets were resuspended in RPMI 1640 and used to infect AGS cells at a multiplicity of infection of 1:50. *S. flexneri* was centrifuged onto cells at 200 × *g* for 5 min. Following a 20 min incubation, cells were washed three times with PBS and fresh medium containing gentamicin (50 μg/ml) (Pfizer) was added to wells. This was considered to be time 0 h.

Preparation of H. pylori *outer membrane vesicles (OMVs)*

OMVs were prepared from *H. pylori* 251 Δ*cag*PAI (90). In brief, bacteria were grown in brain heart infusion broth, supplemented with 1.8% (w/v) β-cyclodextrin (Sigma-Aldrich), with shaking at 125 rpm for 16 h. Bacteria were pelleted for 10 min at 3220 × *g* and the culture supernatants vacuum filtered through a 0.22 μM pore stericup filter (Millipore). OMVs were collected by ultra-centrifugation at 100,000 × *g* for 2 h at 4°C and quantified by Bradford protein assay (Bio-Rad).

Detection of MAPK phosphorylation

AGS si*NOD1* and si*EGFP* cells were incubated with *H. pylori* or *S. flexneri* for the appropriate times. Cells were lysed by the addition of 100 μl boiling Laemmli buffer and subjected to SDS-PAGE. Proteins were transferred to 0.4-μm Transblot nitrocellulose membrane (Bio-Rad), followed by membrane blocking using 5% (w/v) skim milk in TBS. Immunodetection of phosphorylated or total MAPKs was performed by incubating membranes with respective anti-p38, -ERK or -Jnk primary Abs (Cell Signaling Technology) at a dilution of 1/1000 in 5% BSA (Roche), prepared in TBST. Secondary goat anti-rabbit (Chemicon/Millipore) Ab was used at a dilution of 1/1000 in 5% skim milk in TBST. Detection of actin was performed using primary actin and secondary goat anti-rabbit (Chemicon) Abs at a dilution of 1/10000 in 5% skim milk in TBST. Western blots were developed using ECL detection reagent (GE Healthcare) and exposed to Super RX film (Fuji).

MAPK densitometry

Densitometry was performed on Western blots to quantify MAPK activation. Blots from three independent biological replicates were analyzed using ImageQuant software (v7.0). Bands representing either phosphorylated p38, Jnk, or ERK MAPKs were standardized to their corresponding actin bands. Samples were ranked and statistical significance determined using a Mann-Whitney *U* test.

Preparation of nuclear extracts and EMSA

AGS si*NOD1* and si*EGFP* cells were grown in six-well tissue culture plates and stimulated with *H. pylori*. Cells were washed twice in PBS and resuspended in ice-cold PBS containing protease (Roche) and phosphatase (Sigma-Aldrich) mixture inhibitors. Cells were lysed in 1× hypotonic buffer (20 mM HEPES (pH 7.9), 0.1 mM EDTA, 0.1 mM EGTA, 10 mM KCl, and 1 mM DTT), supplemented with 1% Nonidet P-40. Nuclear pellets were washed in 1× low-salt buffer (10 mM HEPES (pH 7.9), 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 25% (v/v) glycerol), then resuspended in 1× high-salt buffer (10 mM HEPES (pH 7.9), 400 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 25% (v/v) glycerol), supplemented with 4.5 μl of 1 M NaCl. Nuclear extracts were collected by centrifugation at 15,000 × *g* and protein concentrations determined using the Bradford protein assay. Oligonucleotides to NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) and AP-1 (5′-CGCTTGATGAGTCAGCCGGAA-3′) consensus sequences (Promega) were [γ-^{32}P]dATP-labeled with T4 kinase (Promega). Nuclear extracts (5 μg) were incubated with 5× binding buffer (2.5 mM HEPES (pH 7.8), 50 mM NaCl, 0.5 mM EDTA, 25% (v/v) glycerol), 1 mg/ml poly dI-dC (Sigma-Aldrich) and labeled oligo for 30 min at room temperature. For supershift assays, Abs specific for NF-κB p50 and p65 subunits and to c-Jun, c-Fos, and ribosomal-S6 (all from Cell Signaling Technology), were incubated with nuclear protein extracts on ice for 20 min, then incubated with labeled probes for 30 min at room temperature. The DNA-protein complexes were resolved by electrophoresis using 6% nondenaturing polyacrylamide gels, which were vacuum dried and exposed to x-ray film.

IL-8 ELISA

IL-8 concentrations in cell culture supernatants were quantified by ELISA (BD Biosciences), according to the manufacturer's instructions. The minimum detection limit of the assay was 3.125 pg/ml.

Statistical analysis

The Student *t* test was used for numerical data, whereas the Mann-Whitney *U* test was used for categorical data as appropriate. p values of <0.05 were considered statistically significant.

Results

NOD1 is required for rapid MAPK activation during H. pylori *infection*

Previous studies have reported rapid MAPK activation within epithelial cells in response to *H. pylori* stimulation (19, 21). To determine the kinetics of activation, a time course of MAPK phosphorylation was performed at 15, 30, 45, and 60 min following stimulation with wild-type *H. pylori* and Δ*cagA* and Δ*cagM* mutants on the G27 strain background (37). Experiments were performed using AGS cells that stably express siRNA to either *NOD1* (si*NOD1*) or an irrelevant gene, *EGFP* (si*EGFP*). p38, ERK, and Jnk MAPKs were rapidly phosphorylated in AGS si*EGFP* cells within 30 min of stimulation with *H. pylori* and activation was sustained after 60 min (Fig. 1, *A–F*). Activation of p38, Jnk, and ERK was dependent on a functional type IV secretion system, as stimulation with an *H. pylori* G27 Δ*cagM* mutant, which has a nonfunctional secretion apparatus, induced significantly lower levels of MAPK phosphorylation than wild-type bacteria at the appropriate times (i.e., 30, 45, and 45 min, respectively; $p < 0.05$) (Fig. 1, *A–F*). From experiments with *H. pylori* Δ*cagA* mutant bacteria, it was shown that CagA was required for maximal p38 and ERK phosphorylation in AGS si*EGFP* cells at 30 and 45 min poststimulation, respectively (Fig. 1, *A–F*; supplemental Fig. 1).[4] In contrast, CagA translocation was dispensable for Jnk phosphorylation in these cells (Fig. 1, *C* and *D*).

NOD1 was previously shown to be essential for Jnk activation in response to *S. flexneri* in HEK293 epithelial cells and for p38 activation by *L. monocytogenes* in HUVEC endothelial cells, respectively (29, 30). To assess whether NOD1 was involved in *H. pylori*-induced MAPK activation, we determined p38, ERK, and Jnk phosphorylation in AGS si*NOD1* cells. In all experiments outlined here and below, IL-8 production was measured to indirectly verify a reduction in *NOD1* expression (data not shown). In addition, Western blot analysis was performed using whole cell extracts to confirm that NOD1 synthesis had been "knocked down" in these cells (Fig. 1*G*). Wild-type *H. pylori* G27 bacteria induced maximal p38 activation within 30 min in si*EGFP* cells, whereas parallel stimulation of si*NOD1* cells resulted in significantly reduced p38 phosphorylation ($p < 0.05$) (Fig. 1, *A* and *B*; supplemental Fig. 1). After 45 min, activation in the si*NOD1* cell line was restored to levels seen in the si*EGFP* cells. Similar findings were observed in cells stimulated with *H. pylori* 251 wild-type and mutant strains, thereby excluding possible strain-specific NOD1-dependent p38 activation (supplemental Fig. 2). In the case of ERK, NOD1 appeared to be important for phosphorylation of this MAPK at 45 min poststimulation, but not earlier (Fig. 1, *E* and *F*). Interestingly, *H. pylori* was found to induce Jnk phosphorylation in a NOD1-independent, but T4SS-dependent manner (Fig. 1, *C* and *D*).

NOD1 is required for Jnk phosphorylation in response to S. flexneri, *but not* H. pylori *stimulation*

Previous reports demonstrated that NOD1 is required for rapid Jnk activation following *S. flexneri* infection of HeLa cells (29). As we found that this did not appear to be the case during *H. pylori* stimulation (Fig. 1, *C* and *D*), AGS si*NOD1* cells were cocultured with virulent *S. flexneri* M90T to exclude cell-specific effects. As reported previously in HeLa cells (29), stimulation with *S. flexneri* M90T resulted in rapid Jnk phosphorylation within 15 min in the si*EGFP* cells, however, phosphorylation was substantially diminished in the si*NOD1* cells at both time points examined (Fig. 1*H*). When stimulated with avirulent *Shigella* BS176, Jnk was not activated in either cell line at 15 or 30 min (Fig. 1*H*). As above (Fig. 1, *C* and *D*), wild-type *H. pylori* induced strong Jnk phosphorylation after 30 min, irrespective of whether NOD1 had been knocked down or not (Fig. 1*H*). Jnk phosphorylation in *S. flexneri* M90T-infected si*EGFP* cells was substantially reduced at 30 min, which is consistent with previous findings that *Shigella* actively inhibits host signaling via the action of a phosphothreonine lyase, OspF, which dephosphorylates MAPKs (43). The differences in NOD1 signaling between *H. pylori* and *S. flexneri* may reflect the different mechanisms (bacterial secretion systems vs invasion) used by these bacteria to activate the NOD1 pathway.

Purified H. pylori *OMVs activate MAPKs in a NOD1-dependent manner*

Recent work in our laboratory has shown that *H. pylori* can also induce NOD1 signaling via a *cag*PAI-independent mechanism involving OMVs, which are released during normal bacterial growth (44). Purified *H. pylori* OMVs contain peptidoglycan and enter nonphagocytic cells to induce NOD1-dependent, but TLR-independent, host cell responses in vitro and in vivo (90). Given that *H. pylori* OMVs appear to selectively induce NOD1 signaling in host cells, we therefore wished to determine whether these structures could induce NOD1-dependent MAPK phosphorylation, in a manner analogous to *cag*PAI$^+$ *H. pylori* bacteria. Stimulation of AGS

[4] The online version of this article contains supplemental material.

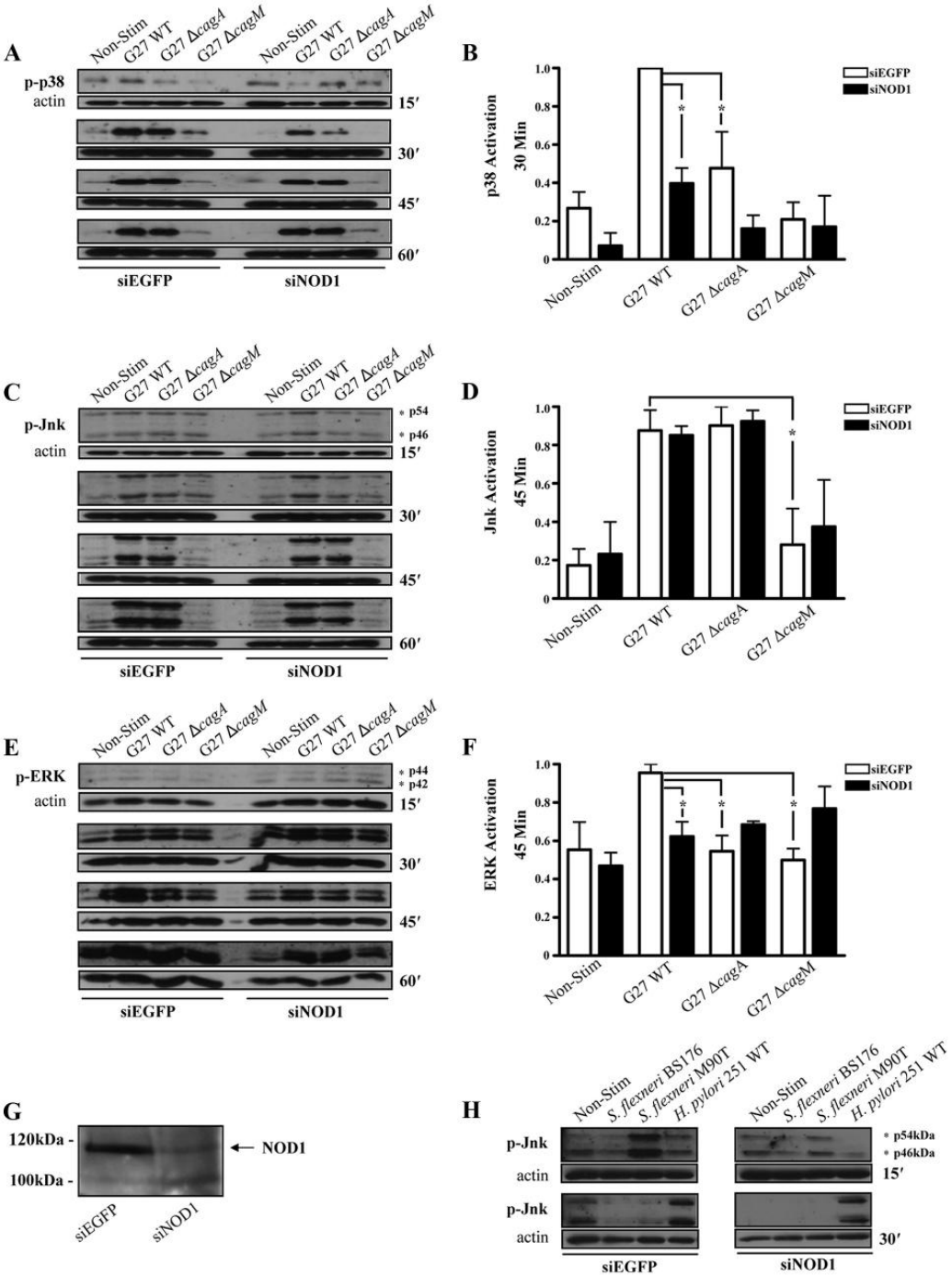

FIGURE 1. NOD1 is required for rapid MAPK activation during *H. pylori* infection. AGS si*EGFP* or si*NOD1* cells were stimulated with wild-type *H. pylori* G27 or the isogenic mutants Δ*cagA* and Δ*cagM* for 15, 30, 45, or 60 min. Cells were lysed in boiling Laemmli buffer and samples analyzed by immunoblot (*A*, *C*, and *E*) and densitometry (*B*, *D*, and *F*) to assess phosphorylation of: p38, Jnk, and ERK. *G*, NOD1 expression was assessed by immunoblot in AGS si*EGFP* and si*NOD1* cells to confirm NOD1 knock down. *H*, AGS si*EGFP* or si*NOD1* cells were stimulated with wild-type *H. pylori* 251, *S. flexneri* M90T or *S. flexneri* BS176 for 15 and 30 min. Cells were lysed in boiling Laemmli buffer and samples analyzed by immunoblot to assess phosphorylation of Jnk MAPK. All membranes were reprobed with anti-actin Abs to ensure equal loadings. Blots are representative of three independent experiments. Statistical analysis of densitometry was performed by normalizing band intensity across replicate experiments. Statistical significance was determined using the Mann-whitney test. *, $p < 0.05$.

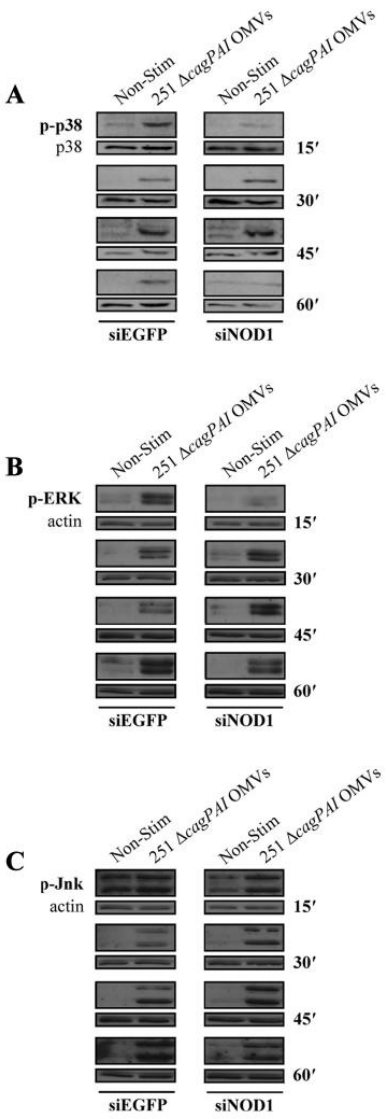

FIGURE 2. Purified *H. pylori* OMVs activate MAPKs in a NOD1-dependent manner. AGS si*EGFP* or si*NOD1* cells were stimulated with *H. pylori* 251 Δ*cag*PAI OMVs for 15, 30, 45, or 60 min. Cells were lysed in boiling Laemmli buffer and samples were analyzed by immunoblot to assess phosphorylation of p38 (*A*), ERK (*B*), and Jnk (*C*) MAPKs. Membranes were re-probed with anti-actin Abs to ensure equal loadings. Blots are representative of two independent experiments.

si*EGFP* cells with OMVs, which had been purified from a Δ*cag*PAI *H. pylori* strain to exclude *cag*PAI-specific effects, induced the rapid phosphorylation of p38, ERK, and Jnk at 15 min poststimulation (Fig. 2). In comparison, reduced levels of p38 and ERK phosphorylation were observed in AGS si*NOD1* cells at this time-point, but not at later time-points (Fig. 2). As observed for live *H. pylori* bacteria, Jnk phosphorylation in response to OMV stimulation occurred as rapidly in si*NOD1* cells and to the same extent as that observed in si*EGFP* cells (Fig. 2*C*). These data demonstrate that *H. pylori* OMVs, which signal via NOD1 (90), are also able to activate p38 and ERK in a NOD1-dependent manner.

NOD1 is required for activation of the transcription factors NF-κB and AP-1

Previous studies demonstrated NOD1-dependent activation of NF-κB using reporter assays (10, 45); however, the nuclear translocation of this transcription factor was not investigated. Additionally, the role of NOD1 in *H. pylori*-induced AP-1 activation has not been investigated before. Thus, EMSA were performed using nuclear extracts isolated from infected cells to confirm the effect of NOD1 on downstream transcription factor activation. Strong NF-κB and AP-1 binding activity was observed in AGS si*EGFP* cells at 2 h poststimulation with wild-type *H. pylori* (Fig. 3, *A–C*). This activity was not observed in nonstimulated cells and was reduced in those stimulated with *H. pylori* Δ*cag*PAI mutant bacteria. Moreover, siRNA inhibition of *NOD1* expression resulted in reduced levels of NF-κB nuclear translocation and completely abrogated AP-1 translocation in response to *cag*PAI$^+$ *H. pylori*. This finding confirms that NOD1-dependent recognition of *H. pylori* directs signaling via the downstream activation and nuclear translocation of both transcription factors.

The transcriptional activity of NF-κB and AP-1 can vary substantially depending on their subunit composition (46–48). To assess the composition of these transcription factors in response to *H. pylori*, supershift assays were performed using nuclear extracts from infected cells. Previous work has demonstrated that *cag*PAI$^+$ *H. pylori* strains induce NF-κB complexes composed of p50/p65 subunits (14, 49). The addition to nuclear extracts of Abs to p50 caused a shift in the NF-κB complexes induced in response to NOD1 signaling (Fig. 3*B*). p65 Abs did not shift the complex, though a substantial decrease in the corresponding NF-κB band can be observed, which is evident of a specific interaction between the Ab and NF-κB complex. The failure to result in a supershift suggests that the binding of the Ab prevented the labeled oligonucleotide from binding to the complex, which has been reported previously for EMSAs (49). We next determined the role of NOD1 in *H. pylori*-induced AP-1 activation. As for *H. pylori*-mediated NF-κB activation, this pathogen was shown to induce the nuclear translocation of AP-1 complexes by a NOD1-/*cag*PAI- dependent mechanism. Supershift assays performed using Abs directed against c-Jun and c-Fos, two known components of the AP-1 complexes induced by *cag*PAI$^+$ *H. pylori* (11, 14), partially shifted the AP-1 complex (Fig. 3*C*). When used together, the c-Jun and c-Fos Abs resulted in a complete shift of this complex (Fig. 3*C*). This indicates that *H. pylori*-induced NOD1 activation induces the formation of AP-1 complexes composed of c-Jun/c-Fos proteins.

To confirm the findings for AP-1 activation by EMSA (Fig. 3*C*), we performed luciferase reporter assays in HEK293 cells. Both *H. pylori* wild-type and Δ*cagA* mutant bacteria induced similar levels of AP-1 reporter activity after 4 h infection (Fig. 3*D*). The *H. pylori* Δ*cagM* mutant, however, was unable to induce AP-1 activation ($p < 0.01$), thus confirming the requirement of a functional T4SS for AP-1 responses to *H. pylori* infection. In agreement with these data, as well as those obtained by EMSA (Fig. 3*C*), *H. pylori*-induced AP-1 reporter activity in AGS cells occurred in a T4SS-/NOD1-dependent manner (Fig. 3*E*). Additionally, si*NOD1* cells stimulated with OMVs produced significantly less AP-1 reporter activity than OMV-stimulated si*EGFP* cells ($p < 0.001$). These results indicate that the relatively subtle and transient influence of NOD1 on MAPK activation has significant effects on the ability of MAPKs to mediate downstream AP-1 activation.

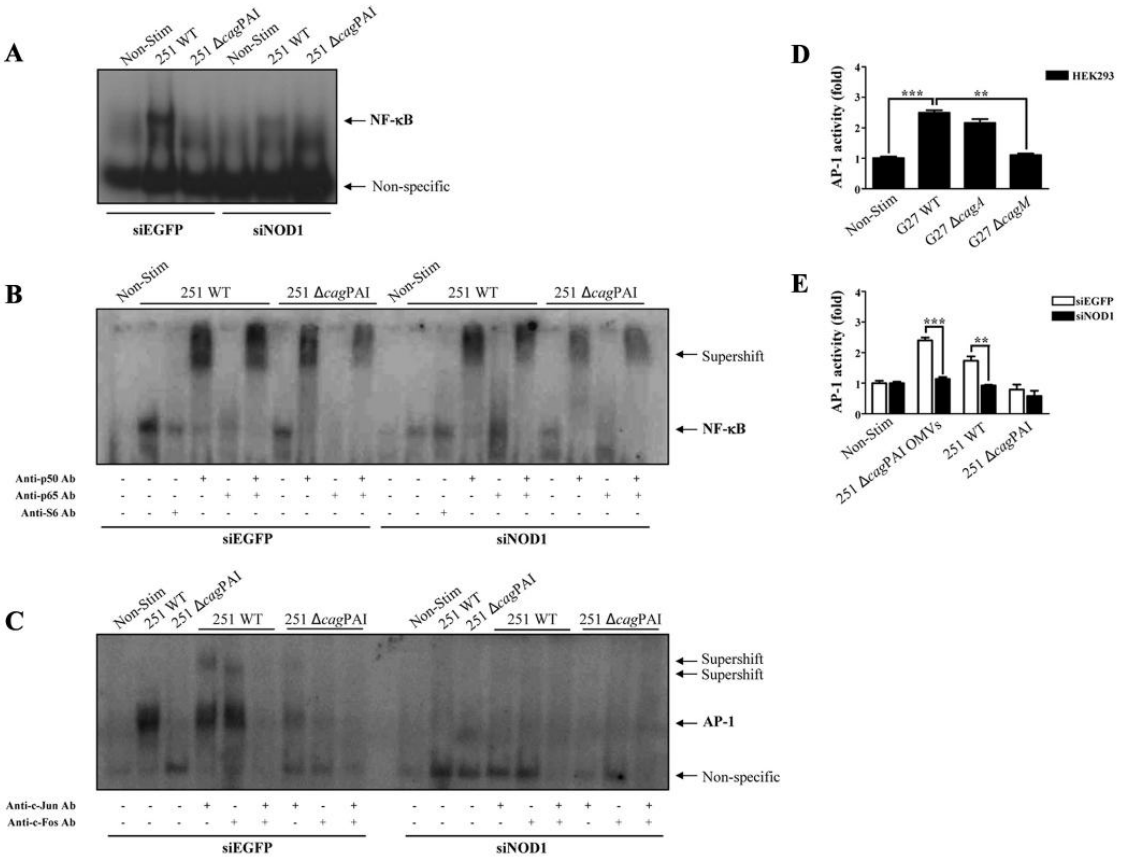

FIGURE 3. NOD1 is required for activation of NF-κB and AP-1. *A*, AGS siEGFP and siNOD1 cells were stimulated with wild-type *H. pylori* 251 or 251 Δ*cag*PAI for 2 h. Nuclear protein extracts were evaluated for NF-κB DNA binding activity by EMSA, using a radiolabeled oligo NF-κB probe. Reaction products were separated using 6% nondenaturing polyacrylamide gels. *B*, Supershift analysis with Abs to NF-κB subunits, p65, p50, or an irrelevant protein, ribosomal protein-S6, were performed using nuclear protein extracts. Extracts were preincubated with Abs, followed by incubation with the radiolabeled NF-κB probe. *C*, Nuclear protein extracts were evaluated for AP-1 DNA binding activity by EMSA, using a radiolabeled double-stranded oligonucleotide AP-1 probe. Supershift analysis with Abs to known AP-1 components, c-Jun and c-Fos, were also performed using nuclear extracts. *D*, HEK293 cells were transfected with a luciferase construct driven by an AP-1 promoter and incubated for 16 h. Cells were stimulated with wild-type *H. pylori* G27, isogenic Δ*cagA* or Δ*cagM* mutants for 4 h, before cell lysis and measurement of luciferase activity. Mean ± SEM are shown. *E*, AGS si*EGFP* and si*NOD1* cells were transfected with a luciferase construct driven by an AP-1 promoter and incubated for 16 h. Cells were stimulated with wild-type *H. pylori* 251, 251 Δ*cag*PAI, or 251 Δ*cag*PAI OMVs for 4 h. Mean ± SEM are shown. All the data presented here are representative of three independent experiments. Statistical significance was determined using the Mann-Whitney test. **, $p < 0.01$; ***, $p < 0.001$.

MAPK inhibitors significantly impair IL-8 responses to H. pylori *infection*

To assess the role of MAPKs in IL-8 production, AGS cells were treated with inhibitors of either p38, ERK, or both, before stimulation with *H. pylori* or, as a control, TNF-α. Cells pretreated with the p38 MAPK inhibitor, SB203580, produced significantly less IL-8 in response to *H. pylori* ($p < 0.05$) and TNF-α ($p < 0.01$), as did cells pretreated with the ERK inhibitor, U0126 followed by stimulation with *H. pylori* ($p < 0.01$) or TNF-α ($p < 0.01$) (Fig. 4*B*). Pretreatment with both inhibitors, resulted in a further reduction in the levels of IL-8 produced in response to *H. pylori* stimulation ($p < 0.001$). Treatment with SB203580, however, did not noticeably reduce the levels of p38 phosphorylation (Fig. 4, *C* and *D*). It was previously reported that the effects of this inhibition may be exerted downstream on the ability of p38 to phosphorylate signaling molecules (50). SB203580 treatment also slightly augmented ERK and Jnk phosphorylation (Fig. 4*B*), which can occur due to antagonistic effects between the MAPKs (51, 52). Likewise, U0126 treatment augmented p38 phosphorylation (Fig. 4*A*) and caused a modest decrease in ERK phosphorylation. This compensatory MAPK activation may explain why IL-8 production is not completely abrogated following pretreatment with inhibitors. The impairment of IL-8 production following MAPK inhibition, confirms the importance of MAPKs in *H. pylori*-induced IL-8 production, which was not fully appreciated in previous studies that instead focused on the role of NF-κB. Additionally, the data indicate that NOD1 may stimulate proinflammatory responses to *cag*PAI$^+$ *H. pylori* through signaling pathways other than NF-κB.

The effects of NOD1 over-expression on MAPK activation in response to H. pylori

To evaluate the role of the NOD1 signaling pathway on MAPK activation, AGS cells were transfected to express either wild-type NOD1; RICK, a serine/threonine kinase that has been reported to interact with NOD1 (27, 28, 53–57); or a dominant-negative form

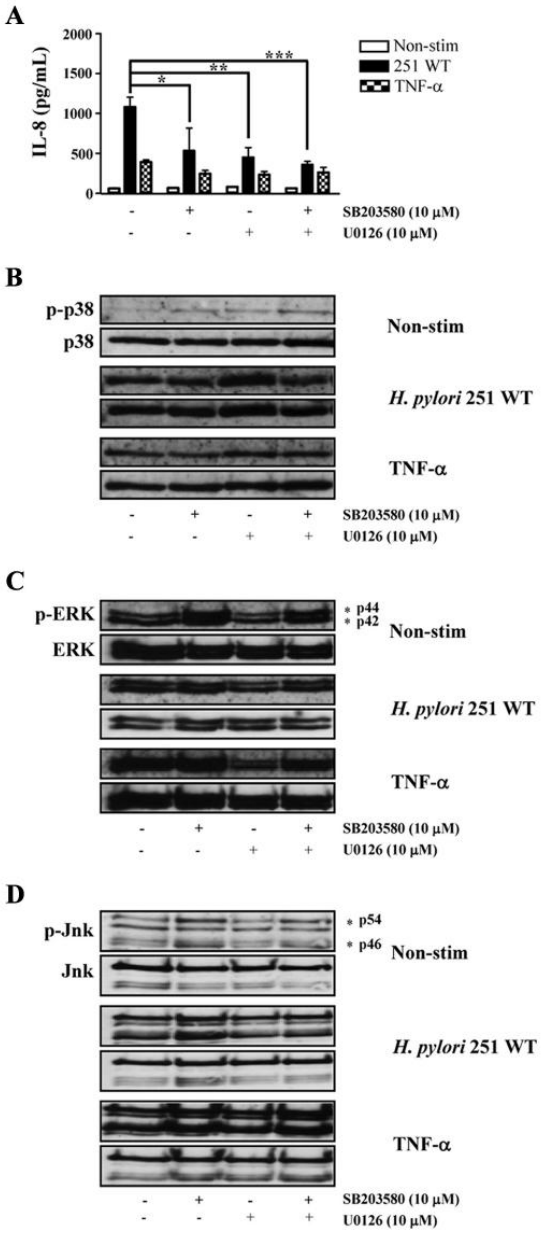

FIGURE 4. MAPK inhibitors significantly impair IL-8 responses to *H. pylori* infection. *A*, AGS cells were preincubated with 10 μM of indicated MAPK inhibitor for 1 h. The cells were washed with PBS and stimulated with wild-type *H. pylori* 251 or TNF-α (10 ng/ml) for 1 h, then washed and incubated for 24 h. After incubation, IL-8 in the culture medium was measured by ELISA. Mean $\pm$ SEM are shown. Statistical analysis was performed using the Student's *t* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *B–D*, To assess MAPK phosphorylation, AGS cells were preincubated with 10 μM of the indicated MAPK inhibitor for 1 h. Cells were washed and stimulated with wild-type *H. pylori* 251 or TNF-α for 30 min, lysed, and analyzed by immunoblot to assess levels of total or phosphorylated p38 (*B*), ERK (*C*), or Jnk (*D*) MAPKs. Results are representative of three independent experiments.

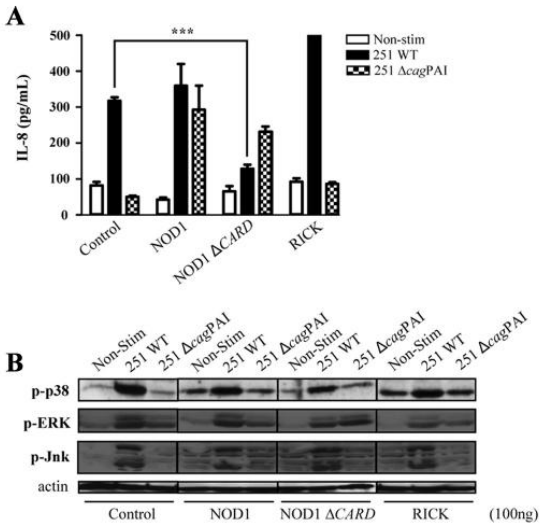

FIGURE 5. The effects of NOD1 over-expression on MAPK activation in response to *H. pylori*. AGS cells were seeded in 24-well tissue culture plates and transfected with 100 ng/well of either NOD1, NOD1 Δ*CARD*, or RICK. Cells were incubated for 16 h then stimulated with wild-type *H. pylori* 251 or 251 Δ*cag*PAI. *A*, Cells were stimulated with bacteria for 1 h then washed with PBS and further incubated for 24 h. The amount of IL-8 secreted into the culture medium was measured by ELISA. Mean $\pm$ SEM are shown. Statistical analysis was performed using the Student's *t* test. ***, $p < 0.001$. *B*, AGS cells were stimulated with bacteria for 30 min, lysed in Laemmli buffer and phosphorylation of p38, ERK, and Jnk MAPKs assessed by immunoblot. Membranes were reprobed with anti-actin Abs to ensure equal loadings. Results are representative of three independent experiments.

of NOD1 (Δ*CARD*) (35). Over-expression of NOD1 in AGS cells did not increase IL-8 production (Fig. 5*A*), nor did it enhance MAPK phosphorylation in response to wild-type *H. pylori* (Fig. 5*B*). Interestingly, however, NOD1 over-expression significantly increased IL-8 production in cells stimulated with a *H. pylori* Δ*cag*PAI mutant (Fig. 5*A*), in addition to modest increases in p38 and Jnk phosphorylation (Fig. 5*B*). This may suggest that an abundance of NOD1 enhances the ability of the cell to respond to the peptidoglycan of *cag*PAI$^-$ *H. pylori* bacteria, which are otherwise unable to inject bacterial components into the cell via a T4SS.

Over-expression of RICK, a cytosolic signaling molecule known to interact with NOD1 during *Listeria monocytogenes* (55, 57) and *Chlamydia trachomatis* (58) infections, significantly augmented IL-8 production above the limit of detection in response to wild-type *H. pylori*. This is the first direct evidence that RICK may also be important in NOD1 signaling to *H. pylori*. Nevertheless, similar to the results for NOD1 over-expression, RICK over-expression enhanced IL-8 production but did not increase the levels of phosphorylation in any of the studied MAPKs (Fig. 5*B*). This may suggest that although we have demonstrated the importance of NOD1 in p38 and ERK phosphorylation, endogenous levels of NOD1 and RICK are sufficient to induce MAPK phosphorylation, or that the effects of over-expression are exerted downstream on NF-κB and AP-1 activation. Of interest is the fact that over-expression of any construct had no effect on Jnk phosphorylation in response to wild-type *H. pylori* or the *H. pylori* 251 Δ*cag*PAI mutant (Fig. 5*B*), however, IL-8 production varied substantially (Fig. 5*A*). This further supports our data that NOD1-dependent proinflammatory responses are occurring independently of Jnk,

which as previously shown, is activated in a T4SS-dependent, but NOD1-independent manner (59).

To further investigate the effect of blocking NOD1 signaling on MAPK activation, we over-expressed the NOD1 Δ*CARD* mutant, which has a deleted CARD domain (35), thus preventing interaction with downstream signaling partners. As reported previously (10), significantly decreased IL-8 production was observed in response to wild-type *H. pylori* ($p < 0.001$) (Fig. 5*A*). Additionally, this decrease in IL-8 production coincided with decreased levels of p38 MAPK phosphorylation (Fig. 5*B*) and a modest reduction in the phosphorylation of ERK, but not Jnk in response to wild-type bacteria (Fig. 5*B*). These data are consistent with the findings reported above for the role of p38 and ERK in NOD1-dependent induction of IL-8 production, demonstrating that in addition to NF-κB activation, NOD1-dependent recognition of *cag*PAI$^+$ *H. pylori* induces p38 and ERK phosphorylation and subsequent IL-8 production.

Discussion

H. pylori rapidly activates MAPKs upon contact with gastric epithelial cells (19, 21). A number of bacterial factors have been implicated in MAPK activation, including VacA (22, 23) and CagA (19, 21). However, it is clear that a T4SS is crucial for complete phosphorylation of p38, ERK, and Jnk MAPKs (19, 21). Despite the knowledge that a functional T4SS is required, the mechanism(s) of T4SS-dependent MAPK activation are not well understood. Previous studies reported that CagA is capable of activating ERK MAPK (24, 60–62), though ERK can also be activated via CagA-independent mechanisms (19, 21, 63). Consistent with these data and with the reported time-dependent effects of CagA on ERK phosphorylation (63), we found that a *H. pylori* Δ*cagA* mutant with a functional T4SS was still capable of inducing wild-type levels of ERK phosphorylation in *siEGFP* cells at 30 min poststimulation (Fig. 1*E*), but not at 45 min (Fig. 1, *E* and *F*). Of more interest, however, we found that following stimulation with the *H. pylori* Δ*cagA* mutant, the levels of p38 activation in siEGFP cells were significantly reduced at 30 min poststimulation (Fig. 1, *A* and *B*). Moreover, this effect was more pronounced when NOD1 was knocked down (Fig. 1, *A* and *B*). To our knowledge, this is the first report to describe CagA-dependent p38 phosphorylation during *H. pylori* infection.

Opitz et al. (30) reported NOD1-dependent p38 activation in endothelial cells following stimulation with *L. monocytogenes*. Likewise, we found that the levels of *H. pylori*-induced p38 and, to a lesser extent, ERK phosphorylation were diminished in AGS cells stably expressing siRNA to *NOD1* (Fig. 1, *A*, *B*, *E*, and *F*). This inhibition in si*NOD1* cells was transient, however, as phosphorylation was restored to levels seen in the si*EGFP* cells, 60 min after stimulation (Fig. 1, *A*, *B*, *E*, and *F*). Interestingly, p38 phosphorylation was dependent only on a functional T4SS after 60 min (Fig. 1*A*), indicating the involvement of an additional NOD1-independent pathway. We found that Jnk phosphorylation was unaffected at any time point in the AGS si*NOD1* cells, though its activation was still dependent on a functional TFSS (Fig. 1, *C* and *D*). These findings are in contrast to those from in vitro kinase assays, showing that *S. flexneri* activated Jnk in a NOD1-dependent manner in HeLa cells (29). However, when AGS si*NOD1* cells were stimulated with the same virulent *S. flexneri* M90T strain used by those authors (29), we confirmed the NOD1-dependent Jnk activation they reported (Fig. 1*H*), indicating that the differences in the findings could not be attributed to the different cell culture models used. This indicates that the NOD1-independent Jnk phosphorylation during *H. pylori* infection is specific to this pathogen. In confirmation of our findings, Snider et al. (59) reported that *H. pylori*-induced Jnk phosphorylation occurred independently of NOD1 and other known upstream kinases such as, Cdc42, Rac1, PI3K, MKK4, and MKK7. Instead, Jnk was found to be activated via Src family kinases (59), which are activated when the TFSS-associated CagL protein binds to $\alpha_5\beta_1$ integrin on host cells (64). Thus, Src kinase activation of p38 during *H. pylori* infection may be responsible for the T4SS-dependent/NOD1-independent phosphorylation seen 60 min poststimulation (Fig. 1, *A* and *B*).

In confirmation of previous reports (10), we found that *H. pylori* induces NF-κB reporter activity in a NOD1-dependent manner, however, this is the first report to demonstrate that the nuclear translocation of the transactivating NF-κB complex is also dependent on NOD1 (Fig. 3, *A* and *B*). In addition, *cag*PAI$^+$ *H. pylori* induce higher levels of NF-κB translocation, however, Δ*cag*PAI mutants can still induce NF-κB activation, though to a lesser extent (Fig. 3*B*). This has been described previously (65, 66), with non-*cag*PAI-encoded proteins, such as OipA and VacA, known to play a role in NF-κB-dependent proinflammatory responses (22, 67). Interestingly, the *H. pylori* 251 strain used in many of the experiments herein expresses an "on" form of the *oipA* gene, required for OipA functionality (data not shown). Nevertheless, the composition of the NF-κB complexes, composed primarily of proinflammatory p50/p65 heterodimers, remained unchanged after *NOD1* knock down. Additionally, we have shown that NOD1 is required for AP-1 activation, as siRNA to *NOD1* abrogated nuclear translocation of AP-1 during *H. pylori* stimulation (Fig. 3*C*). This finding was of particular interest, as although NOD1 was initially required for complete MAPK activation, it was not required for phosphorylation 60 min poststimulation (Fig. 1, *A–F*). This suggests that multiple signaling events activated during *H. pylori* infection are collectively required to initiate a global transcriptional response to infection. The AP-1 complex activated during *H. pylori* infection was primarily found to be composed of c-Jun/c-Fos heterodimers (Fig. 3*C*), which is in agreement with previous findings for *H. pylori* T4SS-dependent AP-1 activation (11, 15, 16).

Many proinflammatory cytokines are dually regulated by NF-κB and AP-1, which can act in concert to amplify the transcriptional response. Examples of this include IL-8 (68, 69), for which maximal transcription in response to *H. pylori* was also shown to require the binding of both NF-κB and AP-1 to upstream promoters (11, 13, 17, 19, 20). Likewise, in this study, we have shown that pretreatment of AGS cells with p38 and ERK inhibitors significantly inhibited IL-8 production in response to *H. pylori* stimulation (Fig. 4*A*). These data reinforce our findings that the dual activation of both NF-κB and AP-1 by NOD1 rapidly induces a robust proinflammatory response upon recognition of *H. pylori*.

NOD1 over-expression was previously found to affect modest increases in NF-κB activation (26, 54). Interestingly, we found that IL-8 production was not significantly elevated in AGS cells transfected with NOD1 before wild-type *H. pylori* stimulation (Fig. 5*A*), though the reasons for this are unclear. Alternatively, the lack of amplified IL-8 production or MAPK activation following NOD1 and/or RICK over-expression may suggest the involvement of additional signaling molecules, which are required for initial host responses to *H. pylori*. Indeed, Fukazawa et al. (70), recently found that the guanine nucleotide exchange factor was critical for the NOD1-dependent signaling responses to *S. flexneri*. It may be that molecules such as guanine nucleotide exchange factor are also required for the initiation of NOD1-mediated proinflammatory signal cascades during *H. pylori* infection.

Interestingly, over-expression of RICK substantially increased IL-8 production in cells stimulated with wild-type bacteria (Fig. 5*A*), suggesting that RICK over-expression may be amplifying

NF-κB-dependent responses to *H. pylori*. Although previous reports have demonstrated that over-expression of RICK in mouse embryonic fibroblasts and HEK293 cells led to amplified NF-κB responses (54, 71, 72), this is the first study to show a potential link between RICK and NOD1 responses to *cag*PAI$^+$ *H. pylori*. The association of NOD1 with RICK has been reported to activate the TGF-β associated kinase, TAK1, resulting in NF-κB activation (27). TAK1 is also known to activate p38 and Jnk (73, 74) and this pathway may represent a mechanism through which NOD1 induces MAPK activation during *H. pylori* infection.

In contrast to the data for over-expression of NOD1 and RICK, expression of the dominant-negative NOD1 ΔCARD construct not only affected IL-8 production in cells, but also resulted in diminished levels of p38 and to a lesser extent, ERK phosphorylation in response to *H. pylori*. No effect, however, was observed for Jnk phosphorylation. These results are in agreement with those of the *NOD1* siRNA studies (Figs. 1 and 2), showing the importance of p38 and ERK in NOD1-dependent IL-8 responses to *H. pylori*.

NOD1 has been shown to be an important regulator of innate immune responses to bacterial pathogens (10, 25, 26, 29–32, 55–58, 70, 75–84), however, research has mainly focused on the role of NOD1 in activating NF-κB-mediated proinflammatory responses. In this study, we show that NOD1 is critical, not only for NF-κB activation, but also for the activation of MAPKs and AP-1 during *H. pylori* infection. This in turn may induce the transcription of a novel subset of proinflammatory genes that are exclusively activated in an AP-1-dependent manner during *H. pylori* infection. One example of this is the decay-accelerating factor (DAF), a *H. pylori* cellular receptor previously shown to be upregulated following contact with *H. pylori* (85). Although the *DAF* promoter contains a κB response element, it was found that *DAF* expression in response to *H. pylori* was dependent on p38 phosphorylation and totally independent of NF-κB (86–89). Further studies are therefore warranted to investigate the role of NOD1 in *H. pylori*-induced DAF expression, as well as in other MAPK regulated host factors.

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Disclosures

The authors have no financial conflict of interest.

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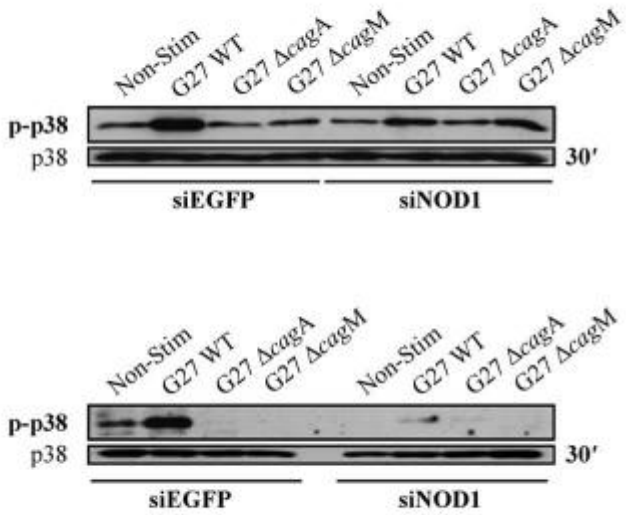
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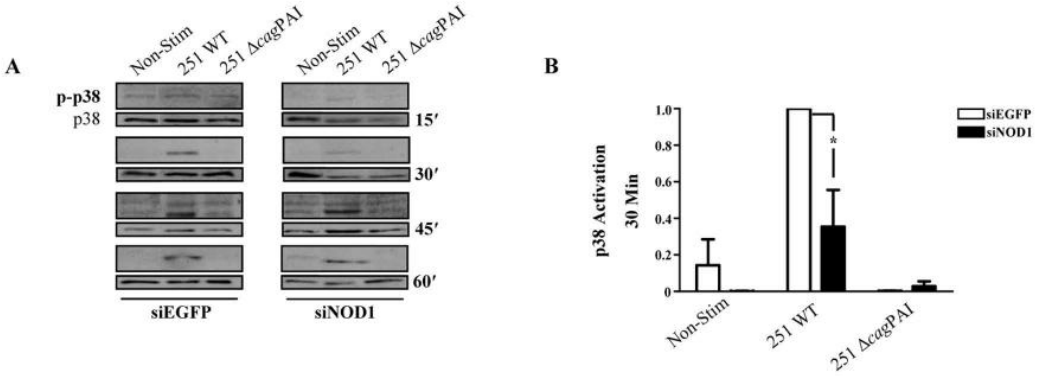
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SUPPLEMENTARY FIGURE 1. *H. pylori* activates p38 MAPK via a NOD1-dependent mechanism. Immunoblots from replicate experiments in which AGS si*EGFP* or si*NOD1* cells were stimulated with wild-type *H. pylori* G27 or the isogenic mutants Δ*cagA* and Δ*cagM* for 30 min. Cells were lysed in boiling Laemmli buffer and samples analyzed by immunoblot to assess phosphorylation of *A* and *B*, p38 MAPK in separate experiments.

SUPPLEMENTARY FIGURE 2. *H. pylori* 251 activates p38 MAPK in a NOD1-dependent manner. AGS si*EGFP* or si*NOD1* cells were stimulated with wild-type *H. pylori* 251 or the isogenic Δ*cag*PAI mutant for 15, 30, 45 or 60 min. Cells were lysed in boiling Laemmli buffer and samples analyzed by immunoblot and densitometry (30 min) to assess phosphorylation of *A* and *B*, p38 MAPK. All membranes were re-probed with total anti-p38 Abs to ensure equal loadings. Blots are representative of 3 independent experiments. Statistical analysis of densitometry was performed by normalizing band intensity across replicate experiments. Significance was determined using the Mann-Whitney test. $*$, $p < 0.05$.

Declaration for Thesis Chapter 2

Declaration by candidate

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
My contribution involved performing experiments pertaining to one minor aspect of the publication.	10%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Jared Snider	Project concept, experimental design and execution, reagent preparation	90%
Brian Bellaire	Experimental design, reagent preparation	
Richard Ferrero	Experimental design and Reagents	
James Cardelli	Project concept, experimental design	

Candidate's Signature	[redacted]	Date 30-9-10

Declaration by co-authors

The undersigned hereby certify that:

1. the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
2. they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
3. they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
4. there are no other authors of the publication according to these criteria;
5. potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
6. the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	Lab of James Cardelli, Louisiana State University Health Sciences Center, 1501 King's Hwy, Shreveport, Louisiana 71130 USA

[Please note that the location(s) must be institutional in nature, and should be indicated here as a department, centre or institute, with specific campus identification where relevant.]

Signature 1	[redacted]	Date 9/13/10
Signature 2		9/17/10
Signature 3		29/9/2010.
Signature 4		

The β_1 Integrin Activates JNK Independent of CagA, and JNK Activation Is Required for *Helicobacter pylori* CagA$^+$-induced Motility of Gastric Cancer Cells*[S]

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The *Helicobacter pylori* CagA protein is translocated into gastric epithelial cells through a type IV secretion system (TFSS), and published studies suggest CagA is critical for *H. pylori*-associated carcinogenesis. CagA is thought to be necessary and sufficient to induce the motogenic response observed in response to CagA$^+$ strains, as CagA interacts with proteins involved in adhesion and motility. We report that *H. pylori* strain 60190 stimulated AGS cell motility through a CagA- and TFSS-dependent mechanism, because strains 60190Δ*cagA* or 60190Δ*cagE* (TFSS-defective) did not increase motility. The JNK pathway is critical for *H. pylori*-dependent cell motility, as inhibition using SP600125 (JNK1/2/3 inhibitor) or a JNK2/3-specific inhibitor blocked motility. JNK mediates *H. pylori*-induced cell motility by activating paxillin, because JNK inhibition blocked paxillin$^{Tyr\text{-}118}$ phosphorylation, and paxillin expression knockdown completely abrogated bacteria-induced motility. Furthermore, JNK and paxillin$^{Tyr\text{-}118}$ were activated by 60190Δ*cagA* but not 60190Δ*cagE*, demonstrating CagA-independent signaling critical for cell motility. A β_1 integrin-blocking antibody significantly inhibited JNK and paxillin$^{Tyr\text{-}118}$ phosphorylation and cell scattering, demonstrating that CagA-independent signaling required for cell motility occurs through β_1. The requirement of both Src and focal adhesion kinase for signaling and motility further suggests the importance of integrin signaling in *H. pylori*-induced cell motility. Finally, we show that JNK activation occurs independent of known upstream kinases and signaling molecules, including Nod1, Cdc42, Rac1, MKK4, and MKK7, which demonstrates novel signaling leading to JNK activation. We report for the first time that *H. pylori* mediates CagA-independent signaling that promotes cell motility through the β_1 integrin pathway.

Helicobacter pylori infects one-half of the world's population, establishing a chronic infection in the gastric mucosa that persists for the lifetime of the host (1, 2). Although most infections only manifest as superficial gastritis, many progress to mucosal necrosis, ulceration, and atrophic gastritis, a precursor lesion of gastric adenocarcinoma (3). In animal studies, 37% of gerbils infected with virulent strains of *H. pylori* developed stomach tumors, demonstrating a direct link between *H. pylori* and gastric carcinogenesis (4). Additionally, epidemiological studies suggest that *H. pylori* infection increases the risk of developing gastric cancer 6-fold, emphasizing the importance of this bacterium in gastric carcinogenesis (5).

H. pylori pathogenesis varies based on the expression of virulence factors used for bacterial colonization and disease progression. The *vacA* gene is encoded by virtually all *H. pylori* strains, but the intense vacuolation caused by VacA varies based on genetic mosaicism (6). Peptic ulceration strongly correlates with strains encoding the most active forms of VacA (6).

The *cag* pathogenicity island (*cag* PAI)[3] contains 32 genes, many of which encode components of a putative type IV secretion system (TFSS). The only known protein transported by the TFSS is CagA, which is also expressed from the *cag* locus (7). During infection, CagA translocates into gastric epithelial cells via the TFSS and is phosphorylated at multiple sites by Src family kinases and c-Abl (8–10). CagA then influences signal transduction pathways by docking with host signaling proteins (11–14). Patients infected with CagA-positive *H. pylori* strains exhibit higher grades of gastric inflammation, atrophic gastritis, and an increased risk of the development of gastric adenocarcinoma (15–17).

In vitro experiments show that epithelial cells cultured with CagA$^+$ bacteria transition from the unstimulated "cobblestone" morphology to the "hummingbird" phenotype indicative of motile cells (18–21). Additionally, *H. pylori* stimulates gastric cancer cell invasion through *in vitro* basement membranes, suggesting a role for *H. pylori* in cancer progression to metas-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–6.

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[3] The abbreviations used are: PAI, pathogenicity island; TFSS, type IV secretion system; JNK, c-Jun NH_2-terminal kinase; FAK, focal adhesion kinase; GFP, green fluorescent protein; PI3K, phosphatidylinositol 3-kinase; siRNA, short interfering RNA; shRNA, short hairpin RNA; m.o.i., multiplicity of infection; MAP, mitogen-activated protein; PBS, phosphate-buffered saline; MKK, MAP kinase kinase; EGFP, enhanced green fluorescent protein; IL, interleukin.

tasis (22–24). The mechanism of *H. pylori*-induced cell motility and invasion are unclear, although recent studies show that CagA associates with key regulators of cell adhesion, motility, and invasion, including c-Met, Grb2, SHP-2 phosphatase, and ZO-1 (11, 12, 14, 22, 25). The critical role of CagA in cancer cell motility was emphasized by Higashi *et al.* (11) who showed that CagA transfection of AGS cells was sufficient to induce the motile phenotype. These data suggest that CagA stimulates all signaling necessary to induce cell motility, although this hypothesis is not universally accepted (22).

In this study, we identified JNK as a key mediator of *H. pylori*-stimulated cell motility, and we found that JNK was activated through a CagA-independent but still TFSS-dependent mechanism. We then evaluated the molecular mechanism of CagA-independent JNK activation, and we determined that CagA-independent activation of JNK occurs through β_1 integrin and Src signaling. Furthermore, we identified paxillin as a downstream target of *H. pylori*-dependent JNK activation and that paxillin activity is required for *H. pylori*-stimulated gastric cancer cell motility. These data show for the first time a mechanism of CagA-independent signaling that promotes cell motility, and we conclude that a combination of CagA-dependent and CagA-independent cell signaling is required for *H. pylori*-induced gastric cancer cell motility.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—AGS gastric adenocarcinoma cells (ATCC, Manassas, VA) were cultured in Ham's F-12 media (Mediatech, Herndon, VA) supplemented with 10% fetal calf serum (Gemini, West Sacramento, CA), 100 μg/ml streptomycin, 100 IU/ml penicillin (Mediatech, Herndon, VA), and 0.0375% (w/v) sodium bicarbonate (Mediatech, Herndon, VA) at 37 °C in 5% CO_2. Cells were cultured to 75% confluency and split either 1:5 or 1:10 using 0.025% EDTA to gently detach cells from plastic. To prevent spontaneous cell scattering associated with increased passage number, fresh stocks were thawed out monthly.

Pharmacological inhibitors LY294002, SP600125, JNK2/3 inhibitor (IX), and Bay11-7082 were obtained from EMD Biosciences (San Diego). Cycloheximide and PP2 were obtained from Sigma. The β_1 blocking antibody AIIB2 was obtained from Developmental Studies Hybridoma Bank (Iowa City, IA). Prior to experiments, subtoxic concentrations of each inhibitor were selected, and Western blots to detect phosphorylated proteins were performed to confirm the functionality of the inhibitors at the concentrations used. For experiments, cells were pretreated with inhibitors for 30 min (LY294002, 50 μM; SP600125, 40 μM; cycloheximide, 10 μg/ml), 2 h (inhibitor IX, 625 nM), 2 h (PP2, 25 μM), or 3 h (Bay11-7082, 0.5 μg/ml) prior to the addition of bacteria.

Bacterial Strains and Culture Conditions—*H. pylori* strains 60190 (ATCC 49503, *cag* PAI$^+$, *vacA* s1/m1) and Tx30a (ATCC 51932, *cag* PAI$^-$, *vacA* s2/m2) were obtained from ATCC (Manassas, VA) and grown on trypticase soy agar plates supplemented with 5% adult defibrinated bovine blood (Gemini, West Sacramento, CA) at 37 °C in 5% CO_2 overnight prior to use in experiments. *H. pylori* mutant strains with disrupted *cagA* (60190Δ*cagA*), *cagE* (60190Δ*cagE*), and *vacA* (60190Δ*vacA*) genes were a kind gift from Dr. Richard Peek (Vanderbilt University, Nashville, TN). These strains were grown on the same plates as wild-type bacteria but under kanamycin selection (50 μg/ml). Bacteria were passaged daily, and fresh bacteria were thawed on a monthly basis.

The *H. pylori* strain G27 was used in this study, and isogenic mutants, *cagA* and *cagM*, were constructed by natural transformation of a kanamycin cassette flanked by regions homologous to the disrupted genes (26, 27). *H. pylori* G27 and mutant strains were routinely cultured on horse blood agar (blood base agar number 2, 8% (v/v) horse blood (Bio-Lab, Victoria, Australia)) supplemented with antibiotics (27). Bacteria were grown at 37 °C for 1–2 days under microaerobic conditions in an anaerobic jar containing a Campygen gas mix of 5% O_2, 10% CO_2, and 85% N_2 (Oxoid, Hampshire, UK). Liquid broth cultures were incubated in 25-cm^3 tissue culture flasks (Iwaki, Japan) in a final volume of 10 ml of brain heart infusion broth containing 10% (v/v) fetal bovine serum (Thermo Electron, Melbourne, Australia) and with shaking at 125 rpm and 37 °C overnight, prior to use in experiments. *H. pylori* isogenic mutants with disrupted *cagA* (G27Δ*cagA*) and *cagM* (G27Δ*cagM*) were grown on the same plates as wild-type bacteria but under kanamycin selection (20 μg/ml).

Infection of AGS Cells—At least 1 day prior to infection, AGS cells were seeded at a density of 2.5–6 $\times$ 10^4 cells/ml in antibiotic-free media. For each experiment, 1 day-old bacteria were suspended in warmed, CO_2-charged antibiotic-free media, and bacterial density was measured by spectrophotometer at 600 nm. Bacteria were then added to cells at a multiplicity of infection (m.o.i.) of 100. Bacterial contact with cells was synchronized by centrifugation at 600 $\times$ *g* for 4 min, after which cells were maintained at 37 °C and 5% CO_2 throughout each experiment. Control cells were prepared under identical conditions.

Scatter Assays—Cells were cultured alone or with bacteria ± inhibitors for 18–20 h, and monolayers were fixed with 4% paraformaldehyde. After three washes with PBS, F-actin was fluorescently labeled with Oregon Green 488 phalloidin (Molecular Probes, Eugene, OR) suspended in BSP (bovine serum albumin, saponin, PBS). Fluorescent and phase images were acquired by wide field fluorescent microscopy.

Western Blot Analysis—Lysates from bacteria alone or cells co-cultured with bacteria were collected in boiling protein loading buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 0.13 mM bromphenol blue, 1 M sucrose). Proteins were separated by 7.5–12% acrylamide gel electrophoresis and transferred to nitrocellulose or polyvinylidene difluoride membranes (Pall, Pensacola, FL). Membranes were blocked with 5% milk or 0.1% gelatin in TBST (20 mM Tris, 137 mM NaCl, 0.1% Tween 20, pH 7.5) and incubated overnight with antibodies specific for tubulin (NeoMarkers, Fremont, CA), CagA (Austral Biologicals, San Ramon, CA), β-actin (Sigma), Rac1 (BD Biosciences), phospho-JNK, phospho-AKT, JNK, Cdc42, phospho-MKK4, MKK4, phospho-MKK7, MKK7, phospho-paxillin$^{Tyr\text{-}118}$, and paxillin (Cell Signaling Technology, Beverly, MA) and phospho-paxillin$^{Ser\text{-}178}$ (EMD Biosciences, San Diego) in 5% bovine serum albumin or 0.1% gelatin in TBST. Blots were followed with horseradish peroxidase-conjugated secondary antibodies

(Amersham Biosciences), and proteins were detected by ECL (Amersham Biosciences).

Colloidal Gold Motility Assay—Assay was derived from colloidal gold phagokinetic assay as described previously (28). Briefly, 12-mm coverslips were immersed in a gelatin solution (Sigma, 300 bloom; 500 mg in 300 ml of water) and heated at 90 °C for 10 min. The gelatin was then removed, and the coverslips were dried at 70 °C for 45 min. After the coverslips cooled, they were aseptically transferred to 24-well plates. The colloidal gold suspension was prepared by mixing 11 ml of water, 6 ml of a 36.5 mM Na_2CO_3 solution, and 2 ml of a 14.5 mM $AuHCl_4$ solution (Fisher). The mixture was gently swirled high over a Bunsen burner until the first sign of boiling, after which the flask was immediately removed from heat, and 2 ml of a 0.1% formaldehyde solution was quickly added.

The hot solution was allowed to slightly cool on the bench top as the flask was swirled, during which time the solution turned moderately brown in color. After the gold solution changed color, 2 ml were added atop each coverslip in the 24-well plate, and the plates were incubated at 37 °C and 5% CO_2 for 3 h to allow the gold particles to settle onto the coverslips. Coverslips were then gently washed and stored in PBS at 4 °C until use.

For motility assays, 1×10^4 cells were seeded onto prepared coverslips and spun at $600 \times g$ to maximize cell attachment to the substratum.[4] After 6–12 h of recovery time, bacteria were added as described above. In inhibition assays, the inhibitors were added 30 min prior to the addition of bacteria, except for SU11274, which was added overnight following a 6-h recovery period.

After 18–22 h, cells were fixed with 4% paraformaldehyde and permeabilized with BSP for 1 h at room temperature without agitation. Coverslips were rinsed three times with PBS and mounted onto slides with Slowfade Gold antifade reagent with 4′,6-diamidino-2-phenylindole (Molecular Probes, Eugene, OR). Phase and fluorescent images were taken of each field, and the area was cleared by single or small colonies of cells measured using ImageJ software (National Institutes of Health). The area was then divided by the number of nuclei in the corresponding fluorescent image to give the average area cleared per cell. Fifteen to 30 fields were visualized in this manner for an average of 100 cells per coverslip, and between one and three coverslips were used per experimental condition. Data for two to three separate experiments were compared and presented as the fold change over control or area cleared per cell. Standard deviation and error were included, and *p* values were calculated by either the paired two-sample *t* test for means (Microsoft Excel) or analysis of variance with Tukey's HSD test (GraphPad Prism).

Short Interfering RNA (siRNA) Transfection—Transfection protocol was modified from Chan *et al.* (29). Briefly, 1.25×10^4 AGS cells were seeded into a 24-well plate, and the following day were transfected with Lipofectamine 2000 (Invitrogen) according to product protocol using 1.5 μl of stock Cdc42 or luciferase siRNA (20 μM; a kind gift from Dr. Marc Symons, Feinstein Institute for Medical Research, Manhasset, NY). After 2 days fresh medium was added, and cells were either infected with *H. pylori* for scatter assays or collected for Western blot analysis.

Lentiviral Delivery of Short Hairpin (sh) RNA—Stable shRNA cell lines were generated using MISSION shRNA lentiviruses (Sigma) according to manufacturer's protocol. Briefly, 5×10^4 AGS cells were seeded in 6-well dishes, and each well was left untreated or infected with one of the five lentiviral clones provided in each target transcript kit (m.o.i. of 0.5) plus Polybrene (8 μg/ml). The following day, virus was aspirated and replaced with fresh media, and the next day the cells were washed, and media containing puromycin (0.6 μg/ml) was added for selection of stable transfectants. After 3 days of daily washes, stable clones were screened for maximal target protein expression knockdown. The clones selected were as follows: Rac1 (MISSION shRNA TRCN0000004873), MKK4 (MISSION shRNA TRCN0000039916), MKK7 (MISSION shRNA TRCN0000000587), paxillin (MISSION shRNA TRCN-0000123138), and non-target control (MISSION shRNA SHC002V). Stable GFP shRNA cell lines were generated by infecting AGS cells with the lentiviral shRNA vector GFP-FSIPPW (a kind gift from Dr. Andrew Kung, Dana-Farber Cancer Institute, Boston). Stable cells were cultured in puromycin-containing media.

Adenovirus Infection—Transient expression of GFP-tagged FAK-related non-kinase (Ad-GFP-FRNK) or GFP (Ad-GFP) was facilitated through adenoviral delivery. Ad-GFP-FRNK and Ad-GFP viruses were a kind gift of Dr. Joan Taylor (University of North Carolina, Chapel Hill). AGS cells were incubated overnight with virus at an m.o.i. of 10. The following day, cells were then infected with *H. pylori* strains for either 1 h (Western blot analysis) or overnight. After overnight incubation, cells were fixed and stained for F-actin using Alexa Fluor 546 phalloidin (Molecular Probes). GFP and Texas Red images were captured, and merge images were generated using ImageJ (National Institutes of Health).

Nod1KD Cell Analysis—AGS cells stably expressing siRNAs to either the caspase-activation recruitment domain of Nod1 or an irrelevant gene (EGFP) were generated by Dr. J. Viala (Institut Pasteur, Paris) (27). Briefly, AGS cells were transfected with plasmid constructs in which short hairpin RNA to the genes of interest had been cloned into psiRNA-hHIneo (InvivoGen, San Diego). To select for clones that had stably incorporated the respective plasmids into their genomic DNA, the cells were grown in RPMI 1640 medium containing 10% fetal calf serum and 400 μg/ml G418 (Invitrogen). G418-resistant cells were isolated and expanded so as to generate stable knockdown clones for *NOD1* or EGFP genes. The characterization of these clones will be described in detail elsewhere.[5] Successful knockdown of Nod1 expression in these cells was published previously (30).

Chemokine Analysis—IL-8 production by EGFP and Nod1KD cells induced by *H. pylori* co-culture was determined

[4] J. L. Snider, personal observations.

[5] R. L. Ferrero, unpublished data.

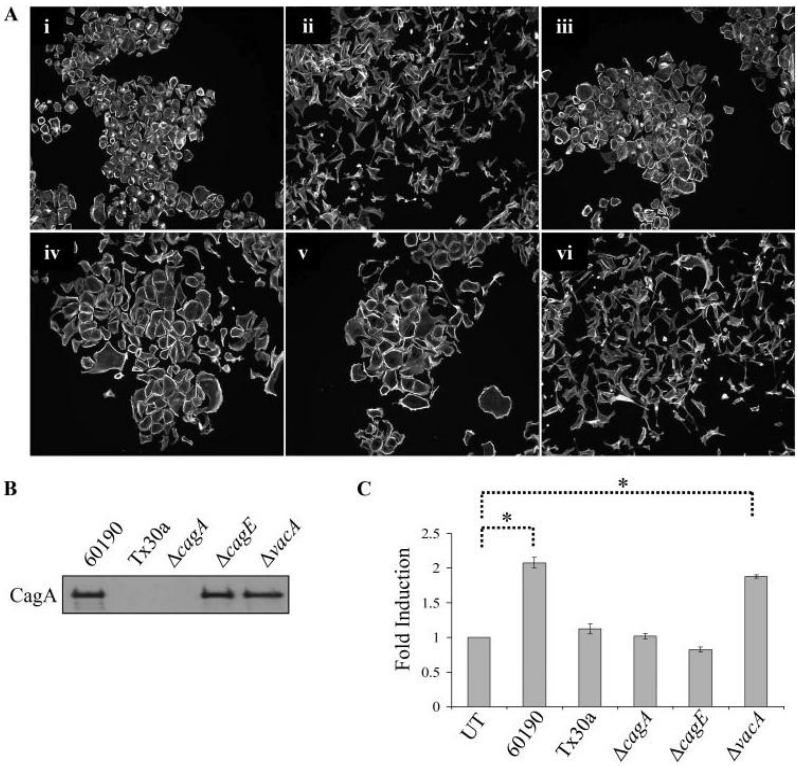

FIGURE 1. ***H. pylori*-stimulated AGS cell scattering and motility are dependent upon CagA and the TFSS.** *A,* AGS cells were cultured alone (*panel i*) or with 60190 (*panel ii*), Tx30a (*panel iii*), 60190Δ*cagA* (*panel iv*), 60190Δ*cagE* (*panel v*), or 60190Δ*vacA* (*panel vi*) for 18 h, after which cells were fixed and stained for F-actin. *B,* Western blot analysis was performed on bacterial lysates using a CagA-specific antibody. *C, graph* represents the effect *H. pylori* strains have on cell motility. Data presented as a fold change compared with control cells in three separate experiments and include the standard error. *UT*, untreated. *, untreated *versus* 60190 or VacA ($p < 0.01$, using paired two-sample *t* test for means). All micrographs, blots, and motility data are representative of multiple experiments.

by collecting 24-h supernatants and using the OptEIA kit (BD Biosciences).

RESULTS

H. pylori-dependent AGS Cell Scattering and Motility Require CagA and the TFSS—Epithelial cell lines co-cultured with Cag PAI$^+$ *H. pylori* strains exhibit the hummingbird phenotype associated with epithelial-mesenchymal transition (14, 18). To demonstrate that a similar phenotype occurred in response to exposure to *H. pylori* in our experimental system, AGS gastric cancer cells were co-cultured with *H. pylori* 60190 (Cag PAI$^+$, vacuolating) or Tx30a (Cag PAI$^-$, nonvacuolating) for 18 h, and cells were fixed and fluorescently labeled for F-actin. As shown in Fig. 1*A, panel ii*, AGS cells cultured with *H. pylori* 60190 exhibited the hummingbird phenotype indicative of motile cells, whereas cells cultured with *H. pylori* Tx30a (Fig. 1*A, panel iii*) showed no morphological changes compared with untreated cells (Fig. 1*A, panel i*).

To determine the bacterial factors required for AGS cell scattering, isogenic mutants of *H. pylori* 60190 with gene disruptions in *cagA* (60190Δ*cagA*), *cagE*, encoding a gene product required for full TFSS functionality (60190Δ*cagE*) or *vacA* (60190Δ*vacA*) were used in co-culture assays. Western blot analysis was first performed on whole bacteria lysates to confirm that the mutant strains 60190Δ*cagE* and 60190Δ*vacA*, but not 60190Δ*cagA*, expressed CagA (Fig. 1*B*). AGS cells were then co-cultured with each of these strains, and the extent of cell scattering was determined by immunofluorescence microscopy. As shown in Fig. 1*A, panels iv* and *v*, 60190Δ*cagA* and 60190Δ*cagE*, respectively, did not induce wild-type cell scattering, demonstrating that the delivery of CagA into host cells is necessary to induce the hummingbird phenotype. The mutant strain 60190Δ*vacA* caused a scattering phenotype similar to wild-type 60190 (Fig. 1*A, panels vi* and *ii*, respectively), demonstrating that the vacuolating cytotoxin of *H. pylori* plays no role in the induction of this morphological response.

Previous studies have measured *H. pylori*-induced cell motility as the percent of cells per field that exhibit the hummingbird phenotype (21, 31–33). To quantitate the level of participation of bacterial and host proteins in cell motility, a modified colloidal gold phagokinetic assay was utilized to investigate the role of CagA, the TFSS itself (using the CagE mutant), and the vacuolating cytotoxin in cell motility. Briefly, cells and bacteria were seeded onto a colloidal gold substrate, and cell motility was measured as a function of the area that cells cleared as they moved during the assay. As shown in Fig. 1*C*, *H. pylori* 60190 stimulated a 2-fold increase in cell motility over untreated cells. Additionally, only the VacA mutant caused a comparable increase in motility over untreated cells, although no significant increase in motility was observed by Tx30a and the CagA or TFSS mutants. These data correlate with our scatter data from Fig. 1*A* and demonstrate that cell motility is a CagA- and TFSS-dependent but VacA-independent event.

H. pylori-induced Cell Scattering and Motility Require JNK Signaling—Recent evidence shows that the JNK pathway is a key mediator of cytoskeletal extensions and cell motility in a number of experimental systems (34, 35). To determine whether JNK plays a role in *H. pylori*-induced cell scattering and motility, AGS cells were pretreated with the pan-JNK inhibitor, SP600125 or DMSO for 30 min prior to the addition of *H. pylori* strains for 18 h (Fig. 2*A*). Cells pretreated with SP600125 did not scatter in response to *H. pylori* compared

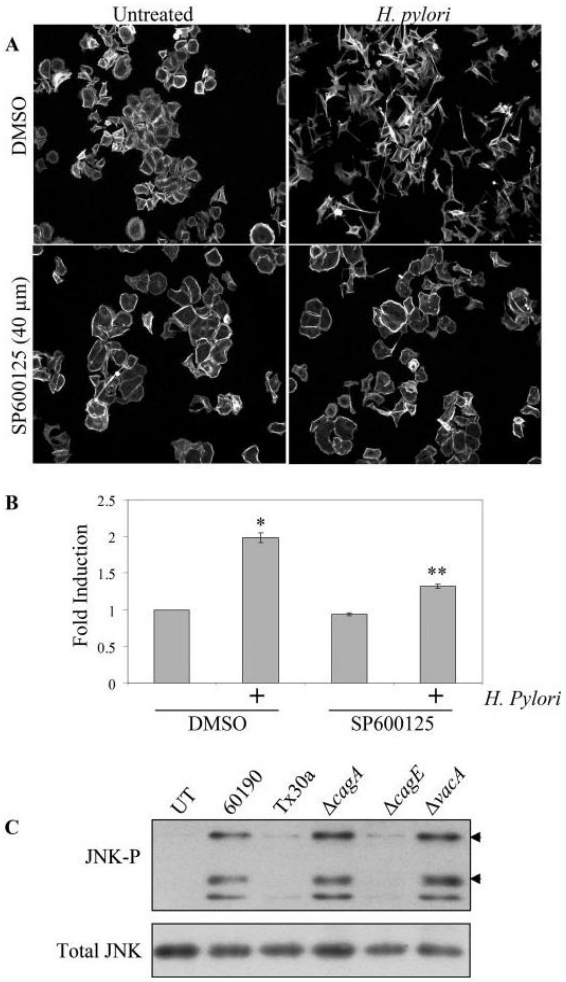

FIGURE 2. ***H. pylori*-induced cell motility requires JNK signaling.** *A*, cells were co-cultured alone (*panels i* and *iii*) or with *H. pylori* strain 60190 for 18 h following pretreatment for 30 min with JNK inhibitor SP600125 (40 μM) or with DMSO as a carrier control. Cells were fixed and stained for F-actin. *B, graph* represents the effects of JNK inhibition on *H. pylori*-induced cell motility. Data are presented as fold change compared with control cells in three separate experiments and include standard error. *C*, AGS cells were co-cultured with the indicated *H. pylori* strains for 2 h, and lysates were collected. Western blot analysis was performed to determine the JNK activation profile using phospho-specific antibodies. Total protein was also probed as a load control. *Arrowheads* indicate the 54-kDa JNK2/3 isoforms and the 46-kDa JNK1 isoform. *UT*, untreated. *, untreated *versus H. pylori* ($p < 0.01$); **, *H. pylori versus H. pylori* + inhibitor ($p < 0.01$, using paired two-sample *t* test for means). All micrographs, blots, and motility data are representative of multiple experiments.

with DMSO control cells (Fig. 2*A*), demonstrating that JNK is required for cell scattering. Motility assays were then employed, and Fig. 2*B* shows that SP600125 significantly decreased AGS cell motility, demonstrating that JNK signaling is required for *H. pylori*-induced cell scattering and motility.

H. pylori Activates JNK in a CagA-independent, TFSS-dependent Manner—The report by Higashi *et al.* (11) suggests that CagA is sufficient to stimulate all pathways required for cell motility. If this hypothesis is true, then all signaling pathways required for *H. pylori*-induced cell motility would require CagA delivery for activation. To test this hypothesis, Western blot analysis was performed on cells co-cultured with the parental and mutant *H. pylori* strains, and JNK phosphorylation in response to different bacterial stimuli was analyzed (Fig. 2*C*). Surprisingly, JNK was phosphorylated in response to co-culture with 60190, 60190Δ*cagA*, or 60190Δ*vacA*, whereas strains lacking a functional TFSS (Tx30a and 60190Δ*cagE*) showed no induction. These data show that JNK signaling, which is required for *H. pylori*-stimulated cell motility, is activated in a CagA-independent but still TFSS-dependent manner.

H. pylori Stimulates JNK through a Nod1-independent Mechanism—Although activation of JNK occurs in a CagA-independent but still TFSS-dependent manner, the mechanism of TFSS-dependent JNK signaling is unknown. A recent report by Viala *et al.* (27) demonstrated that peptidoglycan, a component of the bacterial cell wall, is transported into the cytoplasm and recognized by the pathogen recognition molecule Nod1, causing NF-κB activation and IL-8 secretion. Nod1 is also reported to regulate JNK and p38 activity, and therefore peptidoglycan-mediated Nod1 induction may be the mechanism of CagA-independent JNK activation leading to cell motility (36, 37). To test this hypothesis, cells stably expressing siRNAs targeting Nod1 (Nod1KD) or an irrelevant gene (EGFP) were analyzed for JNK phosphorylation after co-culture with *H. pylori* strain G27 and isogenic mutant strains G27Δ*cagA* or G27 Δ*cagM* (TFSS-defective) (26). Nod1 expression was significantly abolished in the Nod1KD cells, as published previously (30). To confirm a knockdown of Nod1 expression, *H. pylori*-stimulated IL-8 production was analyzed by enzyme-linked immunosorbent assay, and supplemental Fig. 1*A* confirms a significant decrease in IL-8 production by Nod1KD cells in response to *H. pylori* G27. In supplemental Fig. 1*B*, G27 stimulated JNK phosphorylation in a CagA-independent manner in both EGFP and Nod1KD cell lines, which demonstrates two important points. First, CagA-independent JNK activation is not specific to *H. pylori* strain 60190. Second, the bacterium stimulates JNK phosphorylation independent of Nod1 signaling. Furthermore, supplemental Fig. 1*C* shows that Nod1KD cells exhibit the scattered phenotype in response to *H. pylori* similar to EGFP control cells. These observations demonstrate that *H. pylori* activates JNK-dependent cell scattering through a Nod1-independent mechanism.

H. pylori-induced JNK Phosphorylation and Cell Scattering Occur through Integrin Signaling—A recent publication by Kwok *et al.* (38) showed that the TFSS requires the interaction between the TFSS-associated CagL protein and the $\alpha_5\beta_1$ integrin complex to initiate translocation of CagA into the cell. This interaction also activates FAK and Src, both of which play a key regulatory role in integrin signaling (38). This group pretreated AGS cells with a β_1-blocking antibody (AIIB2), which prevented CagA translocation and phosphorylation (38). Evidence also shows that integrin signaling can stimulate JNK activity (39). To determine whether TFSS-dependent β_1 signaling causes activation of JNK leading to *H. pylori*-induced cell motility, AGS cells were pretreated with AIIB2 for 1 h prior to co-culture with *H. pylori* 60190. Western blot analysis was then

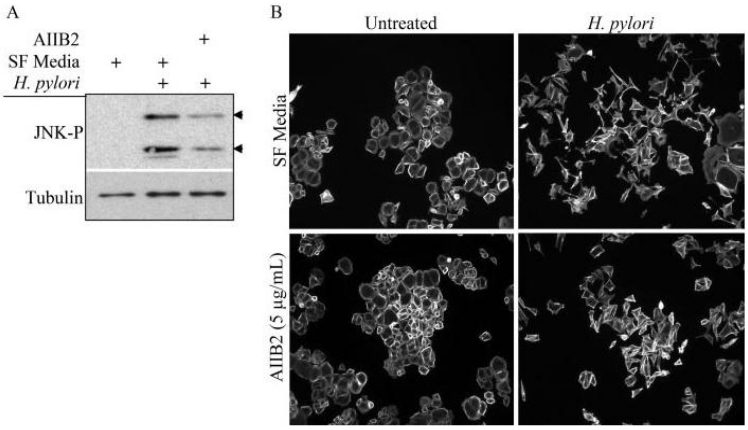

FIGURE 3. **The β_1 integrin is required for *H. pylori*-dependent JNK phosphorylation and cell scattering.** *A*, AGS cells were pretreated for 1 h with the β_1 blocking antibody AIIB2 (5 μg/ml) or an equal aliquot of serum-free media (*SF Media*) prior to co-culture with *H. pylori* 60190, and cell lysates were analyzed for phospho-JNK activity by Western blot analysis using phospho-specific antibodies. Tubulin was also probed as a load control. *B*, AGS cells were pretreated for 1 h as indicated in *A* and then cultured alone or with *H. pylori* 60190 for 18 h. Cells were then fixed and stained for F-actin. All blots and micrographs are representative of multiple experiments.

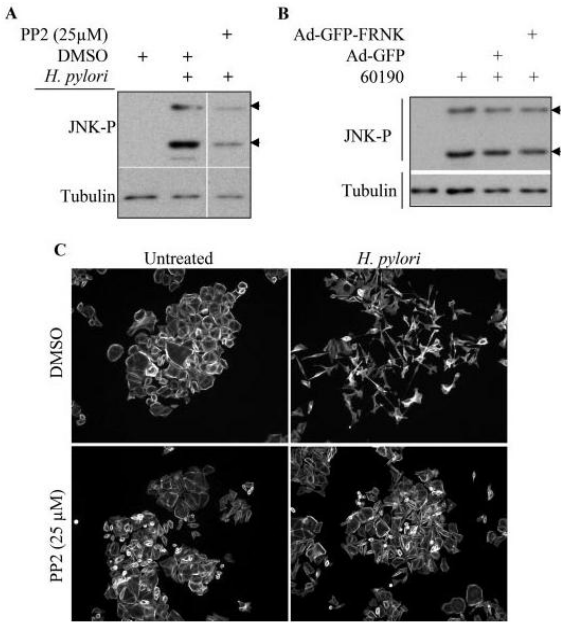

FIGURE 4. **Src, but not FAK, is required for *H. pylori*-induced JNK phosphorylation.** *A*, AGS cells were pretreated with DMSO or PP2 for 2 h before culture alone or with *H. pylori* 60190 for 1 h. Cells were collected and analyzed for phospho-JNK activity by Western blot analysis using phospho-specific antibodies. *B*, AGS cells were incubated overnight with adenovirus encoding GFP-tagged FAK-related non-kinase (Ad-GFP-FRNK) or GFP alone (Ad-GFP) as a control (m.o.i. of 10). Cells were then co-cultured for 1 h with *H. pylori* 60190. Lysates were analyzed for JNK activity. Tubulin was also probed as a load control. *Arrowheads* indicate the 54-kDa JNK2/3 isoforms and the 46-kDa JNK1 isoform. *C*, cells pretreated with either DMSO or PP2 were incubated overnight alone or with *H. pylori* 60190, and cells were fixed and stained for F-actin.

performed, and Fig. 3*A* shows that incubation with the β1 blocking antibody significantly, though not completely, blocked JNK phosphorylation. Scatter assays were also performed, and Fig. 3*B* shows that pretreatment with AIIB2 significantly blocked the robust scattering phenotype induced by *H. pylori* alone, although the cell colonies loosen up, indicative of incomplete inhibition of motility. These data demonstrate, however, that β_1 integrin signaling is required for CagA-independent JNK phosphorylation and cell scattering.

Kwok *et al.* (38) also show that *H. pylori*-mediated β_1 integrin stimulation results in activation of both Src and FAK. These two kinases form a signaling complex that mediates downstream integrin signaling (40). Therefore, we tested if Src and FAK influenced JNK phosphorylation and cell scattering. In Fig. 4*A*, cells pretreated with the Src inhibitor, PP2, showed a significant decrease in *H. pylori*-stimulated JNK phosphorylation compared with DMSO alone. Additionally, PP2 blocked *H. pylori*-induced cell scattering, as shown in Fig. 4*C*. These data demonstrate that Src is required for *H. pylori*-induced JNK activation and cell scattering.

To address the role of FAK in JNK activation and cell scattering, we employed a protein consisting of the carboxyl-terminal noncatalytic domain of FAK, termed FAK-related non-kinase (FRNK). FRNK is a separate protein endogenously expressed which, when overexpressed, inhibits FAK activity (41). Therefore, to inhibit FAK in our studies, GFP-labeled FRNK or GFP alone was expressed in AGS cells using a replication-defective adenovirus construct (Ad-GFP-FRNK and Ad-GFP, respectively) prior to co-culture with *H. pylori* 60190 for 1 h. As shown in Fig. 4*B*, FRNK expression did not inhibit *H. pylori*-induced JNK phosphorylation compared with the GFP control construct, demonstrating that FAK is not required for JNK activity. In Fig. 5, however, cells that expressed GFP-FRNK showed a striking inhibition of *H. pylori*-induced cell scattering. These cells showed high nuclear FRNK localization and loss of cortical actin and stress fibers, compared with GFP control cells, which still scattered in response to *H. pylori*. These data demonstrate that although JNK requires Src activity, JNK phosphorylation occurs independent of FAK. But inhibition of both Src and FAK blocks the *H. pylori*-induced morphogenic response.

H. pylori Stimulates JNK through a PI3K, Cdc42-, and Rac1-independent Mechanism—A key pathway that regulates cancer cell survival is the PI3K pathway, which is known to play a role in integrin signaling and JNK activation (42, 43). To determine whether PI3K regulates JNK activity, AGS cells were pretreated with LY294002 prior to co-culture with *H. pylori* 60190 for 1 h. Western blot analysis was performed on these lysates, and supplemental Fig. 2*A* shows that *H. pylori*-induced JNK activity was not affected by LY294002, which demonstrates that JNK is not regulated by PI3K activity.

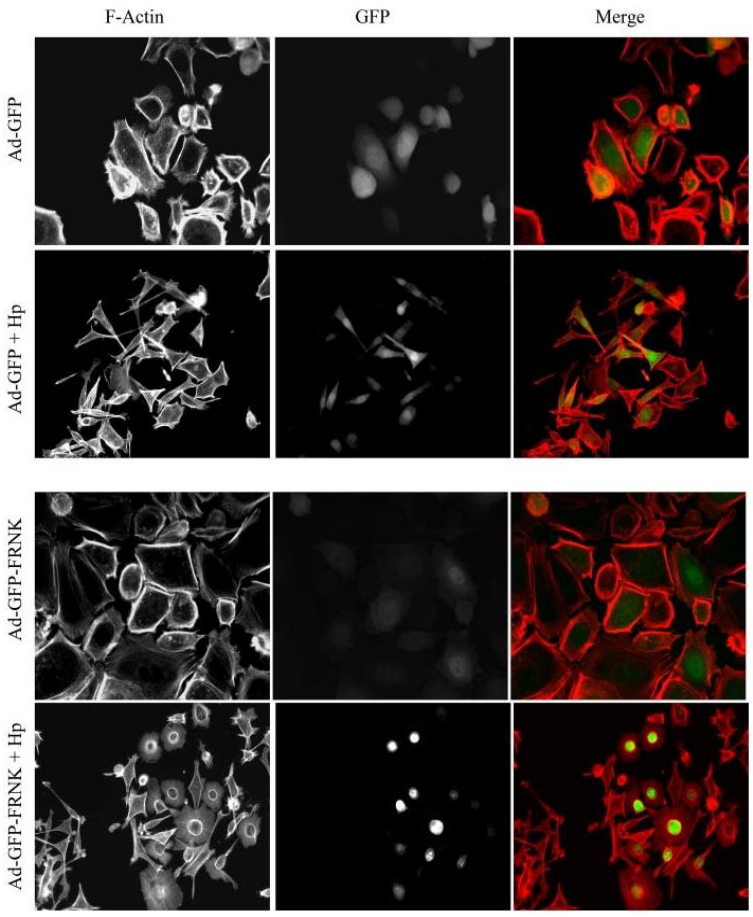

FIGURE 5. ***H. pylori*-induced cell scattering requires FAK activity.** AGS cells were incubated overnight with Ad-GFP-FRNK or Ad-GFP. Cells were cultured overnight alone or with *H. pylori* 60190. Cells were then fixed and stained for F-actin. Images of GFP-labeled and F-actin-labeled cells were then captured.

The Rho GTPases Cdc42 and Rac1 are well known regulators of actin cytoskeletal changes; Cdc42 mediates filopodial protrusions at the leading edge of motile cells, and Rac1 controls the lamellipodial sheets that extend the cell forward (44). Yamauchi *et al.* (45) used dominant-negative constructs to show that Cdc42 and Rac1 also regulate neuronal extension by stimulating JNK activity. Additionally, *H. pylori* is known to activate both Cdc42 and Rac1 (46). To determine whether these Rho GTPases play a role in *H. pylori*-dependent JNK activation and cell motility, siRNA technology was applied to knockdown expression of Cdc42 or Rac1 for Western blot analyses and scatter assays. AGS cells were transiently transfected with Cdc42 siRNAs or control siRNAs (luciferase), co-cultured with *H. pylori* 60190 for 1 h, and lysates were collected for Western blot analysis. The supplemental Fig. 2*B* shows that Cdc42 siRNA (siCdc42)-treated cells show considerable loss of Cdc42 compared with control cells (siLuc), but no difference in *H. pylori*-stimulated JNK phosphorylation was observed between the two transfection conditions. Furthermore, supplemental Fig. 2*C* shows that there was also no difference in *H. pylori*-stimulated cell scattering between siCdc42 (*panel ii*) and siLuc cells (*panel i*), demonstrating that Cdc42 is not required for JNK phosphorylation or cell scattering.

To determine whether JNK activity is regulated by *H. pylori*-dependent Rac1 activation, AGS cells stably expressing Rac1 shRNAs were generated by lentiviral delivery (see "Experimental Procedures"). The supplemental Fig. 2*D* shows that Rac1 shRNA-expressing cells (shRac1) exhibit significant Rac1 protein knockdown compared with GFP shRNA-expressing control cells (shGFP). Additionally, in supplemental Fig. 2, *E* and *F*, respectively, Rac1 knockdown did not affect *H. pylori*-induced JNK phosphorylation or cell scattering. These data demonstrate that *H. pylori* stimulates JNK through a Cdc42- and Rac1-independent mechanism.

H. pylori Stimulates MKK4 Phosphorylation but Activates JNK Independent of MKK4—The MAP kinase kinase 4 (MKK4) is one of two MAP kinase kinases identified as direct upstream JNK kinases (47, 48). To determine whether *H. pylori* activates MKK4 to stimulate JNK activation, AGS cells were co-cultured with the indicated *H. pylori* strains and collected for Western blot analysis. As shown in supplemental Fig. 3*A*, MKK4 was phosphorylated in response to *H. pylori* 60190, *H. pylori* 60190Δ*cagA*, and *H. pylori* 60190Δ*vacA* but not *H. pylori* 60190Δ*cagE*, demonstrating that MKK4 is activated in a CagA-independent, TFSS-dependent manner similar to JNK. To further determine whether MKK4 was required for JNK phosphorylation, AGS cells stably expressing shRNAs against MKK4 (shMKK4) were generated. The supplemental Fig. 3*B* shows successful and efficient MKK4 expression knockdown compared with non-target lentivirus-infected cells (shCtrl). Also, although *H. pylori*-stimulated phosphorylation of JNK isoforms 2 and 3 (JNK2/3) was unaffected in the shMKK4 cells (supplemental Fig. 3*B*, *upper arrowhead*), JNK isoform 1 (JNK1) phosphorylation was significantly reduced in the knockdown cells compared with shCtrl cells (*lower arrowhead*). This suggests that *H. pylori*-stimulated MKK4 demonstrates specificity for the JNK1 isoform. Cells were then co-cultured with *H. pylori* 60190 for scatter assays, and supplemental Fig. 3*C* shows that shMKK4 cells still scattered in response to *H. pylori*, suggesting that neither MKK4 nor JNK1 phosphorylation are required for the *H. pylori*-induced morphological response.

H. pylori Stimulates JNK Phosphorylation and Cell Scattering through an MKK7-independent Mechanism—Besides MKK4, only MKK7 is known to directly regulate JNK activity (48). Therefore, one would predict that if JNK is required for cell scattering and shMKK4 cells show a loss of JNK1 phosphorylation but the cells still scatter, then MKK7 would regulate JNK2/3 activity and be required for *H. pylori*-dependent cell scattering. Indeed, when JNK2/3 activity was pharmacologically inhibited using an inhibitor specific to isoforms 2 and 3 but not 1, *H. pylori*-stimulated cell scattering was significantly blocked, although the normal phenotype was not completely restored; this demonstrates a requirement for JNK2/3 activity in *H. pylori*-stimulated cell scattering (supplemental Fig. 4*A*). Stable AGS cells expressing shRNAs against MKK7 were then generated (shMKK7) to address the role of MKK7 in *H. pylori*-stimulated signaling and cell scattering. Surprisingly, supplemental Fig. 4*B* shows no difference between shCtrl and shMKK7 cells in the phosphorylation of any JNK isoforms in the presence of *H. pylori* 60190, although shMKK7 cells showed significant loss of MKK7 expression. Additionally, shMKK7 cells scattered to a similar extent to control cells in response to *H. pylori* 60190 (supplemental Fig. 4*C*), demonstrating that *H. pylori* induces JNK phosphorylation and cell scattering independent of MKK7.

JNK Phosphorylation and Cell Scattering Occur Independent of Both MKK4 and MKK7—Although the JNK2/3 inhibitor blocked *H. pylori*-dependent cell scattering, MKK7 expression knockdown failed to prevent JNK2/3 phosphorylation. To test the hypothesis that loss of one of these kinases is complemented by the presence of the other, AGS cells stably expressing both MKK4 and MKK7 shRNAs (shMKK4/7) cells were generated by lentiviral delivery and used in co-culture experiments. The supplemental Fig. 5*A* confirms that MKK4 and MKK7 expression is almost completely abrogated in shMKK4/7 cells, and the supplemental Fig. 5*B* indicates that *H. pylori*-induced JNK 2/3 phosphorylation was unaffected in these both control and shMKK4/7 cells. Additionally, shCtrl cells or shMKK4/7 cells were co-cultured with *H. pylori* 60190 for 18 h and fixed and stained for F-actin. The supplemental Fig. 5*C* shows that *H. pylori*-stimulated cell scattering was not blocked, demonstrating that *H. pylori*-stimulated JNK2/3 phosphorylation and cell scattering occur independent of both MKK4 and MKK7.

JNK Mediates H. pylori-dependent Cell Motility through Paxillin—A major function of JNK is to regulate activity of the AP-1 transcription factor, which in turn alters gene expression (48–50). Additionally, recent evidence shows that JNK can influence cell motility by activating downstream effectors that stabilize microtubules and regulate actin reorganization and cell adhesion (35, 51, 52). Cells pretreated with the protein synthesis inhibitor, cycloheximide, or the NF-κB inhibitor, Bay11-7082, were unable to block *H. pylori*-stimulated cell scattering in separate experiments, demonstrating that JNK mediates *H. pylori*-induced cell scattering through a gene expression-independent mechanism (supplemental Fig. 6).

Paxillin is a component of focal adhesions, which facilitate attachment of the actin cytoskeleton to the extracellular matrix

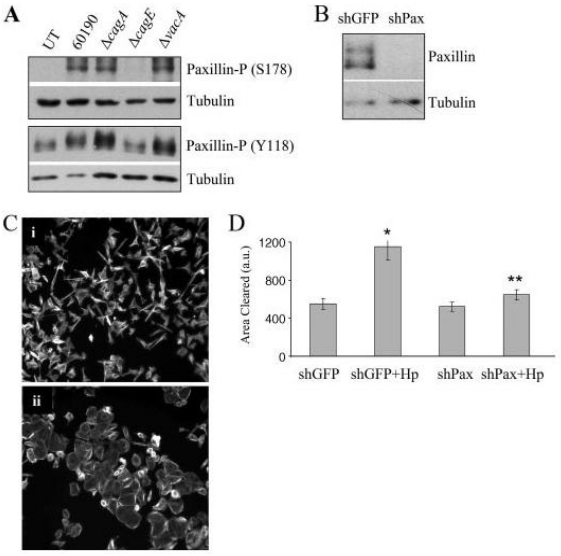

FIGURE 6. ***H. pylori* requires paxillin to induce cell scattering and motility.** *A*, AGS cells were co-cultured with indicated *H. pylori* strains for 1 h, and lysates were collected for Western blot analysis. Paxillin phosphorylation at Ser-178 or Tyr-118 was detected using phospho-paxillin (Ser-178)- or phospho-paxillin (Tyr-118)-specific antibodies. *B*, AGS cells stably expressing shRNAs targeting paxillin (shPax) or GFP (shGFP) were collected for Western blot analysis, and total paxillin was detected using anti-paxillin antibodies. Tubulin was also probed as a load control. *C*, shGFP (*panel i*) or shPax (*panel ii*) cells were co-cultured with *H. pylori* 60190 for 18 h, and fixed and stained for F-actin. *D*, *graph* represents the effects of paxillin expression knockdown on *H. pylori*-induced cell motility compared with control cells in three separate experiments and includes standard error. Data presented as average area cleared per cell from multiple experiments and includes standard error. *, *shGFP+H. pylori versus* shGFP alone ($p < 0.001$); **, *shPax+H. pylori versus* shPax alone ($p > 0.05$; determined by analysis of variance and Tukey's HSD test). *UT*, untreated cells.

(51). Paxillin is phosphorylated at multiple serine and tyrosine residues by different upstream activators to regulate focal adhesion turnover and cell migration (53). Phosphorylation at tyrosine residue 118 (paxillin$^{Tyr-118}$) occurs through Src and FAK in response to growth factors, and serine 178 (paxillin$^{Ser-178}$) is known to be phosphorylated by JNK (35, 51–53). Because of this link between JNK and paxillin, Western blot analyses were performed on AGS co-culture lysates to determine paxillin activation in response to *H. pylori* 60190. As shown in Fig. 6*A*, paxillin was phosphorylated at both Tyr-118 and Ser-178 in a CagA-independent but TFSS-dependent manner. AGS cells stably expressing paxillin shRNAs were generated (shPax) to address the role of paxillin in *H. pylori*-induced cell signaling and motility, and Fig. 6*B* shows loss of detectable paxillin in shPax cells compared with shGFP control cells. Cell scattering and motility assays show a striking decrease in shPax cell scattering and motility compared with shGFP cells in response to *H. pylori* co-culture (Fig. 6, *C* and *D*, respectively), which demonstrates that paxillin is required for *H. pylori*-induced cell scattering and motility.

To determine whether JNK regulates paxillin activity, AGS cells were pretreated with the pan-JNK inhibitor SP600125 (targets isoforms 1–3) prior to co-culture with *H. pylori* 60190 for 1 h. Western blot analysis was then performed on the lysates,

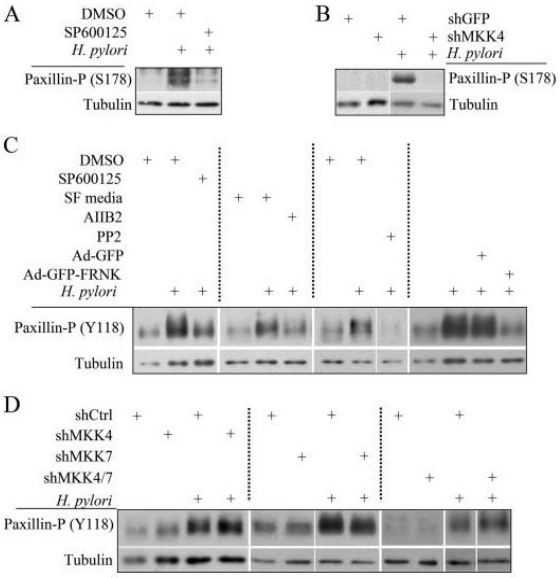

FIGURE 7. ***H. pylori* stimulates β_1-mediated paxillin (Tyr-118) phosphorylation independent of MKK4 or MKK7.** All Western blot analyses were derived from lysates of cells co-cultured with *H. pylori* 60190 for 1 h unless indicated otherwise. *A*, AGS wild-type cells were pretreated with the JNK1/2/3 inhibitor SP600125 (40 μM) or DMSO. *B*, shGFP or shMKK4 cells were cultured alone or with *H. pylori*, and paxillin$^{Ser\text{-}178}$ phosphorylation at was detected using phospho-paxillin$^{Ser\text{-}178}$-specific antibodies. *C*, AGS wild-type cells were pretreated with the DMSO, SP600125 (40 μM), AIIB2 antibody (5 μg/ml), an equal volume of serum-free media (*SF media*), or PP2 (25 μM), or transfected with Ad-GFP-FRNK (m.o.i. of 10) or Ad-GFP (m.o.i. of 10). *D*, untreated shCtrl, shMKK4, shMKK7, or shMKK4/7 cells were co-culture with *H. pylori*, and paxillin$^{Tyr\text{-}118}$ phosphorylation at was detected using phospho-paxillin$^{Tyr\text{-}118}$-specific antibodies. Tubulin was also probed as a load control. All blots are representative of multiple experiments.

and Fig. 7*A* shows that phosphorylation at paxillin$^{Ser\text{-}178}$ was significantly decreased in response to the JNK inhibitor. Next, shGFP or shMKK4 cells were co-cultured with *H. pylori* 60190 for 1 h and collected for Western blot analysis to determine whether paxillin$^{Ser\text{-}178}$ phosphorylation is induced by JNK1. As shown in Fig. 7*B*, shMKK4 cells showed no paxillin$^{Ser\text{-}178}$ phosphorylation compared with strong induction in shGFP cells. This suggests that paxillin$^{Ser\text{-}178}$ may be a substrate of JNK1, but phosphorylation at Ser-178 is not required for *H. pylori*-stimulated cell scattering.

Fig. 7*C* shows that paxillin$^{Tyr\text{-}118}$ phosphorylation was also blocked in the presence of SP600125, demonstrating that paxillin$^{Tyr\text{-}118}$ is regulated by JNK activity. Fig. 7*C* also shows that paxillin$^{Tyr\text{-}118}$ phosphorylation is reduced in response to pretreatment with AIIB (β_1 blocking antibody) or PP2 (Src inhibitor) or FRNK expression (endogenous FAK inhibitor) compared with control conditions, demonstrating that *H. pylori* stimulates paxillin$^{Tyr\text{-}118}$ through the integrin signaling pathway. Western blot analysis also showed no change in paxillin$^{Tyr\text{-}118}$ phosphorylation in response to *H. pylori* in shMKK4, shMKK7, and shMKK4/7 cells compared with shCtrl cells, which shows that paxillin$^{Tyr\text{-}118}$ is phosphorylated independent of known upstream kinases (Fig. 7*D*). These data also suggest that this paxillin$^{Tyr\text{-}118}$ may be important in *H. pylori*-dependent motility.

DISCUSSION

In recent years, CagA has been shown to be sufficient to induce cell scattering (hummingbird morphology) when transiently expressed in cells, and CagA is thought to be the major stimulus of cell morphological changes (11, 12, 21). These previous data conflict with our findings in that the JNK pathway, which is required for *H. pylori*-stimulated cell motility, is induced independent of CagA. When comparing experimental systems, we propose that live bacterial co-culture is more physiologically relevant than overexpression of CagA, because proper expression levels and intracellular location are maintained, and any protein modifications occurring inside the bacteria or during translocation that may influence CagA activity are more likely to occur (54, 55). The report by Al-Ghoul *et al.* (32) demonstrating that Cag PAI gene products independent of CagA were required for cell scattering further suggests that CagA alone is insufficient to stimulate cell scattering.

Our data are consistent with reports of TFSS-dependent, but CagA-independent host responses, such as cyclin D1 expression, NF-κB activation, and the production of cytokines (27, 56, 57). Recent studies also showed that *H. pylori* induced cell invasion through a combination of CagA-dependent and CagA-independent (but TFSS-dependent) signaling (22, 32).

Mechanisms of TFSS-dependent signaling independent of CagA have only recently been reported. The cytoplasmic pattern recognition receptor, Nod1, was shown to be activated in response to peptidoglycan that was transported through the *H. pylori* TFSS to stimulate NF-κB activity (27). Evidence also showed that Nod1 can regulate both JNK and p38 activity, although supplemental Fig. 1 shows that AGS cells lacking Nod1 expression still exhibited *H. pylori*-induced JNK activity and cell scattering, suggesting that the TFSS does not activate JNK signaling through Nod1 (36, 37). More recently, Kwok *et al.* (38) showed that the TFSS activates integrin signaling through interaction of the bacterial CagL protein with the $\alpha_5\beta_1$ integrin heterodimer. Furthermore, this interaction resulted in the activation of Src and FAK, both known to participate in integrin-mediated signaling leading to JNK activation (58). Fig. 3 and Fig. 7*C* strongly suggest that the *H. pylori* CagL-β_1 integrin interaction stimulates CagA-independent JNK-mediated cell motility through paxillin. One may argue that the β_1 blocking antibody inhibits focal adhesions, but because the cells still attach to the substratum, we believe that focal adhesions remain intact.

Upon translocation via the TFSS, CagA is phosphorylated at multiple EPIYA residues by Src family kinases and c-Abl (8–10, 59). CagA then causes dephosphorylation of the activation domain of Src, leading to deregulation of multiple cytoskeletal regulatory pathways and cell motility (60, 61). The inactivation of Src by CagA does not conflict with our data showing Src-mediated JNK phosphorylation, because we detect JNK activation within 30 min of stimulation by *H. pylori,* and CagA-mediated Src inactivation occurs after 3–4 h.

We also show that FAK is not required for *H. pylori*-induced JNK phosphorylation, but it is required for paxillin$^{Tyr\text{-}118}$ phosphorylation and cell scattering, which suggests that FAK

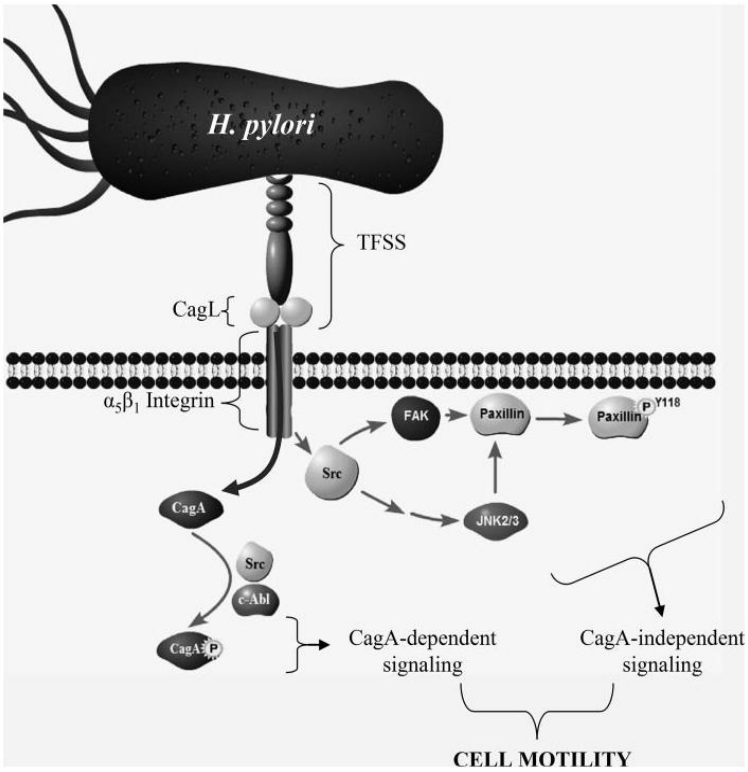

FIGURE 8. ***H. pylori* stimulates cancer cell motility through the combined CagA-dependent and -independent signaling.** CagA is injected into the cell via the $\alpha_5\beta_1$ integrin and associates with known regulators of cell adhesion and motility. CagA-independent activation of the β_1 integrin signaling pathway leads to Src-induced JNK stimulation, which in combination with FAK causes paxillin$^{Tyr\text{-}118}$ phosphorylation. Although both CagA-dependent and CagA-independent signaling pathways are necessary, neither pathway is sufficient by itself for *H. pylori*-induced cell motility.

bypasses JNK to directly target paxillin. This was not surprising, because paxillin$^{Tyr\text{-}118}$ is a known target of FAK (62). Integrin-mediated stimulation of FAK occurs through an undefined mechanism at tyrosine 397, which causes a conformational change that creates a high affinity Src homology 2 domain for Src. Src then mediates further tyrosine phosphorylation of FAK, leading to downstream signaling (40). Therefore, as shown in Fig. 8, we propose that Src-mediated FAK activity leads to paxillin$^{Tyr\text{-}118}$ phosphorylation, which is also stimulated by Src-mediated JNK activation.

Because the integrin-mediated signaling pathway leading to JNK activation is reported to occur independent of Rac1 and PI3K, our data showing that Cdc42, Rac1, and PI3K are not important for *H. pylori*-dependent JNK phosphorylation strengthen our model of JNK activation (39).

H. pylori-dependent JNK2/3 is required for AGS cell scattering (supplemental Fig. 4*A*), but JNK2/3 activation and cell scattering occur independent of MKK4 and MKK7, as shown in supplemental Figs. 3–5. We therefore propose that JNK2/3 phosphorylation and *H. pylori*-dependent cell scattering occur through activation of a third unidentified MAP kinase kinase that targets JNK. Yamauchi *et al.* (58) reported activation of JNK independent of either MKK4 or MKK7 using dominant-negative constructs and termed this unknown kinase MKK-*X*. This hypothesis is conceivable, as JNK isoforms interact with scaffolding proteins that form signaling complexes to promote JNK activation and downstream signaling (48, 63). Because many MAP kinase kinases and MAP kinase kinase kinases, such as MKK1, MKK3, and mixed lineage kinases, are known to be recruited to JNK scaffold proteins, JNK2/3 may be activated by one of these other kinases (48, 63). One of these signaling complexes was reported by Takino *et al.* (63) who showed that the $\alpha_5\beta_1$ ligand, fibronectin, stimulated JNK activity through complex formation of FAK and the JSAP1 scaffold protein. This interaction was enhanced by Src and led to cell migration. These data strengthen the hypothesis of integrin-mediated activation of JNK and the role of scaffold proteins in cell motility.

JNK signaling is emerging as a key mediator in cell migration and invasion. Besides regulating gene expression through the AP-1 transcription factor complex, JNK can activate proteins that regulate microtubule stabilization and focal adhesion turnover (35, 48–50, 64). The supplemental Fig. 6 demonstrates that *H. pylori*-dependent cell scattering does not require *de novo* protein synthesis, which agrees with prior reports of JNK mediating cell migration through a gene expression-independent mechanism (34). We show that *H. pylori*-induced motility requires paxillin expression, and paxillin$^{Ser\text{-}178}$ phosphorylation is dependent upon MKK4 and JNK activity but is not required for cell motility (Figs. 6 and 7 and supplemental Fig. 3). Paxillin$^{Tyr\text{-}118}$ phosphorylation is dependent upon JNK activity, as shown using the pan-JNK inhibitor, SP600125 (Fig. 7*C*). Since the JNK2/3 inhibitor blocked *H. pylori*-induced cell scattering, the fact that paxillin$^{Tyr\text{-}118}$ phosphorylation requires JNK activation and occurs independent of MKK4/7 ablation suggests that JNK2/3 mediates cell motility through Y118 phosphorylation.

Fig. 8 summarizes our model of *H. pylori*-induced cell motility. The process is initiated by the interaction of CagL with the β_1 integrin, which promotes translocation of CagA via the TFSS into tumor cells. β_1 signaling causes Src-mediated CagA phosphorylation (along with c-Abl) to facilitate CagA-dependent signaling that promotes cell motility. Src also stimulates FAK activation, which leads to activation of paxillin. Src also mediates JNK2/3 activation through a mechanism independent of known upstream JNK kinases, and JNK2/3 also stimulates pax-

illin phosphorylation, which promotes focal adhesion turnover necessary for cell motility.

Although these CagA-independent events are necessary, they are insufficient to induce cell scattering and motility without CagA-dependent signaling. Thus, we propose that the combination of CagA-dependent and CagA-independent (but still TFSS-dependent) events is required to stimulate an epithelial-mesenchymal transition-like response in gastric cancer cells in a gene expression-independent manner.

These data demonstrate that the TFSS plays a greater role in host cell physiology than just to deliver CagA from the bacterium into the host cell cytoplasm, and further studies are needed to determine the greater scope of CagA-independent signaling. These studies will also identify additional host cell players that participate in cell motility that may contribute further insight into the mechanisms of gastric cancer progression.

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Note Added in Proof—Since submission, we have generated data demonstrating that the β_1 blocking antibody, AIIB2, does not prevent focal adhesion assembly because we detected no change in the frequency of focal contacts between control and AIIB2-treated cells, as visualized by immunofluorescence microscopy.

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SUPPLEMENTAL FIGURE 1

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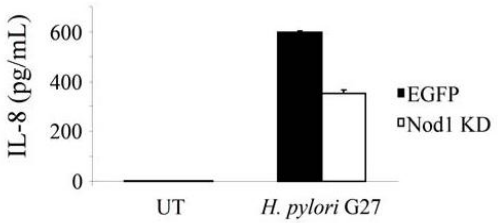

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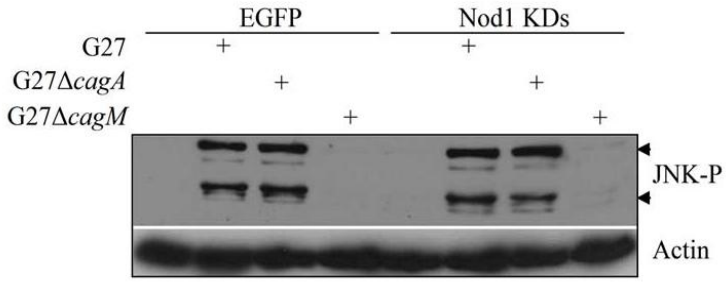

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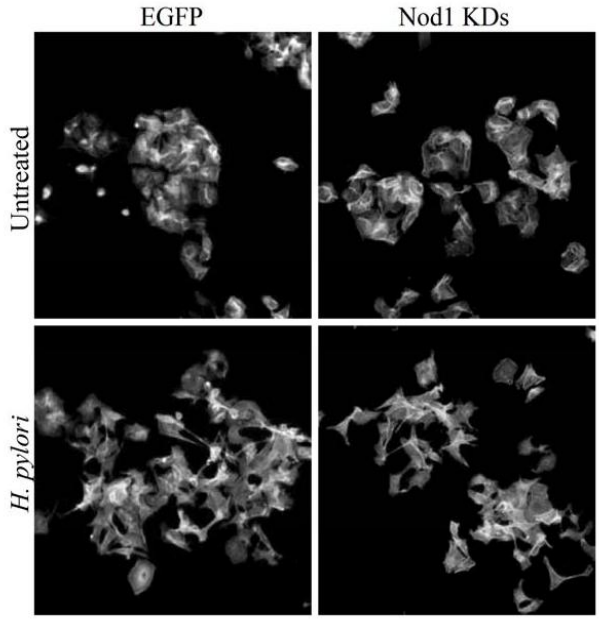

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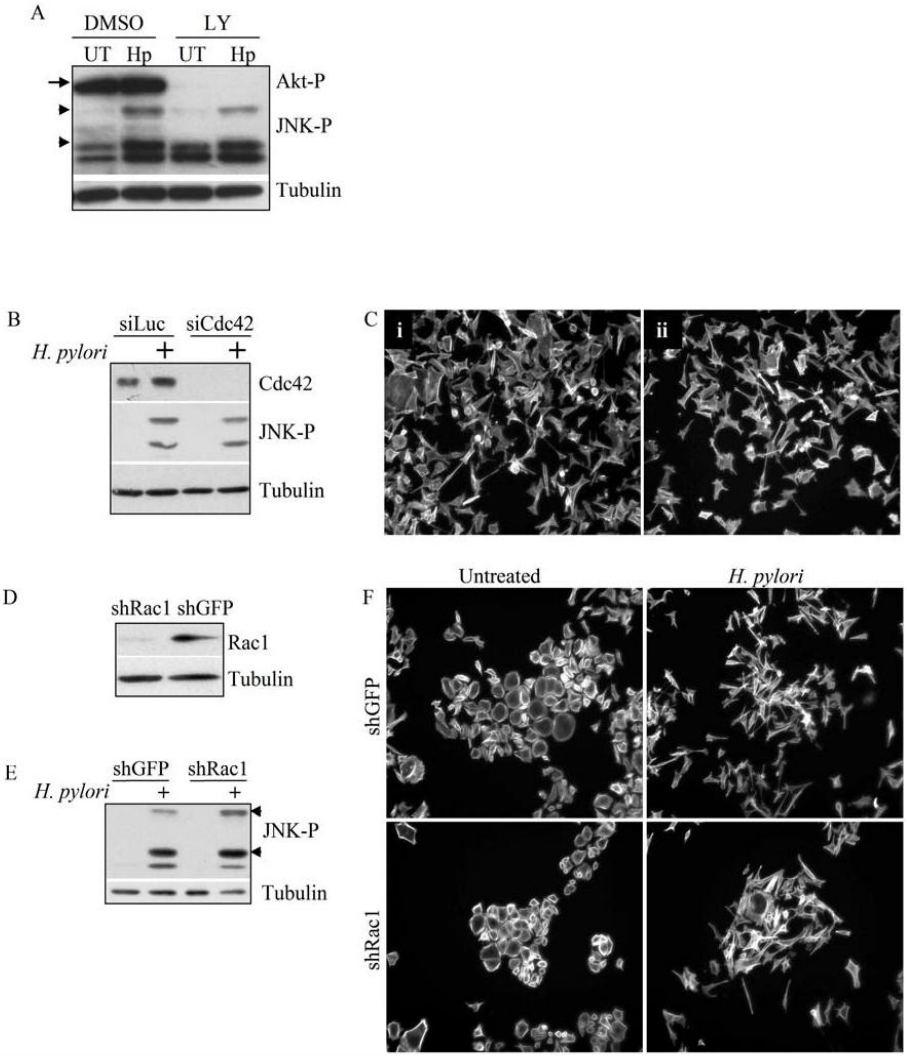

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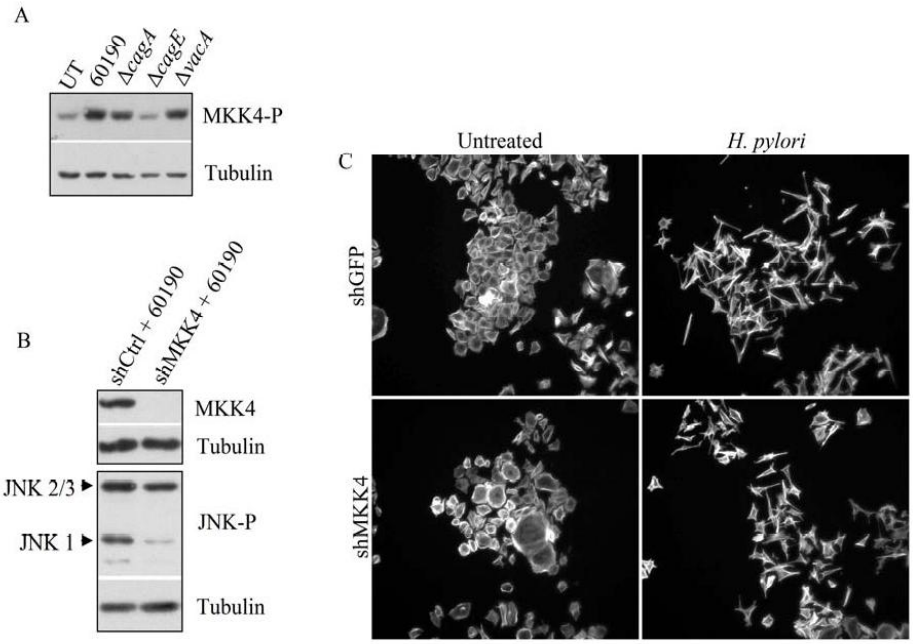

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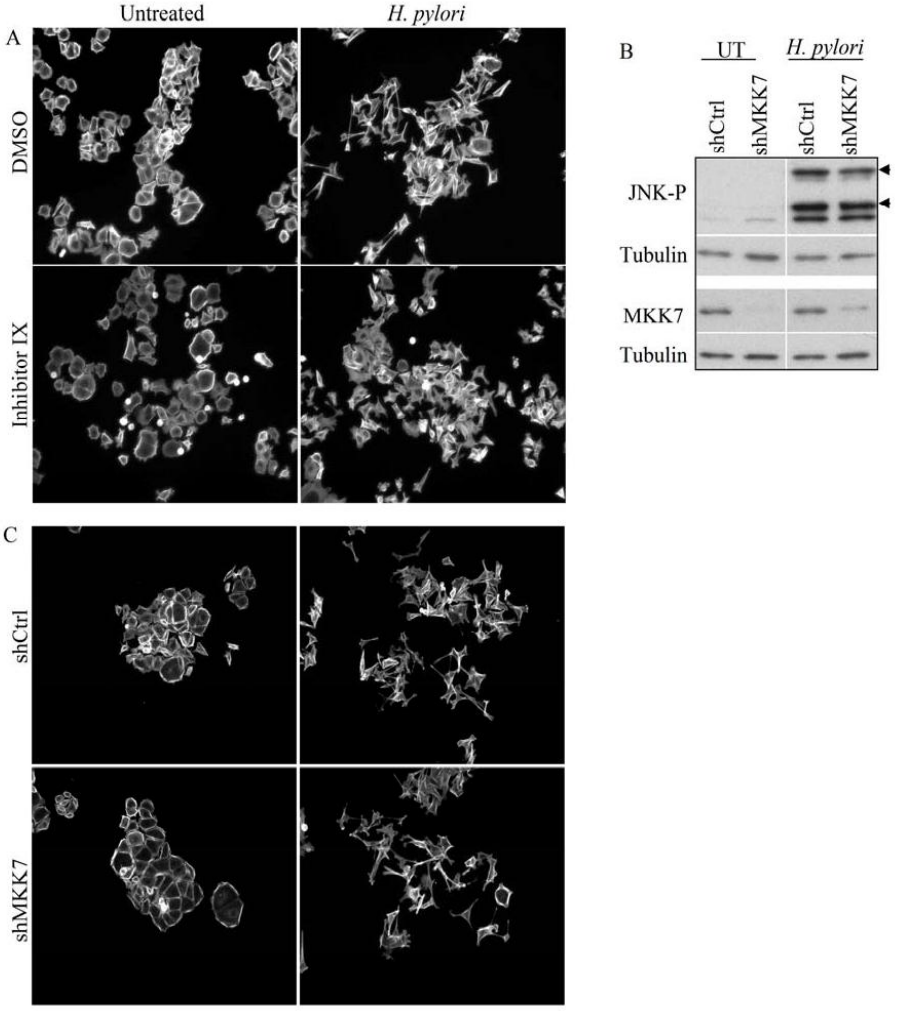

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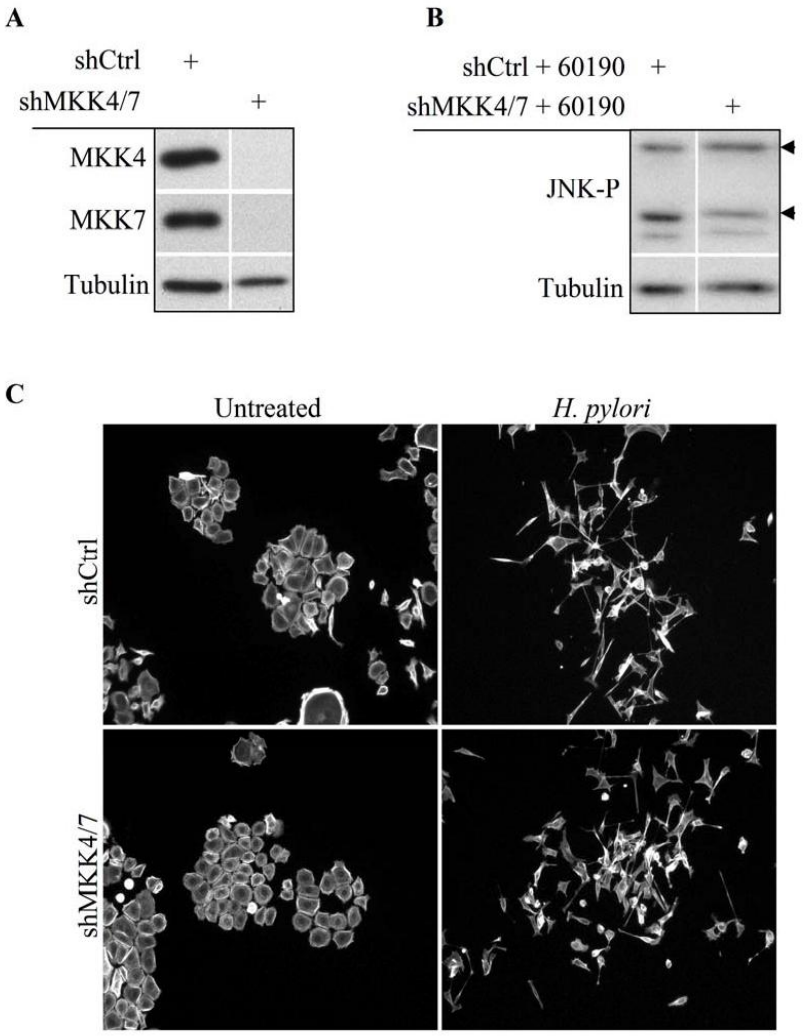

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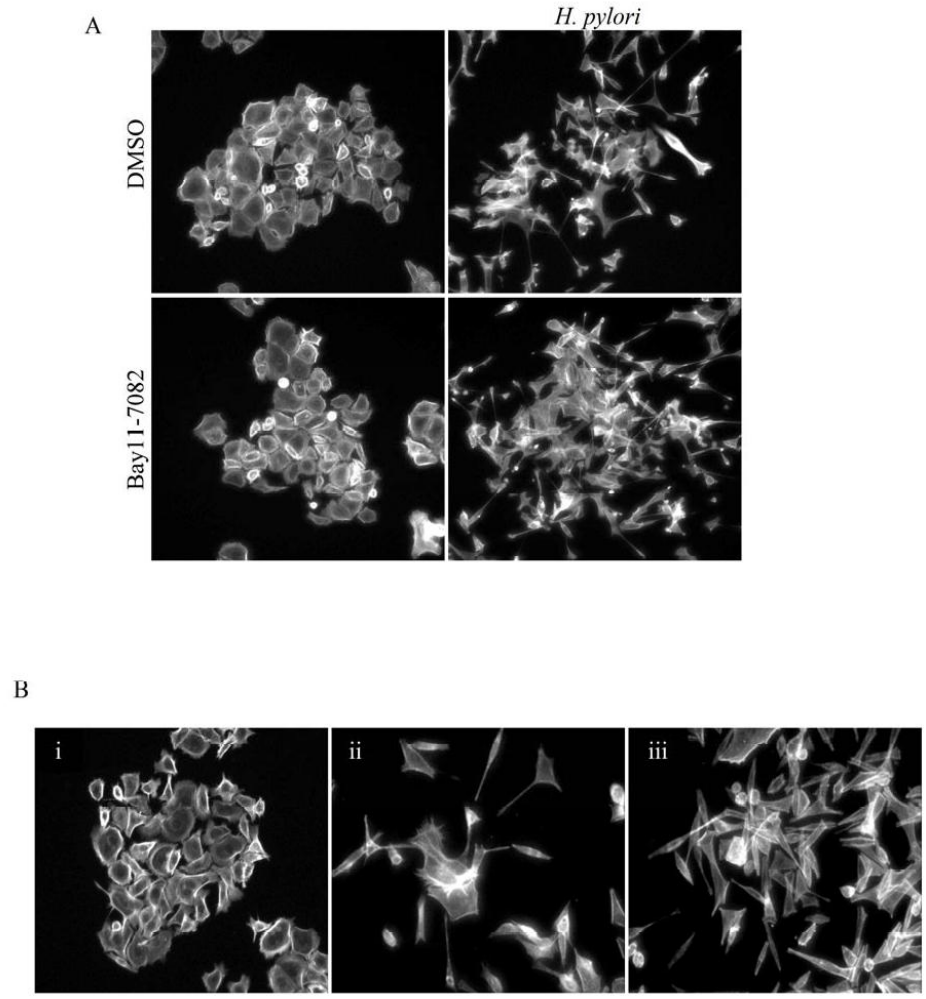


Supplemental Figure 1. Nod1 is not required for *H. pylori*-dependent JNK phosphorylation. (A) EGFP control or Nod1 knockdown (Nod1KD) cells were co-cultured with *H. pylori* strain G27 for 24 hours, and culture supernatant was analyzed for IL-8 secretion. (B) EGFP or Nod1KD cells were co-cultured with indicated *H. pylori* strains for 1.5 hours and lysates were analyzed for JNK phosphorylation by Western blot using phospho-JNK-specific antibodies. F-actin was also probed as a load control. Arrow heads indicate the 54-kD JNK2/3 isoforms and the 46-kD JNK1 isoform. (C) EGFP or Nod1KD cells were cultured alone or with *H. pylori* G27 for 18 hours and fixed and stained for F-actin. ELISA data, blots, and micrographs are representative of multiple experiments.

Supplemental Figure 2. *H. pylori* does not require PI3K, Cdc42 or Rac1 to induce JNK phosphorylation and cell scattering. (A) AGS cells were pretreated with LY294002 or DMSO, and phosphorylated Akt or JNK were detected using phospho-Akt- or –JNK-specific antibodies. (B) AGS cells transiently expressing siRNAs targeting Cdc42 (siCdc42) or luciferase (siLuc) were co-cultured with *H. pylori* 60190. After 1 hour, lysates were collected for Western blot analysis and total Cdc42 or phospho-JNK was detected using anti-Cdc42 or phospho-JNK-specific antibodies. (C) After 18 hours co-culture with *H. pylori* 60190, siLuc (i) and siCdc42 (ii) cells were fixed and stained for F-actin. (D) Western blot analysis was performed on lysates of AGS cells stably expressing shRNAs targeting Rac1 (shRac1) or GFP (shGFP) using anti-Rac1 antibodies. (E) Western blot analysis was performed on shRac1 and shGFP cells co-cultured with *H. pylori* 60190 for 1 hour using phospho-JNK-specific antibodies. Tubulin was also probed as a load control. UT-Untreated cells. Arrow indicates Akt phosphorylation. Arrow heads indicate the 54-kD JNK2/3 isoforms and the 46-kD JNK1 isoform. (F) After 18 hours, cells co-cultured with *H. pylori* 60190 cells were fixed and stained for F-actin. Blots and micrographs are representative of multiple experiments.

Supplemental Figure 3. *H. pylori* induces phosphorylation of MKK4, which is required for JNK1 phosphorylation but not required for cell scattering. (A) Western blot analysis was performed on AGS cell lysates after co-cultured with indicated *H. pylori* strains for 1 hour. Phosphorylated MKK4 was detected using phospho-MKK4-specific antibodies. (B) AGS cells stably expressing shRNAs targeting MKK4 (shMKK4) or non-target shRNAs (shCtrl) were co-cultured with *H. pylori* 60190 for 1 hour and collected for Western blot analysis. Total MKK4 or phosphorylated JNK isoforms were detected using anti-MKK4 and phospho-JNK-specific antibodies. Tubulin was also probed as a load control. (C) Control cells (shGFP) or shMKK4 cells were also co-cultured with *H. pylori* 60190 for 18 hours and fixed and stained for F-actin. Arrow heads indicate the 54-kD JNK2/3 isoforms and the 46-kD JNK1 isoform. UT—untreated cells.

Supplemental Figure 4. *H. pylori* requires JNK2/3 to induce cell scattering, which occurs independent of MKK7. (A) AGS cells were pretreated with DMSO or the JNK2/3-specific inhibitor IX (625 nM) for 2 hour prior to co-culture with *H. pylori* 60190 for 18 hours. Cells were then fixed and stained for F-actin. (B) AGS cells stably expressing shRNAs targeting MKK7 or non-target shRNAs (shCtrl) were co-cultured with *H. pylori* 60190 for 1 hour and collected for Western blot analysis. Total MKK7 and phosphorylated JNK were detected using anti-MKK7 or phospho-JNK-specific antibodies. Tubulin was also probed as a load control. (C) shCtrl and shMKK7 cells were co-cultured with *H. pylori* 60190 for 18 hours and fixed and stained for F-actin. Arrow heads indicate the 54-kD JNK2/3 isoforms and the 46-kD JNK1 isoform. UT—untreated cells.

Supplemental Figure 5. JNK2/3 phosphorylation and cell scattering occur independent of both MKK4 and MKK7. (A) AGS cells stably expressing shRNAs against both MKK4 and MKK7 (shMKK4/7) and shCtrl cells were collected and analyzed for total MKK4 and MKK7 protein by Western blot analysis. (B) shCtrl or shMKK4/7 cells were co-cultured with *H. pylori* 60190 for 1 hour and lysates were analyzed for phospho-JNK activity by Western blot analysis using phospho-specific antibodies. Tubulin was also probed as a load. Arrow heads indicate the 54-kD JNK2/3 isoforms and the 46-kD JNK1 isoform. (C)

shCtrl or shMKK4/7 cells were cultured alone or with *H. pylori* 60190 for 18 hours and then fixed and stained for F-actin. All blots and micrographs are representative of multiple experiments.

Supplemental Figure 6. NF-κB activity and protein synthesis are not required for cell scattering induced by *H. pylori*. (A) Cells were pretreated for 3 hours with DMSO or the NF-κB inhibitor Bay11-7082 (5 μg/mL), followed by co-culture with *H. pylori* strain 60190 for 18 hours. Cells were fixed and stained for F-actin. (B) AGS cells were pretreated with DMSO (i and ii) or cycloheximide (10 μg/mL; iii) prior to co-culture with *H. pylori* 60190 for 18 hours (ii and iii). Cells were fixed and stained for F-actin. Micrographs are representative of multiple experiments.

2.4 Final Discussion

The findings from this chapter support the hypothesis that NOD1 is essential for *cag*PAI-dependent NF-κB activation and translocation during *H. pylori* infection. Furthermore, these data provide evidence for the first time that the serine threonine kinase, RICK, is likely to be involved in NOD1-dependent responses to *H. pylori*. This is consistent with the canonical model of the NOD1/NF-κB signal transduction pathway previously described by other groups. According to that model, NOD1 associates with RICK following peptidoglycan recognition (124, 143-144). Interestingly, however, a recent study reported that RICK was dispensable for NOD1 responses to *H. pylori* (282). In that study, however, the workers also found RICK to not be required for NOD1 signalling to its minimal agonist, iE-DAP, a finding that is not consistent with a large body of work in the field. In any case, RICK was recently shown to phosphorylate TAK1 (283), which in turn induces the activation of both NF-κB and MAPKs (283-284). In this way, TAK1 may be the kinase responsible for downstream NOD1-dependent p38 and ERK MAPK activation during *H. pylori* stimulation.

Intriguingly, the effect of NOD1 knock-down on p38 and ERK phosphorylation was found to be transient. Diminished p38 and ERK MAPK phosphorylation was restored to levels seen in wild-type cells after a period of time, indicating the T4SS may compensate for the lack of NOD1. These findings were supported by another study, which found a direct role for the type-IV pilus in the induction of pro-inflammatory signalling cascades, including Src kinases, via physical interaction with the host cell (285). In this way, work performed with our collaborators identified that *H. pylori*-induced JNK MAPK activation was mediated via the Src kinase (286), which may also explain how the presence of the T4SS was able to activate p38 and ERK at later time-points in cells with reduced NOD1.

Another key finding from our work was the demonstration that AP-1 activation was induced via NOD1-dependent p38 and ERK MAPK phosphorylation. This is the first demonstration of the role of NOD1 in the AP-1 signalling pathway during *H. pylori* stimulation. Furthermore, we found that NOD1-dependent AP-1 activation, in combination with NF-κB, was essential for maximal cytokine production by epithelial cells in response to the pathogen. Interestingly, *in vivo* studies have reported NF-κB activation in the gastric

epithelium of *H. pylori*-infected individuals, which was shown to correlate with IL-8 production, neutrophil infiltration and gastritis (201, 287-289). Furthermore, AP-1-dependent gene expression was also found to be elevated in *H. pylori*-infected patients (290). Consistent with these data, a significant increase in ERK phosphorylation was observed in gastric biopsies from patients infected with $CagA^+$ *H. pylori*, whereas individuals infected with $CagA^-$ isolates had no detectable ERK phosphorylation (291). Whether or not these effects were directly mediated by CagA or the *cag*PAI, however, are unclear. Interestingly, we found that CagA had a small but significant effect on both ERK and p38 MAPK activation at certain time points post-stimulation. While CagA was not required for JNK activation at any stage of stimulation, work performed with our collaborators identified that both CagA and JNK were essential for *H. pylori*-induced epithelial scattering and elongation. Furthermore, given that NOD1 was dispensable for JNK activation by *H. pylori*, we found that NOD1 was also not involved in the induction of the aforementioned 'hummingbird' phenotype.

These data confirm the importance of NOD1 in the rapid induction of pro-inflammatory responses of gastric epithelial cells to *H. pylori*. Furthermore, we have demonstrated for the first time the ability of NOD1 to induce MAPK phosphorylation and downstream AP-1 activation (Figure 2.1). These signalling cascades that are rapidly initiated in the acute phase of infection are critical for the recruitment of immune cells to the site of infection and as a result, are likely to be important for the development of *H. pylori* specific immune responses. Indeed, our *in vitro* data suggest that both the *cag*PAI and NOD1 may be important for transcription factor activation *in vivo* and further studies are warranted to verify this.

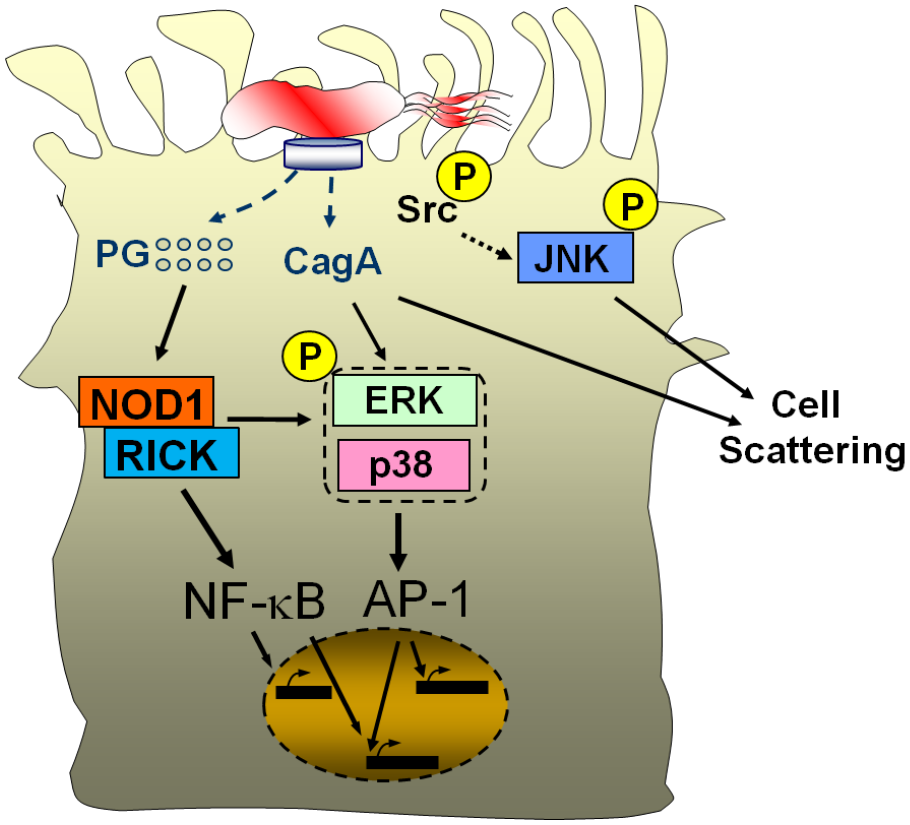


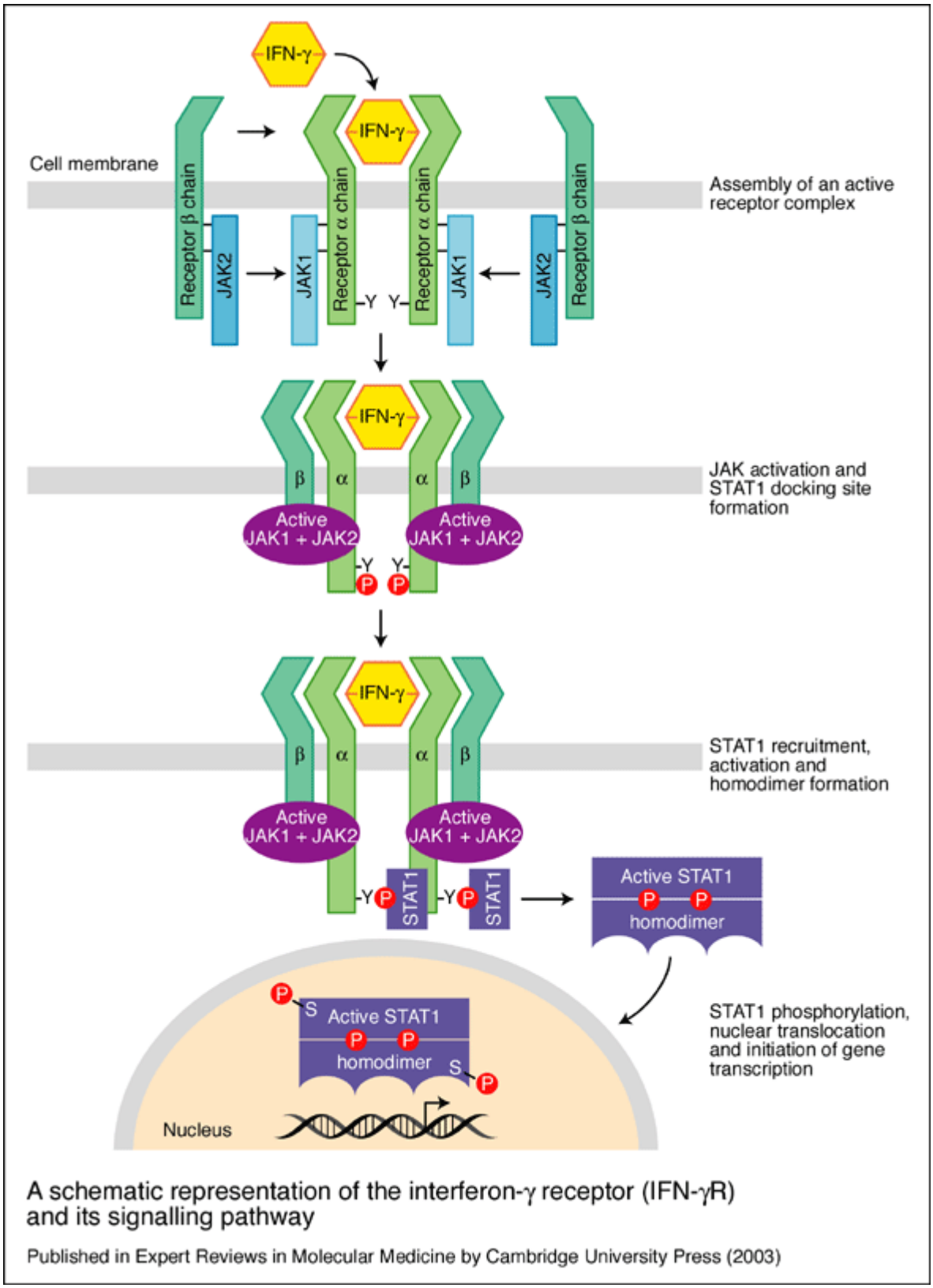
Figure 2.1 NOD1- and *cag*PAI-dependent induction of MAPK signalling pathways in gastric epithelial cells by *H. pylori.*

CHAPTER 3. NOD1 enhances gastric epithelial cell responses to IFN-γ

3.1 Introduction

We have previously shown that in addition to NF-κB activation, NOD1 is essential for the induction of additional signalling cascades during *H. pylori* stimulation, which are required for maximal pro-inflammatory responses by gastric epithelial cells. These NOD1-dependent responses are critical for the recruitment of inflammatory cells to the gastric mucosa and for the induction of adaptive immune responses to the pathogen (24, 292-293). In this way, NOD1 was shown to be essential for the development of T cell Th1-type antibody responses to peptidoglycan adjuvants and *H. pylori* infection respectively (292). Indeed, *H. pylori* infection rapidly polarises host immune responses to a Th1 phenotype, characterised by the infiltration of IFN-γ-producing $CD4^+$ T cells (237-238). IFN-γ has been shown to be responsible for much of the pathology associated with *H. pylori* infection (240, 242, 294) and given that *cag*PAI^+ positive strains are more often associated with severe disease (30-38), NOD1-dependent recognition of these organisms is likely to be crucial for IFN-γ–dependent inflammatory responses.

Global profiling studies of *H. pylori*-infected tissues or cell lines revealed the up-regulation of IFN-γ-responsive genes or genes involved in IFN-γ-signalling (295-297), suggesting that *H. pylori* infection may augment epithelial cell responsiveness to IFN-γ. Furthermore, IFN-γ has been shown to upregulate NOD1 expression in intestinal epithelial cells (298), which was shown to be mediated via the expression and binding of the "Interferon Regulatory Factor 1" (IRF1) to the *NOD1* promoter (298). Likewise, NOD1 protein is elevated in gastric epithelial cells and the lamina propria of patients infected with *H. pylori* (299), an effect probably mediated by the abundance of IFN-γ at the site of infection. Whether enhanced NOD1 expression sensitises epithelial cells to ongoing *H. pylori* stimulation is unknown.



A schematic representation of the interferon-γ receptor (IFN-γR) and its signalling pathway

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Figure 3.1 IFN-γ / STAT1 signalling pathway.

IFN-γ signals via the Jak/STAT pathway following ligand binding to multimerised IFN-γ receptor chains (α and β) (300). This process induces the phosphorylation of receptor-associated Jak1 and Jak2 molecules (301-302), which allows binding and activation of STAT1 (303-304). STAT1 homodimers associate via reciprocal SH2 domains and translocate to the nucleus and bind specific "Gamma-activated sequences" (GAS) in the promoters of a number of genes to induce expression (305). In particular, the *IRF1* promoter contains a GAS sequence and is strongly upregulated upon IFN-γ treatment (306) (Figure 3.1). In the context of *H. pylori* infection, there is conflicting data regarding the role of STAT1 in the induction of gastric epithelial cell responses (191-192). While some investigators have reported that *H. pylori* is capable of inducing STAT1 phosphorylation both *in vitro* and *in vivo*, others have found that *H. pylori* actually inhibits IFN-γ-mediated activation of this pathway (192) (307).

While both NOD1 and IFN-γ are known to be crucial mediators of pro-inflammatory responses during *H. pylori* infection, direct cross-talk between these respective pathways has yet to be thoroughly investigated, despite a potential regulatory link between both factors. To study this, an artificial *in vitro* co-culture system was employed to re-create a basic model of the early stages of infection in gastric epithelial cells. In addition, the expression of various genes involved in NOD1- and IFN-γ-dependent host responses to *H. pylori* were analysed in gastric biopsies from infected and uninfected patients with differing degrees of disease severity. This series of experiments was designed to determine the ability of *H. pylori* to directly activate components of the IFN-γ signalling pathway and investigate the possibility of a positive feedback loop between NOD1-dependent recognition of *cag*PAI^{+} *H. pylori* and Th1/IFN-γ responses in the gastric mucosa.

3.2 Materials and Methods

3.2.1 Cell culture and reagents

The human gastric epithelial cell lines, MKN28 (308) and AGS (309), as well as AGS cells stably expressing shRNA to either the Caspase-activation and Recruitment Domain (CARD) of *NOD1* (AGS siNOD1) (310) or an irrelevant gene, Enhanced Green Fluorescent Protein, *EGFP* (AGS control), were cultured in RPMI 1640 (Gibco, VIC, Australia) containing 10% FCS (Thermo Electron, VIC, Australia). The human embryonic kidney cell line, HEK293, was cultured in DMEM (Gibco) containing 10% (v/v) FBS. All cell lines were supplemented with 1% (v/v) penicillin-streptomycin (Gibco) and 1% (v/v) Glutamax (Gibco) and grown at 37°C, with 5% CO_2. Additionally, AGS siNOD1 and AGS control cells were supplemented with 400 µg/mL Geneticin (Gibco) to maintain selection.

3.2.2 Bacterial strains and isogenic mutants

H. pylori strains 251 (311) and G27 (80) are clinical isolates and the isogenic mutants 251 Δ*cag*PAI (157), (312), G27 Δ*cagA* and G27 Δ*cagM* were constructed by natural transformation as described previously (24, 39). Bacteria were routinely cultured on blood agar medium (40 g/L Blood Agar Base 2, Oxoid, SA, Australia, 8 % Defibrinated Horse Blood, Oxoid; supplemented with Skirrows selective supplement (155 µg/L polymixin B, 6.25 mg/L vancomycin, 3.125 mg/L trimethoprim, 1.25 mg/L amphotericin B; all from Sigma, MO, USA). Isogenic *H. pylori* mutants were grown on blood agar medium supplemented with 10 µg/mL kanamycin, under micro-aerophilic conditions (313). Liquid broth cultures were incubated overnight at 37°C with shaking at 125 rpm in 25-cm^3 tissue culture flasks (IWAKI, Japan,) containing 10 mL of brain heart infusion broth (Oxoid) with 10% (v/v) Newborn Calf Serum (Gibco).

3.2.3 Transient transfection of expression vectors

AGS, MKN28 and HEK293 cells were seeded in 24-well tissue culture plates at a concentration of $1x10^5$ cells/mL and incubated for 18-24 hours. For STAT1 overexpression assays, cells were transfected with 100 ng/well of STAT1 plasmid (314) or pCDNA3. The

total amount of DNA to be transfected was standardised to 1150 ng/well by the addition of pCDNA3 (24). Transfection was achieved using 4 µL polyethyleneimine (PEI) (Polysciences, PA, USA) per µg of total DNA. Cells were cultured in a final volume of 1 mL complete culture media per well and incubated for 16 hours at 37°C in 5% CO_2. All plasmid DNA was prepared using the PureYield^{TM} Plasmid Midiprep System (endotoxin free; Promega, WI, USA).

3.2.4 Stimulation of epithelial cells with bacteria and/or IFN-γ

For transfections, 16 hours after transfection, cells in RPMI or DMEM not supplemented with antibiotics, were stimulated with wild-type *H. pylori* strains 251, G27 and respective isogenic mutants. Briefly, overnight *H. pylori* broths were washed twice in PBS and pelleted at 1250 x g for 10 minutes. Bacterial pellets were resuspended in RPMI or DMEM and added to AGS, MKN28 or HEK293 cells, respectively, at a multiplicity of infection (MOI) of 1:10, a ratio of 1 gastric epithelial cell to 10 bacterial cells.

For IL-8 assays, cell media was replaced with RPMI or DMEM, as appropriate, after 1 hour of co-incubation. After a further 3 hours of incubation, cells were stimulated with 20 ng/mL IFN-γ (Chemicon, VIC, Australia), and incubated for a further 20 hours.

For Western Blotting analysis of STAT1 phosphorylation in response to co-culture with both *H. pylori* and IFN-γ, MKN28 cells were stimulated with *H. pylori* strains, as described above. After 4 hours of co-culture, 20 ng/mL IFN-γ was added, and cells incubated for a further 2 hours. For assessment of STAT1 phosphorylation in response to *H. pylori* alone, MKN28 cells were stimulated with *H. pylori* 251 and isogenic mutant strains for 30 or 60 minutes.

3.2.5 High Content Immunofluorescence Analysis of STAT1 PTyr701 activation in epithelial cells

AGS or MKN28 cells were seeded in black clear bottom Corning Costar 96-well plates (Corning, NSW, Australia) in 100 µl at $1x10^5$ cells/mL, and incubated for 8 hours at 37^{o}C in 5 % CO_2. Media was replaced with serum-free RPMI for 18 hours prior to stimulation. 30 minutes prior to stimulation, media was replaced with 100 µl per well of Hoescht

(Invitrogen) diluted 1:10,000 in RPMI for 30 minutes. Bacterial cultures, prepared as above, were diluted to 10^6 cfu/mL in RPMI. Cell media was replaced with 100 µL per well of the bacterial suspension at a final MOI of 1:10, or 20 ng/mL IFN-γ. 2 hours after stimulation, 8 % formaldehyde in PBS (100 µl) was added directly to wells and incubated for 15 minutes at room temperature. After formaldehyde fixation, cells were incubated for 10 minutes at –20°C with ice-cold 100 % methanol, prior to 3 washes in PBS. Wells were blocked in 100 µL Blocking Buffer (5 % FCS, 0.03 % Triton-X in PBS) for 30 minutes at room temperature. After 3 washes in PBS, cells were stained for 1 hour in 50 µL of rabbit α-P-STAT1 (Tyr701) (Santa-Cruz, CA, USA), diluted 1:200 in Antibody Dilution Buffer (1 % Bovine Serum Albumin; BSA, 0.03 % Triton-X in PBS), then for 30 minutes in 50 µL of goat-α-rabbit IgG conjugated to Alexa Fluor 488 (Invitrogen), diluted 1:500 in Antibody Dilution Buffer. P-STAT1 (Tyr701) activation in cells was viewed using the Cellomics* ArrayScan VTI HCS Reader (Thermo Scientific), capturing at least 1000 cells or 20 fields per well with the 20x objective lens. Data was analysed using the Nuclear translocation analysis algorithm. Cells with a nuclear to cytoplasmic P-STAT1 staining intensity ratio greater than 1.5 were considered to be activated.

3.2.6 Immunoflourescence

MKN28 cells were seeded onto glass coverslips in 24-well tissue culture plates at $1x10^5$ cells/mL and stimulated with *H. pylori* 251 (MOI 1:10) or 20 ng/mL IFN-γ for 2 hours. Cells were stained as above, and coverslips were viewed using a Leica DMR upright fluorescence microscope (Leica) using a 40x objective lens.

3.2.7 Enzyme linked immunosorbent assay (ELISA)

The levels of IL-8, IP-10 and MIG secreted by AGS, MKN28, and HEK293 cells in the culture supernatants were all determined using the respective BD OptEIA™ Human ELISA kits (All from BD Biosciences Pharmingen, CA, USA), in accordance with manufacturer's specifications. Reactions were stopped by addition of 0.5 M sulphuric acid, and absorbances at 450 nm were measured using the BMG FluoStar Optima, and standard curves were constructed to determine cytokine concentrations in test samples. The minimum detection limits of the assays were 3.125 pg/mL, 7.8125 pg/mL and 15.625 pg/mL, respectively.

3.2.8 Luminex ELISA

The levels of RANTES, MCP-1, Mip-1α and Mip-1β secreted by MKN28 cells in the culture supernatants were determined using the Chemokine Human 10-Plex Panel Luminex® Protein Assay (Invitrogen), in accordance with the manufacturer's instructions.

3.2.9 RNA isolation

Human gastric biopsies, stored in RNA later were placed in TRIzol reagent (Invitrogen) and homogenised on ice using a PRO-200 homogeniser (PRO Scientific, CT, USA). RNA from AGS and MKN28 cells that had been grown in 24-well plates (IWAKI) and stimulated with *H. pylori* for 2 or 4 hours, as appropriate, was purified using the Purelink RNA mini kit (Invitrogen) in accordance with the manufacturer's instructions. RNA was eluted in 50 μL of RNase-free H_2O and DNAse treated using Turbo DNA free kit (Ambion,VIC, Australia). RNA concentrations and purity were determined using the Qubit flourometer (Invitrogen) and visualised using agarose gel electrophoresis.

3.2.10 RT-PCR

RNA (1 μg) was reverse transcribed using random hexamers (Invitrogen) and the SuperScript III reverse transcriptase (Invitrogen), according to the manufacturer's instructions. PCR for *cagA* was performed using the primers listed in Table 3.1. PCR reactions contained 200 μM dNTPs, 300 nM forward and reverse primers, 1 unit Taq polymerase, 1.5 mM $MgCl_2$. Cycling conditions consisted of an initial denaturation for 2 minutes at 95^{o}C, followed by 35 cycles of 1 minute at 95^{o}C, and 1 minute at 50^{o}C, and 1 minute at 72^{o}C, followed by a final extension at 72^{o}C for 5 minutes. PCR products were visualised using 1 % agarose gel electrophoresis.

3.2.11 Quantitative RT-PCR (qRT-PCR)

RNA (1 μg) was reverse transcribed using SuperScript III (Invitrogen), according to the manufacturer's instructions. Primers for the genes encoding IRF1 (*IRF1*), IRF3 (*IRF3*), β-actin (*ACTB*), IFN-γ (*IFNG*), NOD1 (*NOD1*), IL-8 (*CXCL8*), IP-10 (*CXCL10*) and 18S RNA (*18S*) were designed using the Primer Express^{TM} primer design software (Applied

Primer name	Primer sequence 5'-3'	Tm
cagA F	ATAATGCTAAATTAGACAACTTGAGCGA	54
cagA R	TTAGAATCAACAAACATCACGCCAT	54
16S F	GGAGTACGGTCGCAAGATTAAA	52.9
16S R	CTAGCGGATTCTCTCAATGTCAA	53.4
IRF1 F	CCCTGCCAGATATCGAGGAG	55.8
IRF1 R	CTCGCACAGCTGAGCTGC	54.7
IRF3 F	GAGGAATTTCGGCTCTGCC	53.1
IRF3 R	TCCTTGCTCCGGTCCTCTG	55.3
IFNG F	TGTCGCCAGCTAAAACAGG	56.6
IFNG R	TGGGATGCTCTTCGACCTCGA	56.2
CXCL10 F	CACCTTTCCCATCTTCCAAGGG	56.6
CXCL10 R	AGGATGGCAGTGGAAGTCCATG	56.6
18S F	CGGCTACCACATCCAAGG	62
18S R	GCTGGAATTACCGCGGCT	58
NOD1 F	ACGATGAACTGGCAGAGAGTT	55
NOD1 R	GGGAGTCCCCTTAGCTGTGA	64

Table 3.1 Oligonucleotides used in this study

Biosystems, VIC, Australia; Table 3.1). Each reaction mix consisted of 0.5 μL of 1 μM forward and reverse primers (Micromon, VIC, Australia), 5 μl SYBR® GREEN PCR Master Mix (Applied Biosystems, Warrington, UK) and 4 μL cDNA template (diluted 1:50) or genomic DNA standards. The optimal amounts of genomic DNA standards were determined to be 16 ng, 640 pg and 25.6 pg per reaction. Each reaction was made to a final volume of 10 μL with ultrapure distilled H_2O. Reactions were performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems) using the following conditions: 2 minutes at 50°C, 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C, and 60°C for 1 minute. Cycle threshold (Ct) values were calculated as the lowest cycle number producing an exponential increase in PCR product amplification. Following PCR, a melting curve analysis was performed as follows: 95°C for 15 seconds, 60°C for 20 seconds, followed by slow heating at 0.03°C/second up to 95°C. The purity of amplicons was determined by visualisation of a single peak in the melting curve. No peaks were observed in the samples without cDNA or in which reverse transcriptase had not been added. All PCR reactions were performed in triplicate in MicroAmp® Optical 384-Well Reaction Plates (Applied Biosystems). To ensure standardisation of amplification efficiencies for all primer sets, standard curves were constructed by plotting average Ct values against the logarithm of the concentrations of the genomic DNA standards. cDNA concentrations of the target genes for each test sample were determined from the standard curve and normalised to expression of *ACTB* or *18S* RNA, as appropriate.

3.2.12 Western Blotting

MKN28 cells were incubated with *H. pylori* or IFN-γ for the appropriate times. Cells were lysed by the addition of 100 μL boiling Laemmli buffer and subjected to 10 % (v/v) SDS-PAGE. Proteins were transferred to nitrocellulose membrane using the iBlot Dry Blotting System (Invitrogen), followed by membrane blocking using 5% (w/v) skim milk in TBS (Tris Buffered Saline: 2.42 g/L Tris, 8 g/L NaCl, pH to 7.6). Immunodetection of phosphorylated or total STAT1 was performed by incubating membranes with respective anti-STAT1 primary antibodies (P-STAT1 (Tyr701); P-STAT1 (Ser727); Total STAT1; Cell Signaling Technology) at a dilution of 1:1000 in 5% Bovine Serum Albumin (Roche, IN, USA), prepared in TBST. Secondary Goat α-Rabbit (Chemicon, Millipore) antibody was used at a dilution of 1:1000 in 5% skim milk in TBST (TBS with 0.01 % Tween 20).

Westerns were developed using ECL detection reagent (GE Healthcare) and exposed to Super RX film (FUJI).

3.2.13 Gastric Biopsies and Histological Grading

A total of 20 patients presenting with a wide range of gastric symptoms attending the Monash Medical Centre Gastrointestinal and Liver Unit (Investigators: Associate Professor William Sievert and Associate Professor Brendan Jenkins; Clayton, Australia) were recruited for this study. Informed consent was obtained prior to enrolment and the local ethics committee approved the study. Findings in endoscopy and results of histopathological examination of gastric biopsies, classified according to the Sydney classification (315-316), were recorded by Dr. Prithi Bhathal. A total of 6 patients presented with normal gastric histology, 8 patients presented with moderate gastritis and 6 patients, with severe gastritis. A total of 11 patients were positive for *H. pylori*, as detected by qRT-PCR for 16S RNA. Of these, 5 patients were *cagA* positive, as determined by PCR for *cagA*. Of the 12 gastric cancer samples analysed in this study, the majority were distal intestinal-type adenocarcinoma. Three adenocarcinoma patients were positive for *H. pylori*, of which one was infected with a $cagA^+$ strain.

3.2.14 Statistical analysis

The Student's *t* test was used for numerical data, whereas the Mann-Whitney test was used for categorical data as appropriate. p values of < 0.05 were considered statistically significant.

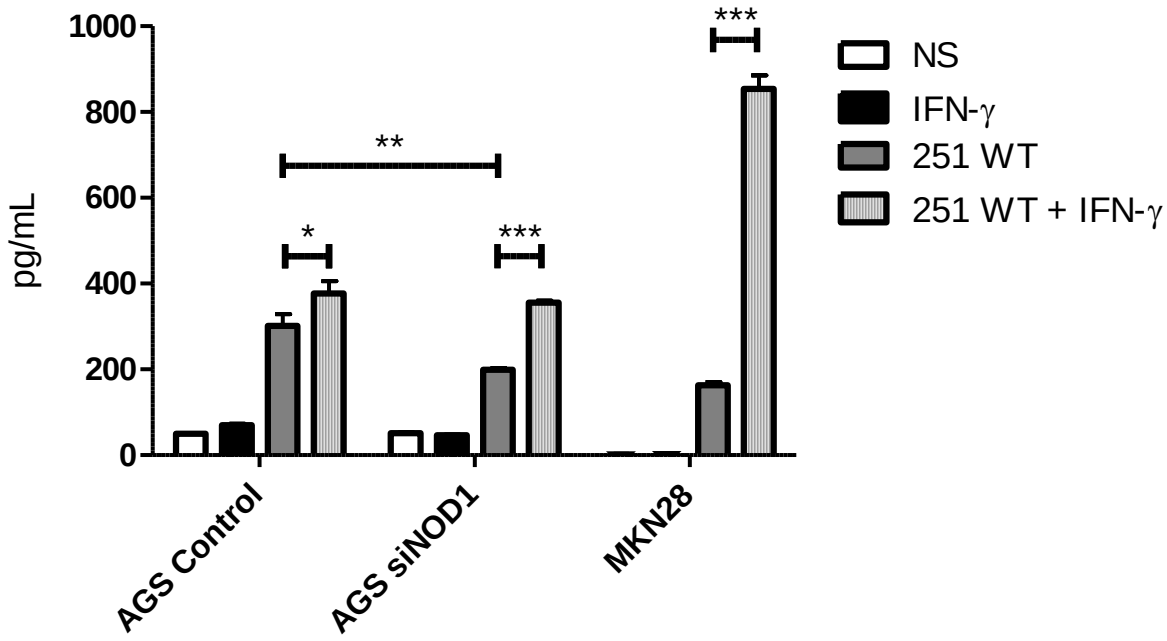
3.3 Results

3.3.1 IFN-γ-dependent responses in different gastric epithelial cell lines.

The first aim of this work was to assess the responses of different gastric epithelial cell lines to *H. pylori* stimulation both with and without combined IFN-γ treatment. Preliminary co-culture experiments were performed to assess the ability of *H. pylori* to induce IFN-γ signalling pathways and/or to augment pro-inflammatory responses to IFN-γ treatment. In addition. AGS siNOD1 cells stably expressing shRNA to knock-down *NOD1* mRNA were employed to determine whether these responses were dependent on NOD1 and the *cag*PAI. In addition, the gastric epithelial cell line MKN28 was also used to further compare responses between gastric cell lines. Briefly, cells were stimulated for 1 hour with wild-type *H. pylori* strain 251 and IFN-γ was added 4 hours later, followed by a further 20 hours incubation. At this point, supernatants were collected and analysed by ELISA to measure the production of pro-inflammatory chemokines. This protocol was designed to artificially mimic an *in vivo* infection, the rationale being that infection of the gastric epithelium with *H. pylori* results in the recruitment of IFN-γ-secreting monocytic cells.

As expected, AGS siNOD1 cells stimulated with wild-type bacteria secreted significantly less IL-8 than AGS control cells (Figure 3.2 a), due to their impaired ability of NOD1-induced NF-κB activation (310, 317). Both AGS control and siNOD1 cells that were co-stimulated with both bacteria and IFN-γ had enhanced IL-8 production as compared to cells treated with either bacteria or IFN-γ alone (Figure 3.2 a, $p<0.05$, $p<0.0001$, and $p<0.0001$ for AGS Control, AGS siNOD1 and MKN28 cells, respectively). Likewise, MKN28 cells stimulated with bacteria and IFN-γ had significantly elevated IL-8 production, which was 5-fold higher in cells stimulated with *H. pylori* alone (Figure 3.2 a). IFN-γ treatment alone failed to stimulate IL-8 production above basal levels in any of the cell lines tested.

a) IL-8 ELISA



b) IP-10 ELISA

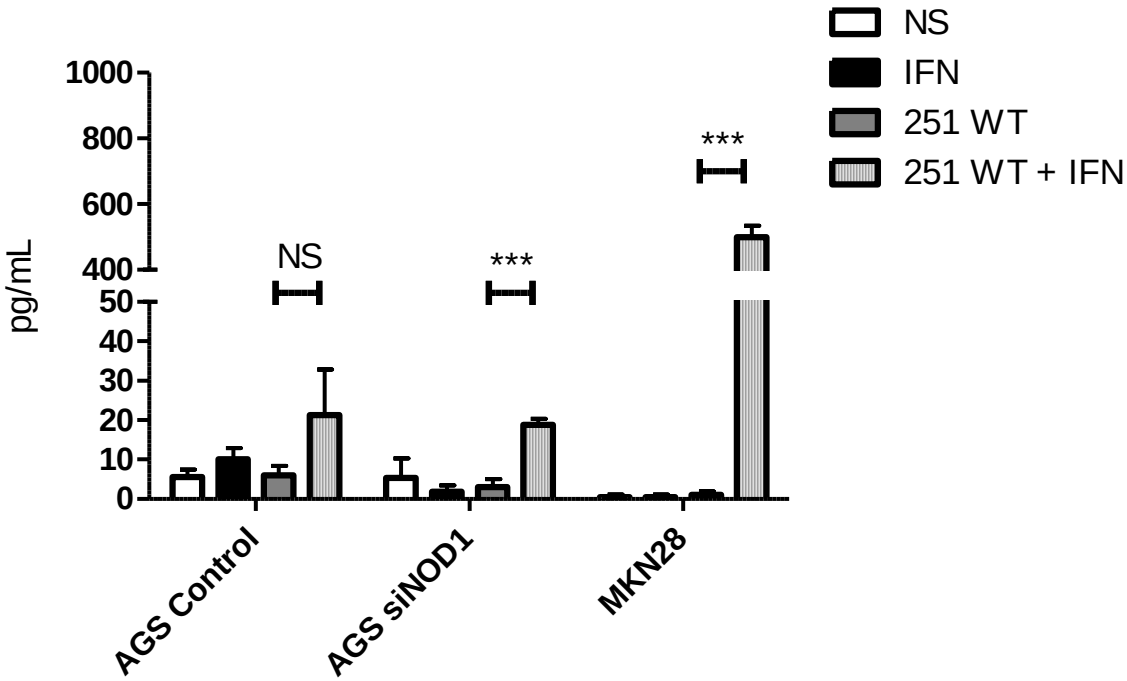


Figure 3.2 Gastric epithelial cell lines respond similarly to stimulation with *H. pylori* and IFN-γ.

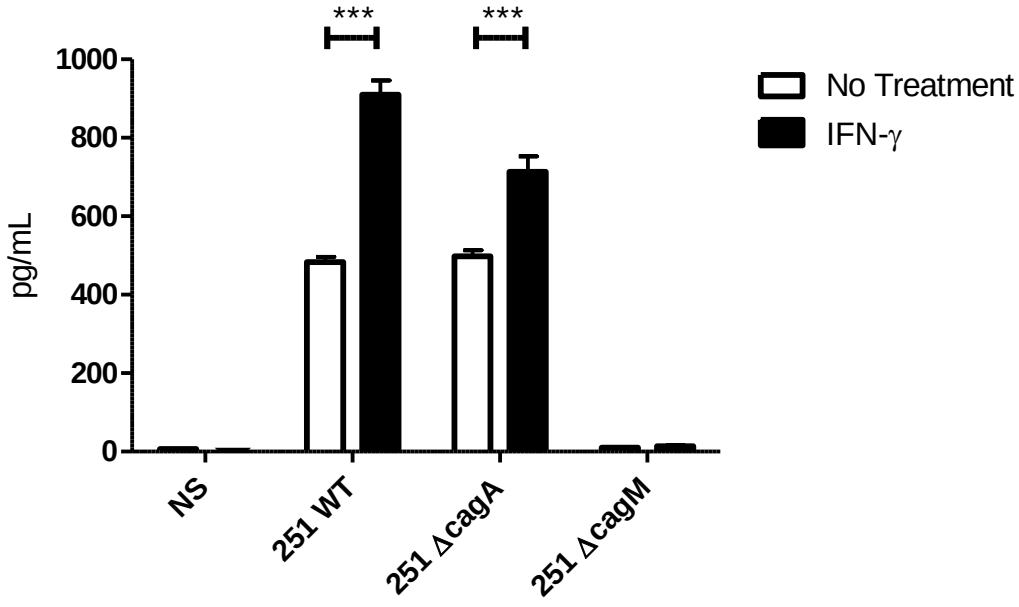
Gastric epithelial cell lines were treated or not with wild-type 251 *H. pylori* for 1 hour. Cells were then washed and replenished with fresh media and incubated for a further 3 hours, followed by the addition of IFN-γ (20 ng/mL). Culture supernatants were collected a total of 24 hours after initial stimulation and analysed by ELISA to determine the secretion of **a)** IL-8 or **b)** IP-10. Error bars indicate standard deviation across samples analysed in triplicate. Results are representative of at least 3 biological replicates. NS: not significant; * $p<0.05$; ** $p<0.01$; *** $p<0.0001$, as analysed by unpaired *t*-test.

We next assessed the ability of these cells to produce "interferon-inducible protein 10" (IP-10) in response to *H. pylori* and/or IFN-γ stimulation. IP-10 is a potent chemoattractant for monocytes and activated T cells (318) and is therefore likely to play an important role in the recruitment of immune cells to the gastric mucosa during *H. pylori* infection. Interestingly, secretion of IP-10 did not rise above basal levels in any of the cell lines following stimulation with either bacteria or IFN-γ alone (Figure 3.2 b). In contrast, combined stimulation with *H. pylori* and IFN-γ resulted in IP-10 production in all cell lines (Figure 3.2 b), indicating that multiple stimuli are required for the expression of this chemokine in gastric epithelial cells. This finding is consistent with that of a previous report (307). In comparison to MKN28 cells, however, AGS control and siNOD1 cells secreted much less IP-10 when co-stimulated with bacteria and IFN-γ (Figure 3.2 b, $p<0.0001$), suggesting that AGS control and siNOD1 cells may be less responsive to IFN-γ stimulation.

3.3.2 IFN-γ augments epithelial cytokine responses in a *cag*PAI-dependent manner.

As MKN28 cells were shown to induce robust chemokine responses to IFN-γ and *H. pylori* co-stimulation, the following series of experiments in this cell line were designed to examine the role of the *cag*PAI in IFN-γ signalling responses. Both wild-type and isogenic *ΔcagA* mutant *H. pylori* bacteria were equally able to stimulate the production of large quantities of IL-8, whilst the isogenic *ΔcagM* mutant, which lacks a functional T4SS (21, 157), was unable to do so (Figure 3.3 a). These results confirm the requirement of the *cag*PAI and T4SS for cytokine production by epithelial cells, whilst demonstrating that CagA is not required for these responses. Co-stimulation of cells with *H. pylori* and IFN-γ resulted in significant increases in IL-8 production, although this effect too was dependent on the *cag*PAI, as combined stimulation of cells with *H. pylori* Δ*cagM* and IFN-γ failed to induce IL-8 production (Figure 3.3 a).

a) IL-8 Production



b) IP-10 Production

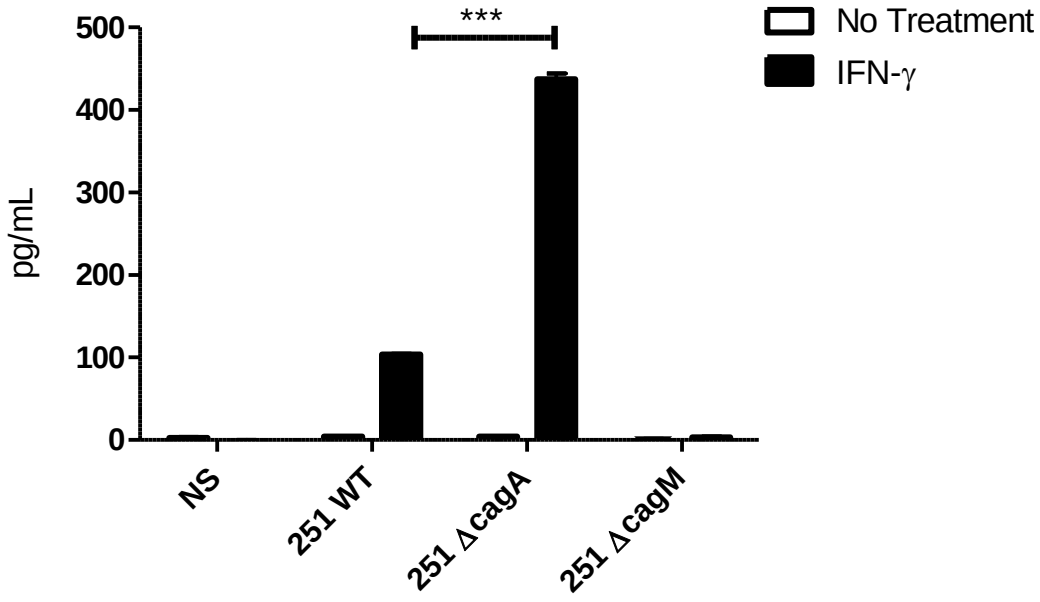
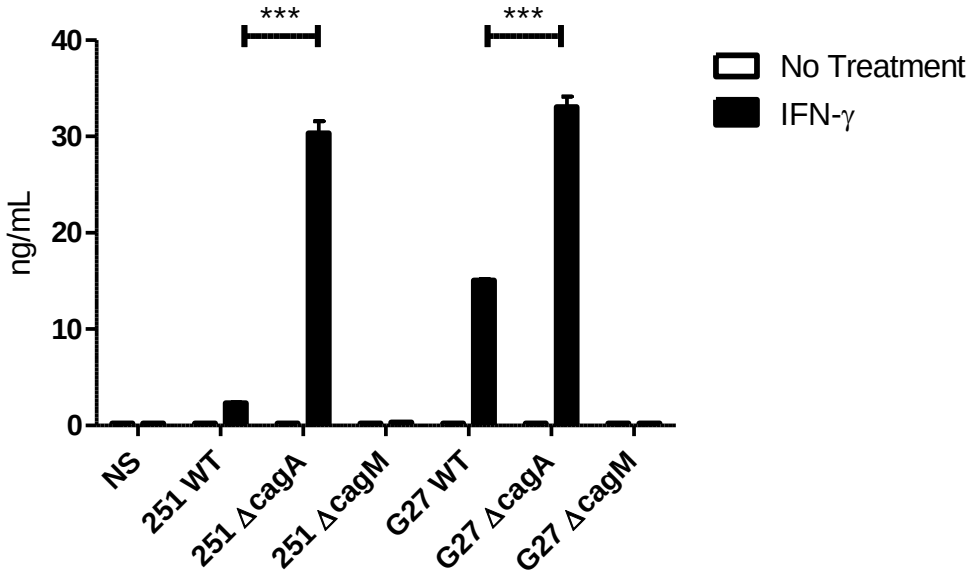


Figure 3.3 *H. pylori* enhances chemokine responsiveness of MKN28 cells to IFN-γ in a *cag*PAI-dependent manner.

MKN28 cells were treated or not with wild-type 251 *H. pylori* or isogenic *ΔcagA* or *ΔcagM* mutants for 1 hour. Cell media was then replaced with fresh media and incubated for a further 3 hours, prior to the addition of IFN-γ (20 ng/mL). Culture supernatants were collected a total of 24 hours after initial stimulation and analysed by ELISA to determine the secretion of **a)** IL-8 or **b)** IP-10. Error bars indicate standard deviation across samples analysed in triplicate. Results are representative of at least 3 biological experiments. ***; $p<0.0001$, as determined by unpaired *t*-test.

a) IP-10 Production



b) MIG Production

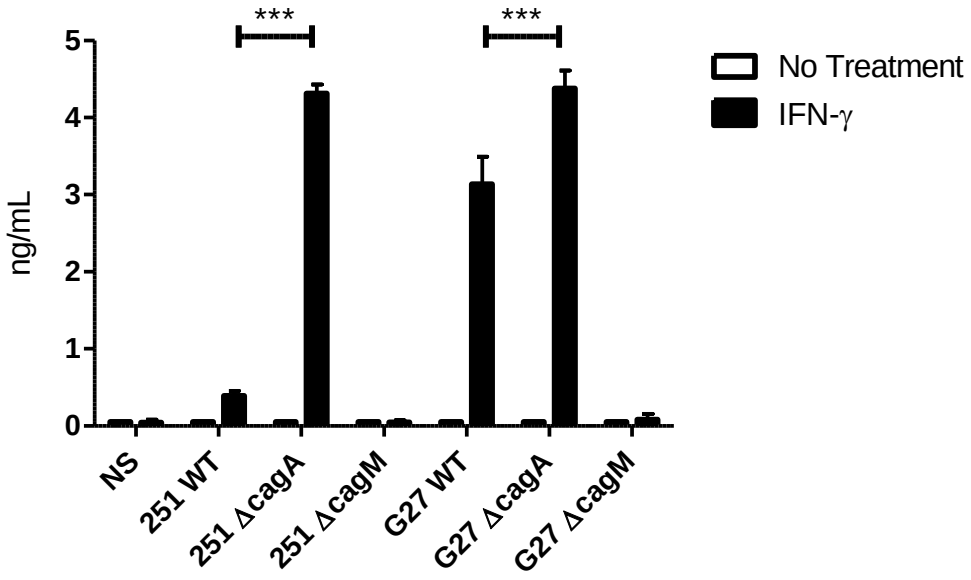


Figure 3.4 *H. pylori*-induced enhancement of MKN28 cell chemokine responses to IFN-γ are not strain specific.

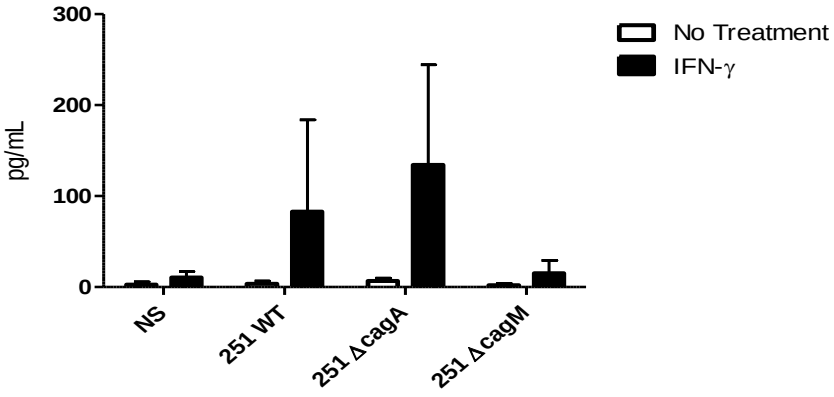
MKN28 cells were treated or not with either wild-type 251 or G27 *H. pylori*, or isogenic *ΔcagA* or *ΔcagM* mutants on either genetic background for 1 hour. Cell media was then replaced with fresh media and incubated for a further 3 hours, prior to the addition of IFN-γ (20 ng/mL). Culture supernatants were collected a total of 24 hours after initial stimulation and analysed by ELISA to determine the secretion of **a)** IP-10 or **b)** MIG. Error bars indicate standard deviation across samples analysed in triplicate. Results are representative of at least 3 biological experiments. ***; $p<0.0001$, as determined by unpaired *t*-test.

As observed in the previous experiment, *H. pylori* infection alone failed to induce detectable IP-10 production in all cell lines tested, regardless of the *cag*PAI status of the bacteria, which was the case with IFN-γ treatment alone (Figure 3.3 b). Whilst combined *H. pylori* and IFN-γ treatment induced IP-10 production, this effect occurred in the presence of bacteria with a functional *cag*PAI, indicating that the activation of *cag*PAI-dependent signalling pathways is required to facilitate the IFN-γ-dependent expression of pro-inflammatory molecules, such as IP-10. A separate finding of interest was that cells co-stimulated with IFN-γ and the Δ*cagA H. pylori* mutant secreted significantly more IP-10 than cells stimulated with IFN-γ and wild-type bacteria (Figure 3.3 b), although the reasons for this are unclear.

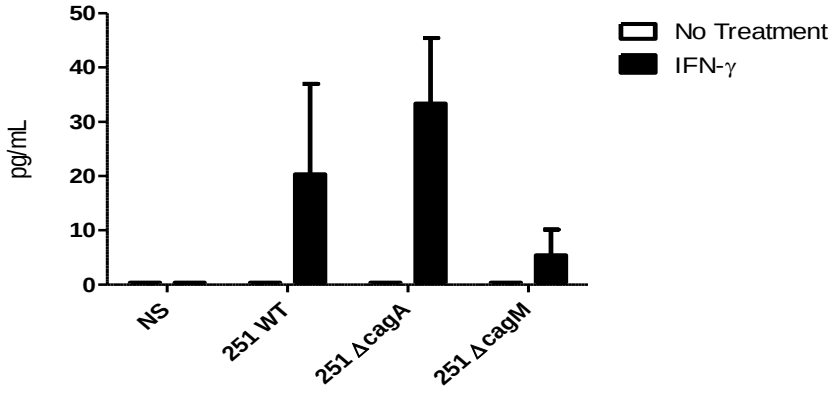
To further confirm the findings above, we performed additional experiments with another set of *H. pylori* parental and isogenic mutant strains. Furthermore, we measured production of another IFN-γ-inducible cytokine, "Monocyte Interferon-gamma-inducible protein" (MIG), which also has chemotactic activity for lymphocytes (319). The same cytokine secretion profile was observed for both IP-10 and MIG in cells stimulated with *H. pylori* strain 251 and strain G27, with respect to the requirement of the *cag*PAI (Figure 3.4 a, b). In addition, IFN-γ treatment alone failed to stimulate MIG production (Figure 3.4 b), indicating that this cytokine is induced in very much the same way as IP-10 in these cells. In agreement with the results obtained using *H. pylori* strain 251, the G27 *ΔcagA* mutant induced significantly more IP-10 and MIG production than the corresponding wild-type strain (Figure 3.4, $p<0.0001$), showing that this too is not a *H. pylori* strain-specific response.

Supernatants from MKN28 cells co-stimulated with *H. pylori* 251 and IFN-γ were also analysed using a multiplex assay to detect the secretion of chemokines: Eotaxin, Gro-α, MCP-1, MCP-2, MCP-3, Mip-1α, Mip-1β and RANTES (Figure 3.5). Some of these chemokines are reportedly upregulated by *cag*PAI^{+} *H. pylori* bacteria [Gro-α (200, 202, 320), MCP-1 (203), RANTES (204)]. Interestingly, stimulation of cells with *H. pylori* alone failed to induce the production of detectable quantities of any of the chemokines

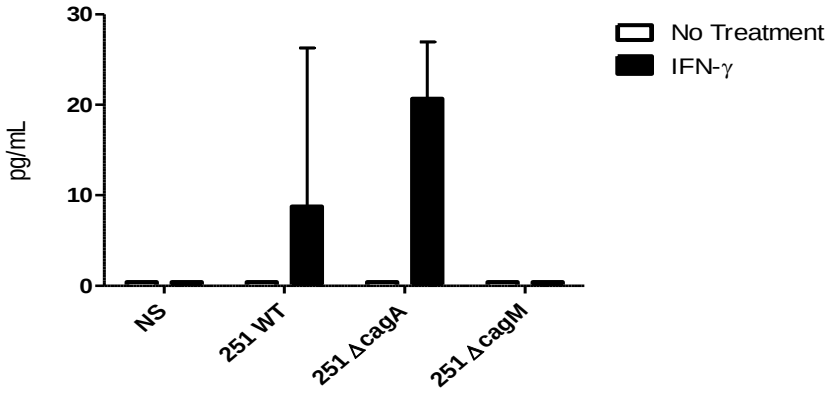
a) RANTES Production



b) MCP-1 Production



c) Mip-1α Production



d) Mip1-β Production

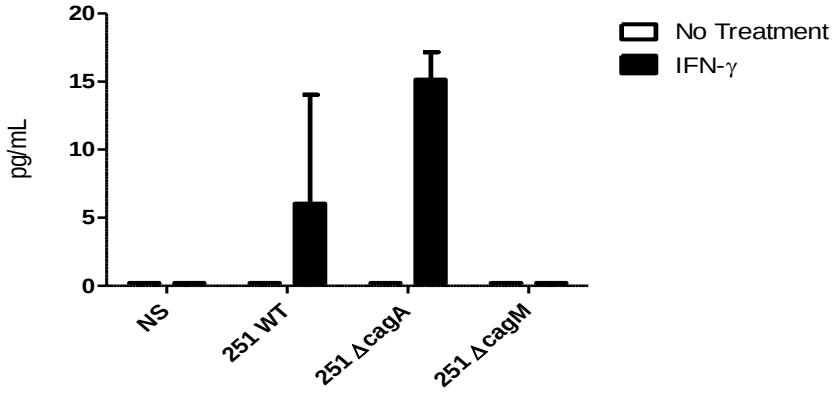


Figure 3.5 *H. pylori* and IFN-γ co-stimulation induce chemokine secretion in MKN28 cells in a *cag*PAI-dependent manner.

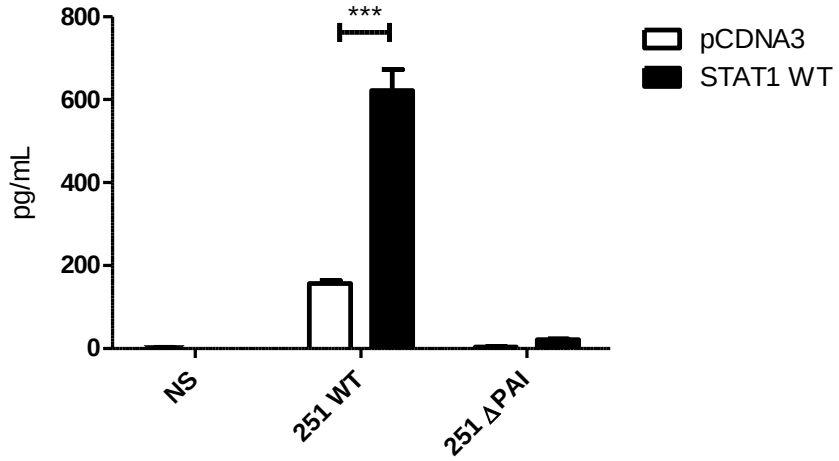
MKN28 cells were treated or not with wild-type 251 *H. pylori* or isogenic *ΔcagA* or *ΔcagM* mutants for 1 hour. Cell media was then replaced with fresh media and incubated for a further 3 hours, prior to the addition of IFN-γ (20 ng/mL). Culture supernatants were collected a total of 24 hours after initial stimulation and analysed by Luminex Multiplex ELISA to determine the secretion of **a)** RANTES, **b)** MCP-1, **c)** Mip-1α, or **d)** Mip-1β. Error bars indicate standard deviation across triplicate biological samples from independent experiments.

tested (Figure 3.5 a-d). In contrast, co-stimulation of cells with IFN-γ and *cag*PAI$^+$ bacteria induced RANTES, MCP-1, Mip-1α and Mip-1β production (Figure 3.5 a-d). Co-stimulation of cells with IFN-γ and *H. pylori ΔcagM* mutant was able to induce the very modest production of RANTES and MCP-1 (Figure 3.5 a, b). As observed with IP-10 and MIG in previous experiments, IFN-γ-treated cells co-stimulated with the *H. pylori* Δ*cagA* mutant produced greater quantities of chemokines than the cells co-stimulated with wild-type bacteria, although these differences did not achieve statistical significance. There was no detectable levels of Eotaxin, Gro-α, MCP-2 or MCP-3 in the supernatants of MKN28 cells, stimulated with either *H. pylori* 251 or G27 strains. Taken together, these findings indicate that stimulation of MKN28 epithelial cells with *H. pylori* alone is insufficient to induce the secretion of numerous pro-inflammatory cytokines, suggesting that their expression in epithelial cells during infection may be delayed and dependent on additional stimuli, such as the presence of IFN-γ.

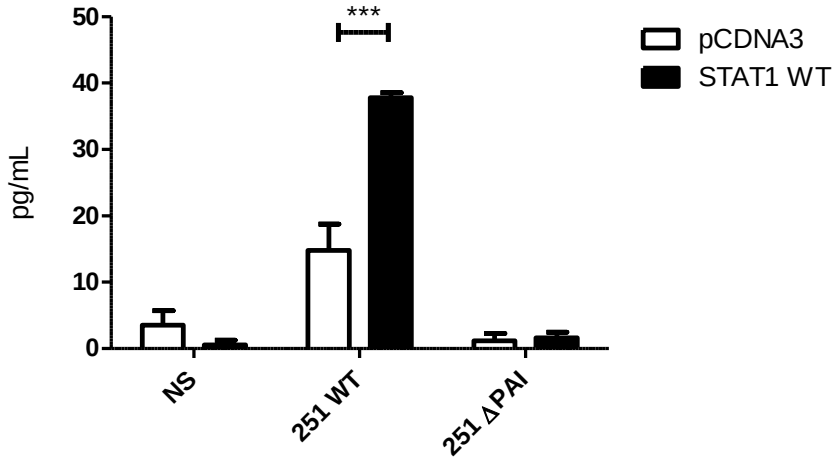
3.3.3 STAT1 over-expression augments epithelial cell responses to *H. pylori*.

IFN-γ induces the phosphorylation and nuclear translocation of STAT1 dimers, which directly bind to the promoters of a number of pro-inflammatory genes (305, 321). Given the ability of IFN-γ treatment to enhance epithelial responses to *H. pylori* stimulation, the following series of experiments were designed to examine whether STAT1 *per se* was sufficient to directly mediate these effects. For these experiments, we used an artificial system to over-express STAT1 in a number of different epithelial cell lines (AGS, MKN28, HEK293), followed by stimulation with *H. pylori*. IL-8 production was significantly upregulated in wild-type *H. pylori*-stimulated MKN28 cells over-expressing STAT1 as compared to stimulated cells that were transfected with the control plasmid (Figure 3.6 a, $p<0.0001$). This effect was a *cag*PAI-dependent event, as cells infected with *H. pylori* mutant bacteria lacking the entire *cag*PAI were unable to induce substantial cytokine production, nor was STAT1 over-expression able to enhance production (Figure 3.6 a). Similar results were observed with respect to IP-10 production, although responses were relatively low, given that cells were not co-stimulated with IFN-γ (Figure 3.6 b).

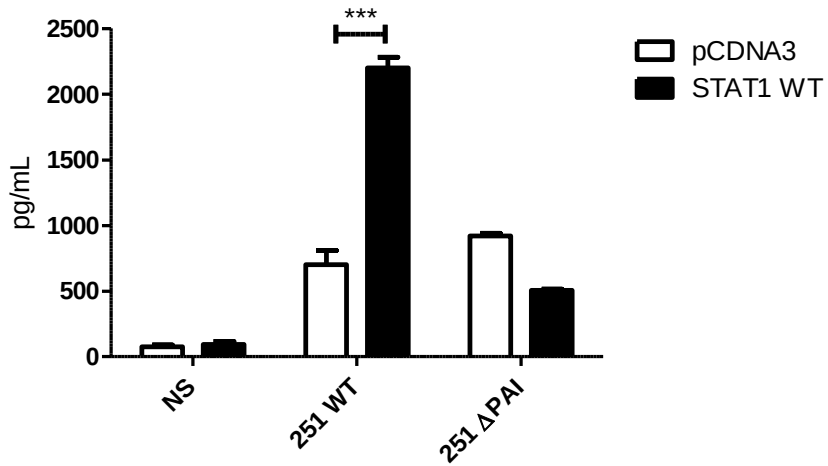
a) IL-8 Production



b) IP-10 Production



c) IL-8 Production



d) IL-8 Production

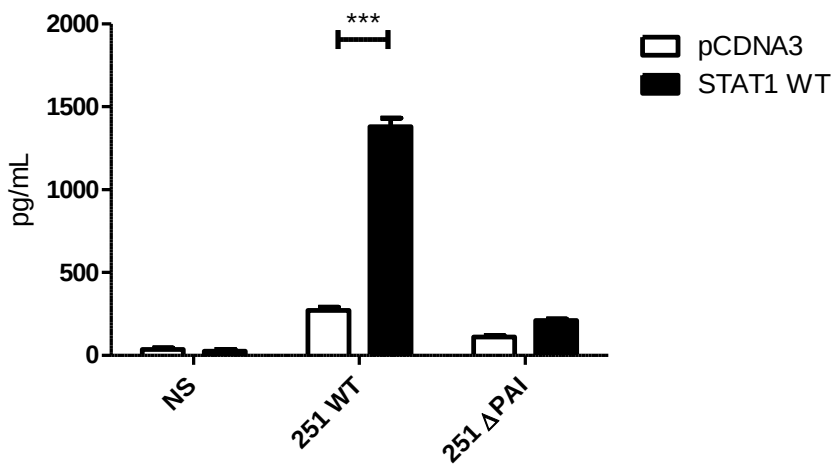


Figure 3.6 STAT1 over-expression enhances chemokine secretion in response to *H. pylori* in gastric epithelial cells.

MKN28 **(a-b),** AGS **(c)**, or HEK293 **(d)** cells were transfected with either pCDNA3 or STAT1-expression constructs for 18 hours prior to stimulation with wild-type 251 *H. pylori* or isogenic *⊿cag*PAI mutants for 1 hour. Cell media was then replaced with fresh media and further incubated. Culture supernatants were collected a total of 24 hours after initial stimulation and analysed by ELISA to determine the secretion of **a, c-d)** IL-8 or **b)** IP-10. Error bars indicate standard deviation across samples analysed in triplicate. Results are representative of at least 3 biological replicates. *** $p<0.0001$, as determined by unpaired *t*-test.

The results from preliminary STAT1 over-expression experiments using AGS and HEK293 epithelials also supported the findings from MKN28 cells (Figure 3.6 c, d). These results confirm that STAT1, a key transcription factor induced by IFN-γ, is capable of synergising with *H. pylori*-induced signalling pathways to enhance IL-8, and to some extent, IP-10 production by gastric epithelial cells. Tyrosine phosphorylated STAT1 is capable of inducing the expression of *IRF1 (306)*, which in turn has been shown to bind to the *NOD1* promoter and upregulate its expression (298). In this way, STAT1 over-expression may increase the total levels of NOD1 in these cells and make them more responsive to *cag*PAI$^+$ bacteria.

3.3.4 *cag*PAI$^+$ *H. pylori* bacteria enhance IFN-γ-induced STAT1 phosphorylation.

The results thus far have demonstrated that IFN-γ treatment synergises with *cag*PAI$^+$ *H. pylori* bacteria to upregulate production of pro-inflammatory cytokines by epithelial cells. In addition, the over-expression of STAT1 alone was shown to be capable of mediating cytokine secretion. A critical event in the downstream activation of pro-inflammatory responses induced by IFN-γ, is the tyrosine phosphorylation of STAT1 (321). Considering that IFN-γ treatment and STAT1 over-expression enhances epithelial responses to *cag*PAI$^+$ *H. pylori*, but not to bacteria lacking a functional T4SS, it was hypothesised that these effects may have occurred via *H. pylori*-dependent STAT1 tyrosine phosphorylation. To investigate this, MKN28 cells were cocultured with *H. pylori* and IFN-γ. Whole cell lysates were then analysed by immunoblot to detect tyrosine phosphorylation of STAT1. As a positive control, IFN-γ treatment alone was shown to rapidly induce the phosphorylation of the STAT1 tyrosine 701 residue (Tyr701). This effect was drastically enhanced in cells that had been co-stimulated with *cag*PAI$^+$ *H. pylori* (Figure 3.7). STAT1 phosphorylation in these cells was observed 6 hours after the initial *H. pylori* infection and 2 hours after the addition of IFN-γ. Despite an increase in phosphorylation levels, however, the levels of total STAT1 protein remained unchanged

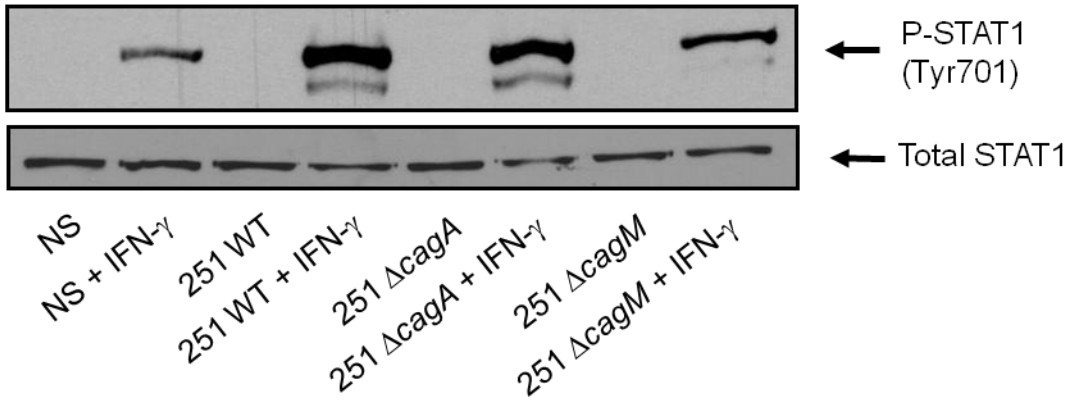


Figure 3.7 *H. pylori* augments IFN-γ-dependent Tyr701 STAT1 phosphorylation in a cagPAI-dependent manner in MKN28 cells.

MKN28 cells were stimulated or not with *H. pylori* 251 wild-type or isogenic *ΔcagA* or *ΔcagM* strains for 4 h. After 4 hours, media was replaced and cells were stimulated or not with 20 ng/mL IFN-γ for a further 2 hours. Cells were lysed in 100 µL boiling Laemmli buffer and subjected to SDS-PAGE and Western Blotting analysis using P-STAT1 (Tyr701) and Total STAT1 antibodies.

(Figure 3.7). In addition, there was a small increase in IFN-γ-induced STAT1 phosphorylation in cells infected with the *H. pylori ΔcagM* mutant, indicating that while the *cag*PAI is primarily responsible for the increase, other non-*cag*PAI-associated *H. pylori* factors may have a minor effect. Intriguingly, cells stimulated only with *H. pylori* displayed no STAT1 Tyr701 phosphorylation at this time-point, regardless of the *cag*PAI status (Figure 3.7).

3.3.5 *H. pylori* induces STAT1 serine 727 phosphorylation, but not tyrosine 701 phosphorylation, in a *cag*PAI-dependent manner.

A previous study reported that *H. pylori* is capable of inducing STAT1 Tyr701 phosphorylation in MKN45 gastric epithelial cells, which peaked 2 hours after stimulation (191). Using MKN28 cells, we were unable to demonstrate STAT1 tyrosine phosphorylation 6 hours after stimulation with *H. pylori* alone (Figure 3.7). Therefore, a co-culture time-course was performed to observe the kinetics of STAT1 phosphorylation, if any, in the earlier stages of *H. pylori* stimulation. As expected, IFN-γ rapidly induced STAT1 Tyr701 and serine 727 (Ser727) phosphorylation within 30 minutes of treatment (Figure 3.8). Serine phosphorylation is essential for the complete functionality of tyrosine phosphorylated STAT1 (322). While the Tyr701 residue is critical for the dimerisation and nuclear translocation of STAT1, Ser727 phosphorylation is required to mediate the maximal transcriptional potential of IFN-γ (322). In contrast to IFN-γ treatment, *H. pylori* stimulation failed to induce STAT1 tyrosine phosphorylation at either 30 or 60 minutes (Figure 3.8), nor at any later time points tested post *H. pylori* stimulation (data not shown). Similar results were obtained when using *H. pylori* strain G27 (data not shown). Intriguingly, while all strains and corresponding mutants were unable to phosphorylate the Tyr701 residue, STAT1 Ser727 phosphorylation was induced by *cag*PAI^{+} *H. pylori* within 30 minutes of stimulation. This activity peaked at 60 minutes post stimulation (Figure 3.8).

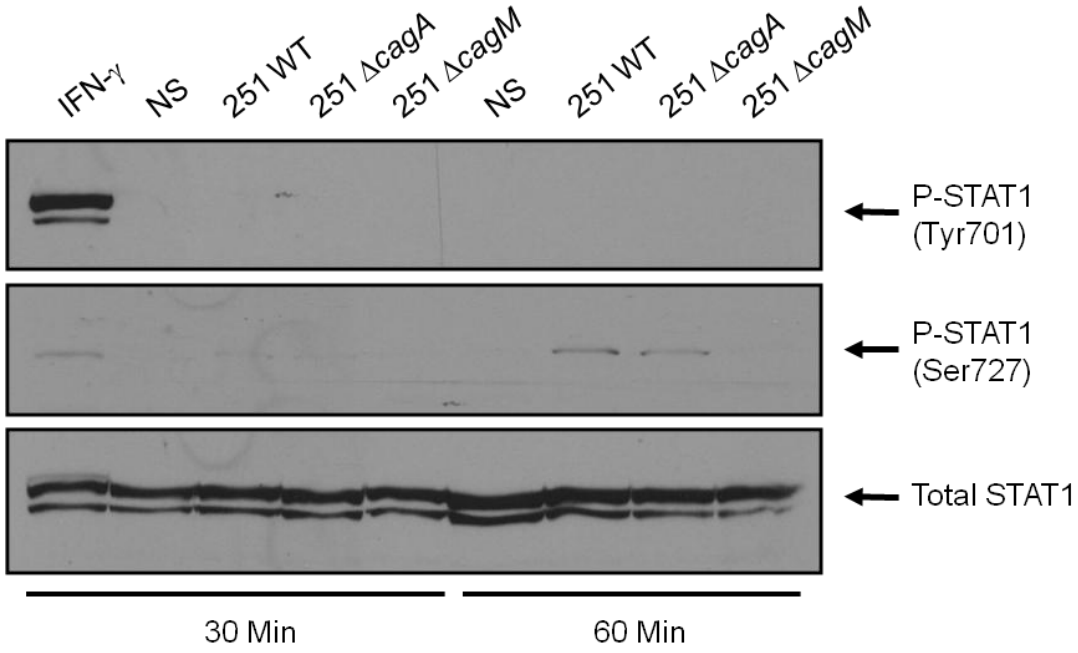


Figure 3.8 *H. pylori* induces STAT1 Ser727, but not Tyr701, phosphorylation in a *cag*PAI-dependent manner in MKN28 cells.

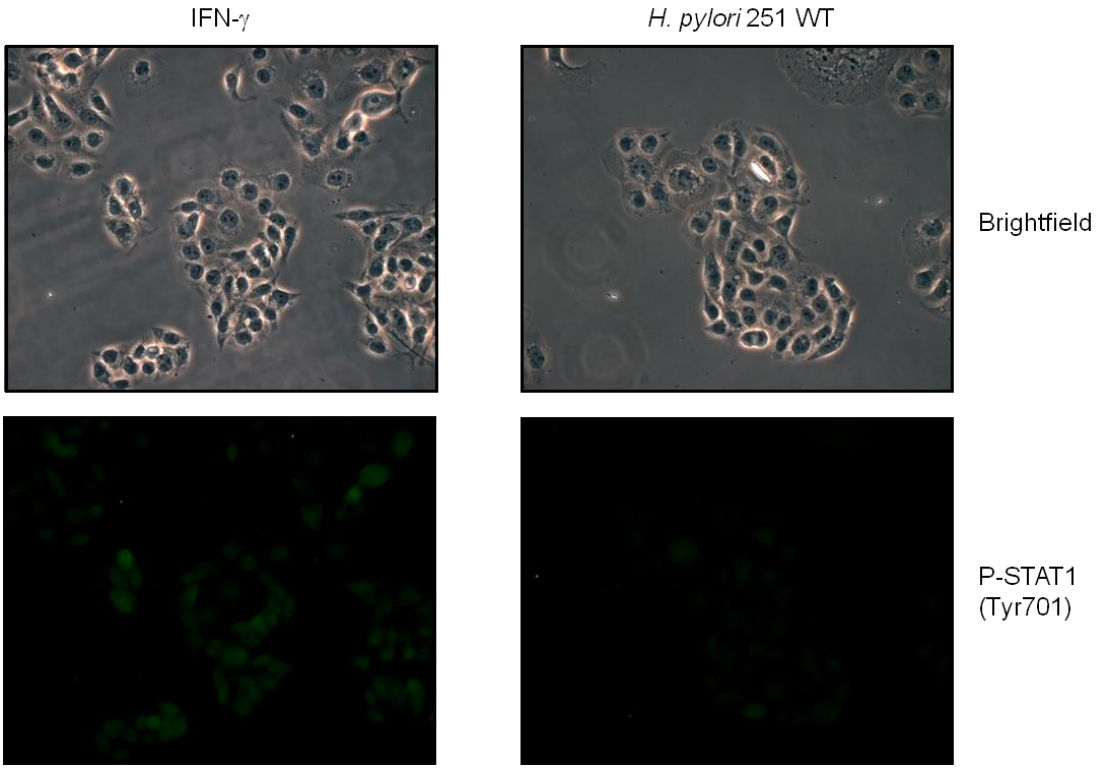
MKN28 cells were stimulated or not with *H. pylori* 251 wild-type or isogenic Δ*cagA* or Δ*cagM* strains for 30 or 60 minutes. As a positive control, cells were also stimulated for 30 minutes with 20 ng/mL IFN-γ. Cells were lysed in 100 µL boiling Laemmli buffer and subjected to SDS-PAGE and Western Blotting analysis using P-STAT1 (Tyr701), P-STAT1 (Ser727)and Total STAT1 antibodies.

To further confirm that *H. pylori* alone was not capable of inducing STAT1 Tyr701 phosphorylation, we employed immunofluorescence techniques in an attempt to directly visualise STAT1 tyrosine phosphorylation in gastric epithelial cells stimulated with *H. pylori* and IFN-γ. In agreement with the data obtained from immunoblotting analysis, *H. pylori*-stimulated cells showed no visible increase in Tyr701 phosphorylation at 2 hours after stimulation, as compared to IFN-γ-treated cells (Figure 3.9 a). High content immunofluorescence was also used to evaluate more subtle phosphorylation changes in *H. pylori*-stimulated cells. Specifically, the fluorescence intensity of cytoplasmic and nuclear localised phosphorylated STAT1, if any, was calculated numerically on a cell to cell basis, enabling the detection of both STAT1 phosphorylation and its nuclear translocation. IFN-γ-treated cells displayed a high nuclear to cytoplasmic intensity ratio as compared to non-stimulated cells 2 hours after stimulation, indicating Tyr701 phosphorylated STAT1 localisation to the nucleus of IFN-γ-treated cells (Figure 3.9 b). In agreement with previous experiments, however, *H. pylori* infection failed to induce the nuclear translocation (or activation) of Tyr701-phosphorylated STAT1. Taken together, these data suggest that although *H. pylori* does not induce STAT1 Tyr701 phosphorylation, the bacterium is capable of enhancing the IFN-γ-induced phosphorylation of this residue. While the precise mechanism(s) through which this occurs is(are) unclear, we show that this process is dependent on the *cag*PAI. Furthermore, co-culture of cells with *cag*PAI$^+$ *H. pylori* bacteria induced rapid STAT1 Ser727 phosphorylation, which may be involved in a previously uncharacterised signalling pathway in *H. pylori*-stimulated gastric epithelial cells.

3.3.6 *H. pylori* induces *IRF1* transcription in a *cag*PAI- dependent manner.

While it is clear that *cag*PAI$^+$ *H. pylori* bacteria can enhance the pro-inflammatory responses of epithelial cells to IFN-γ, a distinct mechanism of action remains elusive. In particular, the expression of many IFN-responsive genes were found to be induced only upon co-stimulation with IFN-γ and *cag*PAI$^+$ bacteria. IFN-γ treatment upregulates

a) Immunofluorescence



b) High Content Immunofluorescence

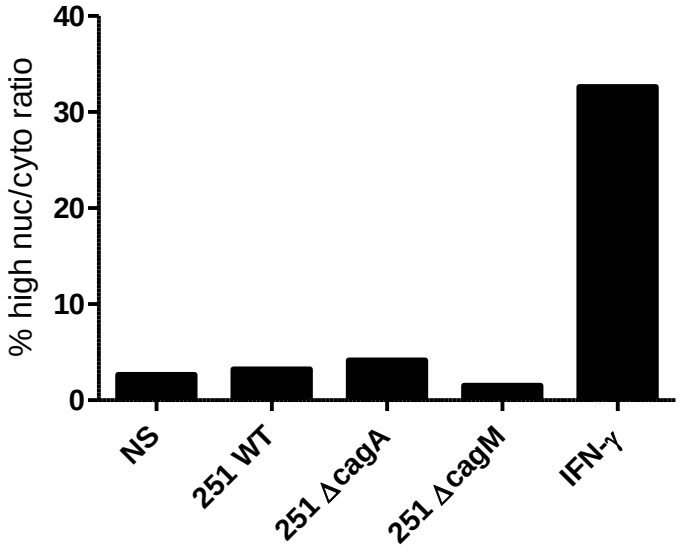


Figure 3.9 *H. pylori* does not induce STAT1 Tyr701 phosphorylation in MKN28 cells.

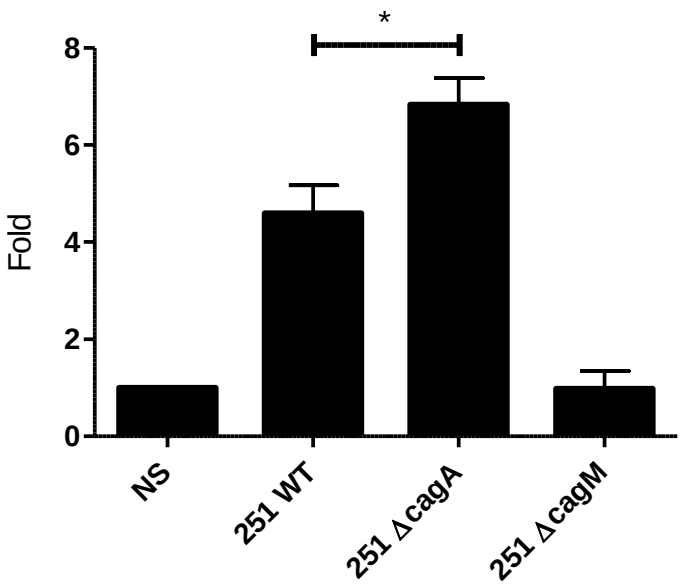
a) MKN28 cells, on glass coverslips, were stimulated with *H. pylori* 251 or 20 ng/mL IFN-γ for 2 hours. Phosphorylated STAT1 was detected using rabbit P-STAT1 (Tyr701) antibodies and secondary anti-rabbit Alexa Fluor 488 antibodies. Cells were viewed using a 40x objective lens. **b)** MKN28 cells, seeded in 96-well clear bottom plates, were stimulated for 1 hour with IFN-γ or *H. pylori* 251 wild-type or isogenic *ΔcagA* or *ΔcagM* mutants. Cell nuclei were stained with Hoescht and STAT1 (P-Tyr701) was stained using rabbit P-STAT1 (Tyr701) antibodies and secondary anti-rabbit Alexa Fluor 488 antibodies. Images were collected using the Cellomics* ArrayScan VTI HCS Reader, and analysed using the Nuclear Translocation algorithm. Nuclear to cytoplasmic fluorescence intensity ratios greater than 1.5 were considered high.

expression of the transcription factor, IRF1, via the binding of phosphorylated STAT1 to specific "gamma-activated sequences" (GAS) elements in the *IRF1* promoter (pine, 1994). NF-κB has also been shown to induce *IRF1* expression (323-324) and given that *cag*PAI$^+$ *H. pylori* bacteria are potent activators of NF-κB, the ability of *H. pylori* to induce *IRF1* transcription was investigated. To examine *H. pylori* induced *IRF1* expression, quantitative real-time PCR analysis (qRT-PCR) was performed on cDNA prepared from MKN28 cells co-cultured with *H. pylori* for varying periods of time. Indeed, both wild-type and *ΔcagA* mutant *H. pylori* strongly upregulated *IRF1* transcription within 2 hours of stimulation, an effect not seen in cells stimulated with *H. pylori ΔcagM* mutants (Figure 3.10 a). As was the case for the production of IP-10, MIG and various other chemokines, *ΔcagA H. pylori* mutants induced significantly more *IRF1* transcription than wild-type bacteria at 2 hours post-stimulation (Figure 3.10 a, $p<0.05$). This difference was reversed at 4 hours post-stimulation, with wild-type bacteria inducing slightly higher levels of *IRF1* expression than *ΔcagA* mutants, although the trend did not reach significance (Figure 3.10 b). As a control, we also analysed the transcription of another IRF family member, IRF3, in order to determine if this is a specific induction of IRF1 or of all IRF family members. While IRF3 has been reported to be induced following stimulation of certain TLRs (325), its expression was not induced by *H. pylori* at 2 hours (Figure 3.10 c), nor at later time-points (data not shown).

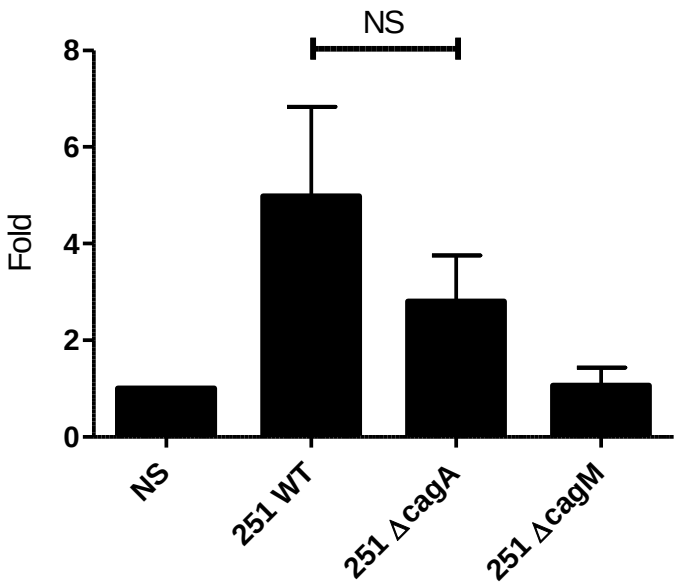
3.3.7 *H. pylori* induces *IRF1* expression in a *cag*PAI- and NOD1-dependent manner.

From the data presented above, we have shown that the *cag*PAI is essential for *H. pylori*-induced *IRF1* expression, which concurs with a previous study (191). Given that NOD1 is a crucial mediator of *cag*PAI-dependent epithelial signalling, we next investigated the role of NOD1 in *H. pylori*-induced *IRF1* expression using AGS siNOD1 cells stably expressing shRNA directed against *NOD1*. As observed in MKN28 cells, both wild-type and Δ*cagA* mutant *H. pylori* induced *IRF1* expression within 2 hours of stimulation (Figure 3.11), although these levels were more modest than those induced in MKN28

a) *IRF1* Expression



b) *IRF1* Expression



c) *IRF3* Expression

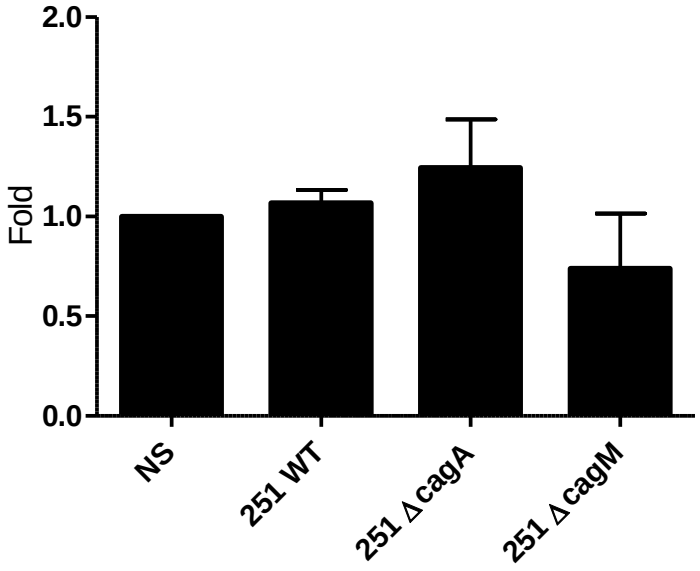


Figure 3.10 *H. pylori* induces *cag*PAI-dependent *IRF1*, but not *IRF3*, expression in MKN28 cells.

a-b) *IRF1* mRNA expression in MKN28 cells in response to stimulation for **a)** 2 hours or **b)** 4 hours with parental *H. pylori* 251 or isogenic *ΔcagA* or *ΔcagM* mutant strains was assessed by qRT-PCR. **c)** *IRF3* mRNA expression in MKN28 cells in response to stimulation for 2 hours with parental *H. pylori* 251 or isogenic *ΔcagA* or *ΔcagM* mutant strains was assessed by qRT-PCR. As a control (NS), cells were left untreated. Target gene expression was normalised to *ACTB* expression. Error bars indicate SEM determinations of 3 separate experiments performed in triplicate. * $p<0.05$; NS: not significant, as determined by the Mann-Whitney test.

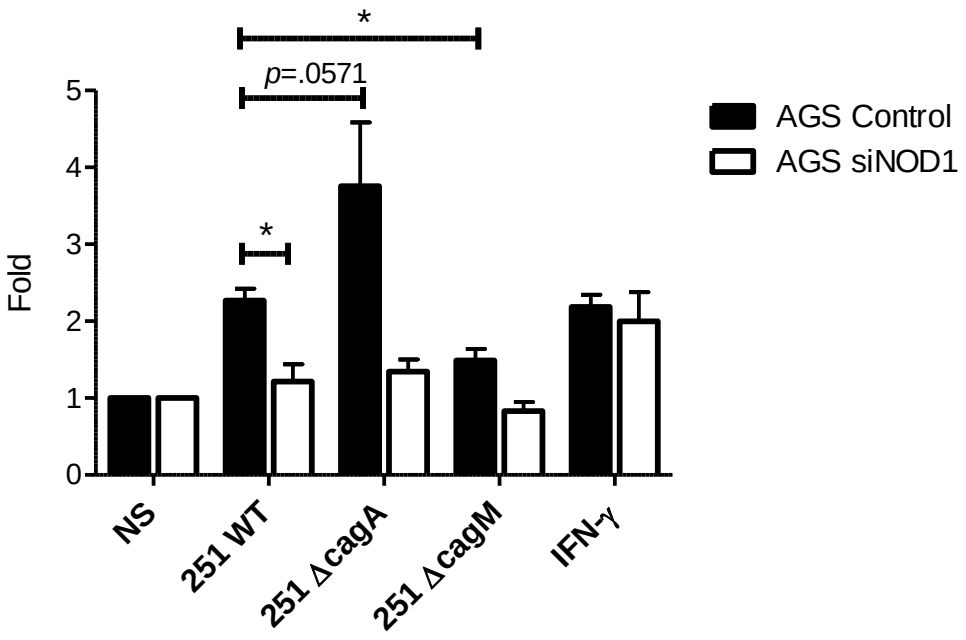


Figure 3.11 *H. pylori* induces *IRF1* expression in AGS cells in a NOD1- and T4SS-dependent manner.

IRF1 mRNA expression in AGS control and AGS siNOD1 cells in response to stimulation for 2 hours with parental *H. pylori* 251 or isogenic *ΔcagA* or *ΔcagM* mutant strains was assessed by qRT-PCR. As a negative control (NS), cells were left untreated. As a positive control, cells were stimulated with 20 ng/mL IFN-γ. Target gene expression was normalised to *ACTB* expression. Error bars indicate SEM determinations of 3 separate experiments performed in triplicate. * $p<0.05$, as determined by the Mann-Whitney test.

cells (Figure 3.10). Expression was again higher in *H. pylori* Δ*cagA*-stimulated cells as compared to those stimulated with wild-type bacteria, although this trend did not reach significance (Figure 3.11, p=0.0571). Importantly, all *IRF1* transcriptional responses were abolished in *H. pylori*-stimulated AGS siNOD1 cells, regardless of the *cag*PAI status of bacteria (Figure 3.11). In addition, corresponding IL-8 production in the AGS siNOD1 cells was significantly reduced as compared to the AGS control cells (data not shown), confirming that NOD1 levels were in fact reduced. Furthermore, these data demonstrate that NOD1, in addition to the *cag*PAI, is critical for the induction of *H. pylori*-induced *IRF1* expression in gastric epithelial cells.

3.3.8 *IFNG*, *IRF1*, *NOD1* and chemokine expression is upregulated in human gastric biopsies during *H. pylori* infection and correlates with disease severity.

We and others have previously demonstrated the importance of NOD1-dependent recognition of *cag*PAI$^+$ *H. pylori* for the induction of pro-inflammatory responses in gastric epithelial cells (24, 310, 317). The data from this chapter also provides evidence for NOD1-dependent *IRF1* expression and the requirement for the *cag*PAI in augmented responses to IFN-γ. IFN-γ has been shown to upregulate *NOD1* expression via IRF1 (298) and previous studies have reported that all three factors are critical for the development of gastritis and Th1-type adaptive immune responses during *H. pylori* infection (24, 161, 235-236). Therefore, we examined the existence of a possible positive feedback mechanism between these factors, chemokine production and gastritis *in vivo*. Gastric biopsies were taken from both the antrum and body of the stomach from 20 patients and assessed by histology, with the degree of gastritis graded as being normal (6 samples), moderate (8 samples) or severe (6 samples) (Table 3.2). Furthermore, biopsies were collected from 12 patients with antral stomach tumours, sampling both tumour and non-tumour tissue for paired analysis. RNA was extracted from the biopsies and analysed for the expression of genes involved in host responses to *H. pylori*. Furthermore, quantitative real-time PCR detection of *H. pylori* 16S RNA and standard PCR detection

Patient	Disease Pathology	*H. pylori* status	*cagA* status	Mononuclear Infiltration	Neutrophil Infiltration	Atrophy	Intestinal Metaplasia
1	**Normal**	-	NA	0	0	0	0
2	**Normal**	-	NA	0	0	1	0
3	**Normal**	-	NA	1	0	0	0
4	**Normal**	-	NA	0	0	0	0
5	**Normal**	-	NA	0	0	0	0
6	**Normal**	-	NA	0	0	0	0
7	**Moderate**	-	NA	2	0	0	0
8	**Moderate**	+	-	2	0	2	0
9	**Moderate**	-	NA	2	0	2	2
10	**Moderate**	+	-	2	0	2	0
11	**Moderate**	+	+	2	1	1	0
12	**Moderate**	+	-	2	0	1	0
13	**Moderate**	-	NA	2	1	1	0
14	**Moderate**	+	+	2	1	2	2
15	**Severe**	+	-	3	2	3	1
16	**Severe**	+	-	2	2	2	1
17	**Severe**	+	+	3	2	2	0
18	**Severe**	+	+	2	1	2	2
19	**Severe**	+	+	2	3	1	0
20	**Severe**	+	-	2	2	0	0

Table 3.2 Disease Pathology and *H. pylori* status of antral gastric biopsy specimens.

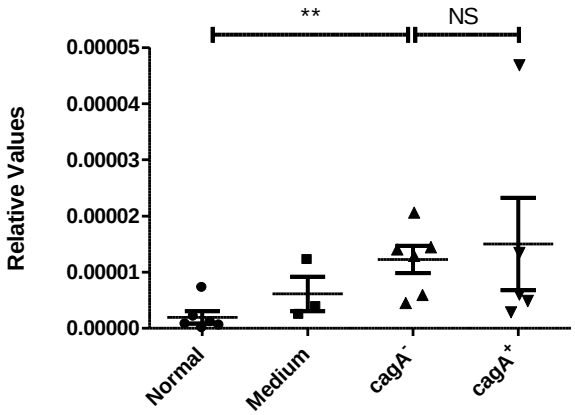
Disease pathology was graded as moderate and severe using a revised version of the Sydney System. Samples that were *H.pylori*-negative with no evidence of lesions were considered normal. *H. pylori* status was confirmed using real-time PCR to detect the presence of pathogen-specific *16S* RNA. In *H. pylori*-positive samples, the presence of *cagA* was determined using standard PCR to detect the 5` conserved region of the *cagA* gene.

of the *cagA* gene amongst these isolates was used to determine the infection status of each individual (Table 3.2). In this way, the relative contributions of *cag*PAI- and non-*cag*PAI-encoded factors in the regulation of host pro-inflammatory gene expression could be evaluated *in vivo*.

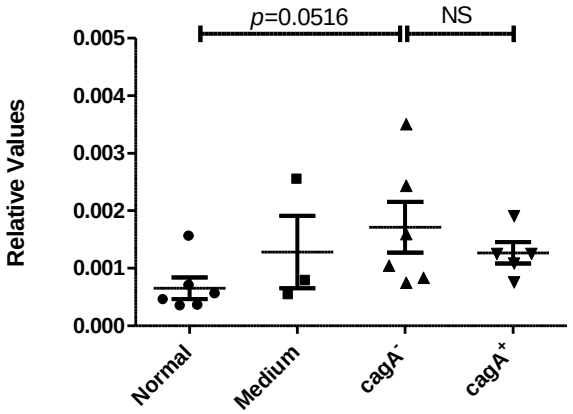
Reports in the literature have demonstrated that *H. pylori* infection induces potent Th1 immune responses, characterised by the production of large quantities of IFN-γ (235-236). Although *cag*PAI$^-$ *H. pylori* strains too were shown to be capable of inducing this type of response, they appeared to do so less effectively (199, 326). Indeed, while *H. pylori*-infected patients analysed in this study tended to express more *IFNG,* there were no significant differences in the *IFNG* levels induced by CagA$^+$ and CagA$^-$ isolates (Figure 3.12 a). As *in vitro* data demonstrated that *cag*PAI$^+$ *H. pylori* induced the expression of *IRF1* in a NOD1-dependent manner (Figure 3.11), we analysed the expression of *IRF1* and *NOD1* to determine if our *in vitro* results were comparable in the context of an *in vivo H. pylori* infection. Although *IRF1* and *NOD1* expression was elevated in *H. pylori*-infected patients, no differences were noted between patients infected with CagA$^+$ or CagA$^-$ isolates (Figure 3.12 b, c, respectively).

We next evaluated the expression of the chemokines, *CXCL8* and *CXCL10 in* the gastric biopsy samples. These chemokines are important in the recruitment of inflammatory cells to the gastric mucosa during *H. pylori* infection. Furthermore, *CXCL8* has previously been shown to be elevated during *H. pylori*-induced gastritis (195, 198-199). Indeed, this was true for the biopsies tested in this report, where substantial *CXCL8* expression was generally only measured in biopsies from *H. pylori*-infected individuals (Figure 3.12 d). While *CXCL8* expression was significantly elevated in the *H. pylori*-infected mucosa, no differences were observed between patients infected with either CagA$^+$ or CagA$^-$ strains (Figure 3.12 d). These results are in contrast to previous reports, which have shown that *H. pylori*-induced *CXCL8* expression in the gastric mucosa strongly correlates with *cag*PAI$^+$ bacteria (53, 200). The fact that this was not observed in our studies, however, may simply reflect the relatively small sample size available for analysis.

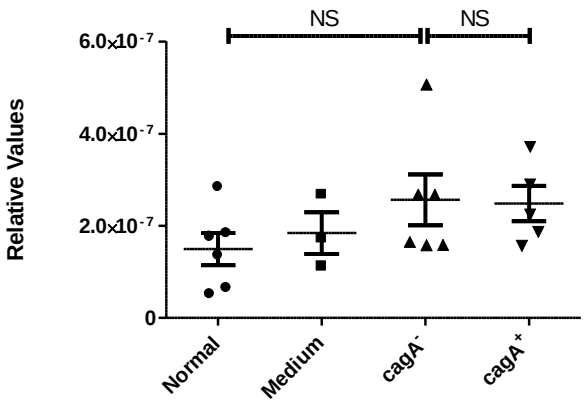
a) *IFNG* Expression



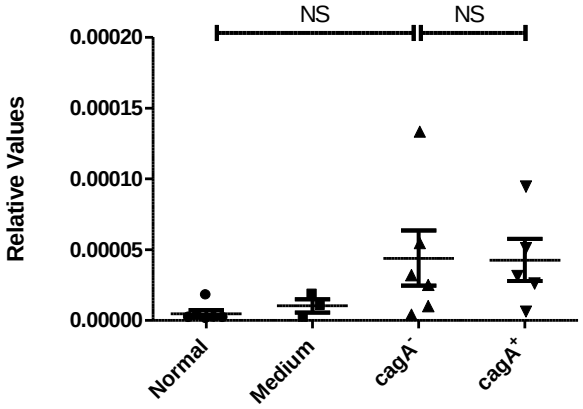
b) *IRF1* Expression



c) *NOD1* Expression



d) *CXCL8* Expression



e) *CXCL10* Expression

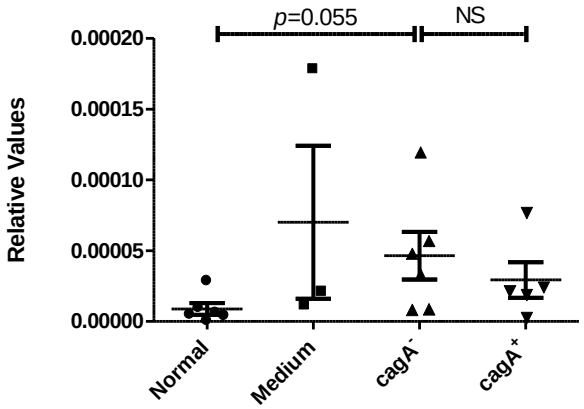


Figure 3.12 *H. pylori* infection induces expression of pro-inflammatory molecules *in vivo*.

Antral gastric biopsies from 20 patients with or without *H. pylori* infection were analysed for expression of **a)** *IFNG*, **b)** *IRF1*, **c)** *NOD1*, **d)** *CXCL8* or **e)** *CXCL10* by qRT-PCR. Patients are grouped based on gastric pathology and *H. pylori* status. Normal indicates patients with normal histology in the absence of *H. pylori* infection. Medium indicates medium gastritis in the absence of *H. pylori* infection. $CagA^-$ indicates patients infected with $CagA^-$ strains of *H. pylori*, whereas $CagA^+$ indicates patients infected with $CagA^+$ *H. pylori* strains, as determined by *cagA*-specific PCR. Target gene expression was normalised to *18S RNA* expression. Individual data points indicate the expression levels for individual patients and error bars indicate SEM determinations. ** $p<0.01$; NS: not significant, as determined by the *t*-test. *cagA*

Although these pro-inflammatory mediators did not correlate with the *cag* status of *H. pylori* in our study, we were able to show an association between their expression and disease status. Accordingly, the expression of *IFNG* in antral biopsies correlated strongly with disease severity, with discernable increases in expression between normal, moderate and severe samples (Figure 3.13 a, $p<0.05$). There was no difference, however, in the expression pattern of *IFNG* between tumour and corresponding non-tumour samples, which were both similar to the levels measured in patients with moderate gastritis (Figure 3.13 a). IFN-γ is known to strongly induce *IRF1* expression (306) and thus, *NOD1* expression (298). Certainly, our data suggests that this is also true in gastritis, where both *IRF1* and *NOD1* expression followed the same trend as seen with *IFNG*, with significant incremental increases in expression correlating with disease severity (Figure 3.13 b, c). Intriguingly, we found that both *IRF1* and *NOD1* expression were modestly, yet significantly elevated in tumour tissue (Figure 3.13 b, c, $p<0.05$, $p<0.0001$, respectively).

As previously mentioned, *CXCL8* expression has been shown to correlate with disease severity during *H. pylori* infection *in vivo*. We were able to detect statistically significant increases in *CXCL8* expression between normal and moderate gastritis samples and again between patients with moderate and severe gastritis (Figure 3.13 d). Furthermore, *CXCL8* expression was drastically elevated in tumour specimens as compared to the non-cancerous surrounding tissue, where *CXCL8* expression was comparable to those in patients with moderate gastritis (Figure 3.13 d, $p<0.05$). The fact that normal tissue samples in many instances expressed no detectable *CXCL8*, indicates that expression of this chemokine is induced virtually only in situations of inflammation and is therefore an accurate indicator of disease severity during *H. pylori* infection. Indeed, neutrophil infiltration scores from antral biopsies (Table 3.2) positively correlate with *CXCL8* expression from those samples (data not shown).

While *CXCL10* expression was also elevated during gastritis as compared to normal tissues, these differences were only statistically significant for cases of severe gastritis (Figure 3.13 e, $p<0.05$). Furthermore, there was no significant up-regulation when comparing moderate and severe gastritis, indicating that the pattern of *CXCL10*

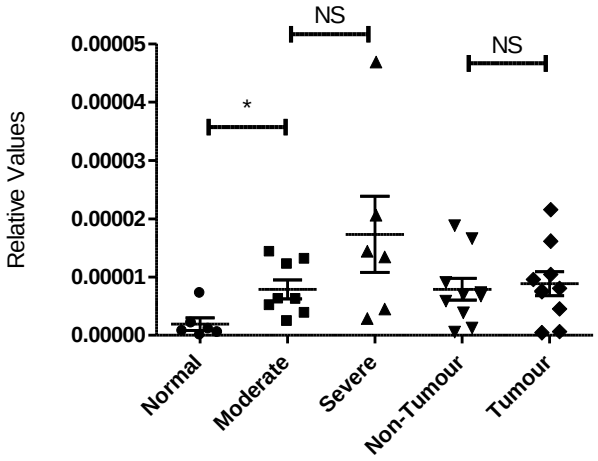
expression does not fully correlate with that of IFN-γ. Finally, *CXCL10* expression is similar in both tumour and non-tumour tissue at levels comparable to those detected in severe gastritis (Figure 3.13 e).

The findings from the *in vivo* expression pattern of *IFNG*, *IRF1*, *NOD1*, *CXCL8* and *CXCL10* in gastric biopsies implicates all in gastritis, with expression generally correlating with disease severity. Intriguingly, while *H. pylori* infection was associated with enhanced expression of these factors, we were unable to demonstrate a correlation with the *cag* status of infecting bacteria. While this result may be attributable to the small sample size available, no study to our knowledge has investigated the correlation between *in vivo* expression of *IFNG*, *IRF1*, *NOD1* or *CXCL10* and the *cag* status of *H. pylori*. A noteworthy finding of the present work was the elevated *IRF1* and *NOD1* expression in gastric tumours. To our knowledge, this has not before been reported in gastric malignancy.

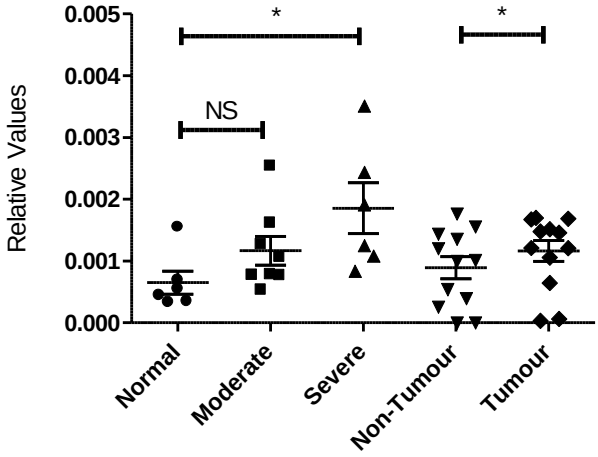
3.4 Discussion

Colonisation of the gastric mucosa by *H. pylori* rapidly induces the recruitment of immune cells to the site of infection (164, 196, 214-219). Although certain *H. pylori*-encoded virulence factors are capable of interacting with and stimulating these inflammatory cells, such responses are likely to be largely initiated by gastric epithelial cells, which are the first point of contact for *H. pylori*. Accordingly, we suggest that NOD1-dependent recognition of *H. pylori* by epithelial cells provides the stimuli that are pivotal in initiating pathogen-specific immune responses. We demonstrated in the previous chapter that NOD1-dependent recognition of *cag*PAI$^+$ bacteria results in NF-κB and AP-1 activation and the secretion of pro-inflammatory chemokines, such as IL-8. Both IL-8 and Gro-α are potent neutrophil chemo-attractants produced in large quantities in the gastric mucosa of *H. pylori*-infected patients, particularly with those infected with *cag*PAI$^+$ isolates (200, 202). Furthermore, the expression of these chemokines has been shown to correlate with increased infiltration of inflammatory cells (198, 200, 202).

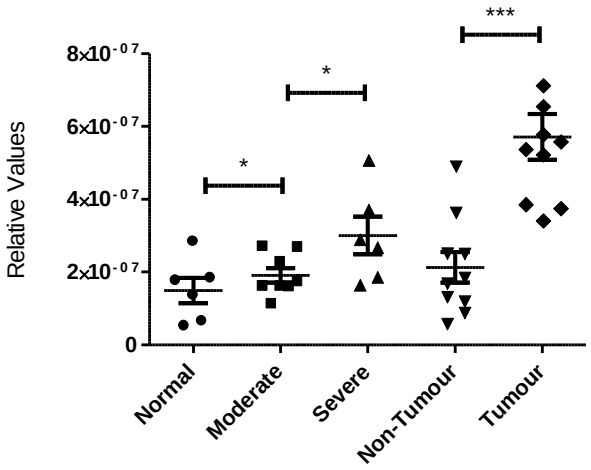
a) *IFNG* Expression



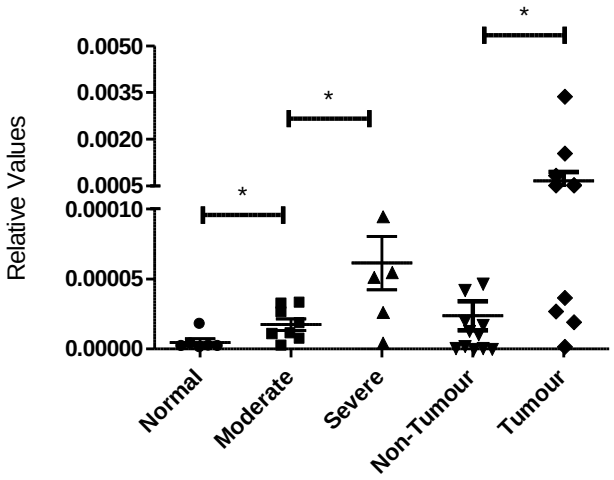
b) *IRF1* Expression



c) *NOD1* Expression



d) *CXCL8* Expression



e) *CXCL10* Expression

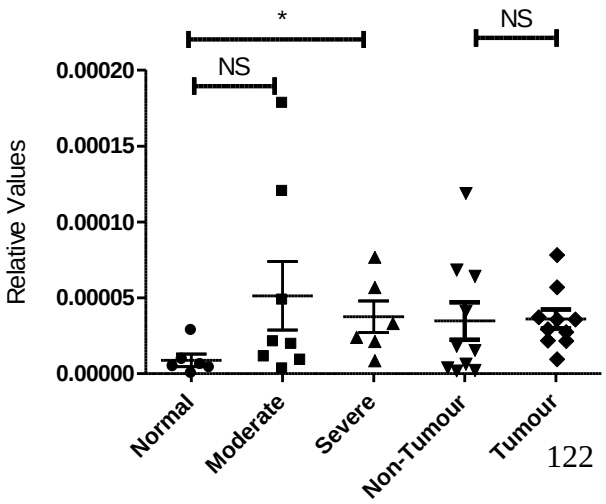


Figure 3.13 Expression of pro-inflammatory molecules correlates with disease severity *in vivo.*

Antral gastric biopsies from 20 patients with or without *H. pylori* infection were analysed for expression of **a)** *IFNG*, **b)** *IRF1*, **c)** *NOD1*, **d)** *CXCL8* or **e)** *CXCL10* by qRT-PCR. Patients are grouped based on disease category. Normal indicates patients with normal histology. Medium and severe indicates medium and severe gastritis, respectively. Tumour and non-tumour groups indicate paired samples taken from the tumour and non-tumour tissue of gastric biopsies containing tumours. Target gene expression was normalised to *18S RNA* expression. Individual data points indicate the expression levels for individual patients and error bars indicate SEM determinations. * $p<0.05$; *** $p<0.0001$; NS: not significant, as determined by the *t*-test.

The expression of the Th1-type cytokines IL-12 and IFN-γ are also correlated with gastritis (92, 260, 327) and the majority of $CD4^+$ T-cells isolated from the infected mucosa produce IFN-γ and display a polarised Th1 phenotype (92, 235-237, 327). This response is associated with the development of gastritis, which is further enhanced in patients infected with *cag*PAI^+ *H. pylori* strains (31, 34, 36-37, 328). While a number of studies have demonstrated that both $CD4^+$ T cells and IFN-γ are the precipitating factors responsible for the development of severe gastritis in the host (238, 242), few studies have investigated the direct effects of IFN-γ on NOD1-dependent epithelial responses to *H. pylori*. The current study not only examined this question, but also the roles of the *cag*PAI in these responses.

We first investigated the ability of *cag*PAI^+ *H. pylori* to induce the gastric epithelial cell secretion of the IFN-γ-responsive T cell chemoattractants, IP-10 and MIG. While *H. pylori* induced robust IL-8 production in a *cag*PAI- and NOD1-dependent manner, bacteria alone were unable stimulate IP-10 and MIG production. Furthermore, IFN-γ treatment alone also failed to induce the production of these chemokines, indicating that multiple stimuli are required to upregulate these genes in gastric epithelial cells, which is consistent with the work of Kraft and colleagues (307). Studies of the *CXCL10* promoter revealed that it contains both NF-κB and "interferon-stimulated response element" (ISRE) binding sites (329). While STAT1 homodimers do not bind ISRE, an interferon-induced complex "interferon-stimulated gene factor 3" (ISGF3) is capable of binding such sites (330). ISGF3 is composed of STAT1, STAT2 and IRF9 and its expression can be induced by IFN-γ treatment (331). In some instances this complex is sufficient to upregulate IP-10 production (329). A recent study found that IFN-γ-dependent IP-10 production is dependent on the action of nuclear histone deacetylases (HDACs) to facilitate gene transcription (332), which may explain the variability in the literature with respect to the ability of IFN-γ alone to stimulate IP-10 production. Interestingly, the *CXCL9* (MIG) promoter contains similar transcription factor binding sites (333) and given that our results show equivalent patterns of MIG and IP-10 production during *H. pylori* stimulation, it is likely that both of these chemokines are regulated in the same

manner. Certainly, in the context of *H. pylori*-stimulated gastric epithelial cells, IP-10 expression appears to be dependent on the dual stimulation with IFN-γ and *cag*PAI-dependent factors, such as NF-κB. This synergism between IFN-γ and NF-κB has been previously shown to augment IL-8, IP-10 and MIG production in gastric epithelial cells (307, 334).

These findings suggest that gastric epithelial cells are unable to induce the production of IP-10 and MIG directly in response to *cag*PAI^{+} *H. pylori* bacteria and thus, may be less able to recruit T cells to the gastric mucosa in the early stages of infection. In contrast, a recent study reported that stimulation of gastric epithelial cell lines with the purified NOD1 agonist, triDAP, induced robust IP-10 production, as did co-culture of AGS cells with *cag*PAI^{+} *H. pylori* bacteria (282). Specifically, they found that NOD1-dependent recognition of *H. pylori* induced an autocrine pathway that led to the production of IFN-β, thereby inducing the formation of ISGF3 and subsequent IP-10 production (282). In this way they found that NOD1-dependent IFN-β induction was essential for the production of IFN-β, IFN-γ and IP-10 in the gastric mucosa during *H. pylori* infection, finding no role for NOD1 in the activation of NF-κB or associated responses (282). Furthermore, they found that while *in vivo* inhibition of NF-κB reduced TNF-α and MIP-2 production in *H. pylori*-infected mice, bacterial burden was only slightly increased, suggesting that NF-κB plays only a minor role in host responses to *H. pylori* (282).

Importantly, however, the vast majority of experiments in the Watanabe study (282), were performed with synthetic NOD1 ligands, used at high doses (10-100 μg/mL). Also, all of the *in vitro* experiments were performed in either a colonic cell line, or in primary intestinal or fibroblast cells. The two sole *in vitro* experiments in which *H. pylori* bacteria were used to stimulate gastric epithelial cells employed the AGS cell line. As reported here, we found that in comparison to MKN28 cells, even combined stimulation of AGS cells with *H. pylori* and IFN-γ induced relatively low levels of IP-10 production. A report published during the course of our studies revealed that AGS cell lines (including the founding strain catalogued in the ATCC) can be persistently and unknowingly infected with the Parainfluenza 5 virus (PIV5) (335). Importantly, the PIV5 virus selectively

targets STAT1 for proteasomal degradation, which would significantly impair IFN-γ-dependent signalling (335). Indeed, preliminary results from our laboratory suggest that the AGS cell lines employed in this study may be infected with PIV5 (data not shown), which would explain the reduced responses observed upon treatment with IFN-γ. It is noteworthy that the AGS cells used in the Watanabe study (282) were obtained from the ATCC.

Although our results indicate that *H. pylori* may not be able to directly stimulate IP-10 or MIG secretion in gastric epithelial cells, it is possible that the recruitment of IFN-γ-producing immune cells to the mucosa may provide the gastric epithelium with the required stimuli to induce production at later time points. An *in situ* hybridisation study analysed the pattern of chemokine expression in the *H. pylori*-infected stomach and reported that both IP-10 and MIG were neither expressed, nor present at the gastric epithelium, instead finding that these molecules were produced exclusively at sites of high T cell density or infiltration (336). While this suggests that epithelial cells may not be a major source of IP-10 and MIG production during *H. pylori*-associated chronic gastritis, the *cag* status of infecting isolates in that study was not determined. Therefore, it is difficult to draw any definitive conclusion since our findings indicate that combined stimulation with IFN-γ and *cag*PAI^{+} *H. pylori* bacteria is required for the production of these chemokines by gastric epithelial cells.

Intriguingly, a separate study reported that certain *H. pylori* membrane and soluble fractions actually inhibit gastric epithelial responses to IFN-γ and TNF-α treatment, suggesting that certain *H. pylori* factors may inhibit IFN-γ-dependent responses during infection (307). Interestingly, we found that co-stimulation of cells with IFN-γ and the *H. pylori Δcag*A mutant induced significantly higher IP-10 and MIG production than cells stimulated with IFN-γ and wild-type bacteria. Similar trends were found for the production of RANTES, MCP-1, Mip-1α and Mip-1β, which are all chemokines involved in the recruitment of immune cells. Importantly, IL-8 production was equivalent between cells stimulated with IFN-γ and either wild-type or Δ*cagA* mutant *H. pylori*.

Therefore, NF-κB activation by *cag*PAI$^+$ *H. pylori*, in combination with the exposure of the gastric epithelium to IFN-γ, could potentially provide the necessary stimulus for the production of these chemokines, whereas CagA may exert an inhibitory effect as a mechanism of curtailing excessive inflammatory responses.

IFN-γ treatment results in the rapid phosphorylation of STAT1, which is an essential transcription factor involved in the expression of a number of pro-inflammatory genes (reviewed in (305)). Indeed, we found that the augmentation of epithelial chemokine responses upon co-stimulation with IFN-γ and *cag*PAI$^+$ *H. pylori* coincided with enhanced STAT1 Tyr701 phosphorylation. Nevertheless, co-stimulation of IFN-γ-treated cells with *H. pylori* Δ*cagA* bacteria did not result in a further enhancement of STAT1 Tyr701 phosphorylation as compared to levels detected in cells co-stimulated with wild-type bacteria, suggesting that the inhibitory effects of CagA are not exerted on STAT Tyr701 phosphorylation. In contrast, a previous studied reported that *H. pylori* was able to directly inhibit IFN-γ-induced STAT1 Tyr701 phosphorylation, nuclear translocation and subsequent gene transcription in gastric epithelial cells (192). These effects were shown to be dependent on the interaction of viable bacteria with the host cell, yet intriguingly were found to occur in a *cag*PAI- and therefore CagA-independent manner. The work, however, was performed using independent *H. pylori* isolates and not with isogenic mutants. Furthermore, this group used only 1 ng/ml of IFN-γ, whereas 20 ng/ml was used to stimulate cells in experiments performed in this chapter, which is in the lower range of the concentration employed in similar studies with gastric epithelial cells (233, 337-339). While this may explain the lack of inhibition of IFN-γ-dependent responses in our hands, it does not explain the observed augmentation of chemokine production and STAT1 phosphorylation induced by co-stimulation with IFN-γ and *cag*PAI$^+$ *H. pylori*. Furthermore, we found that in the absence of IFN-γ, STAT1 over-expression significantly increased IL-8 and IP-10 production upon stimulation with *cag*PAI$^+$ *H. pylori* bacteria, suggesting that STAT1 may be involved in pro-inflammatory gastric epithelial cell responses to *H. pylori*.

Although discrepancies were noted between the study by Mitchell *et al*. and results from this chapter, our findings concur with respect to the inability of *H. pylori* to directly induce STAT1 Tyr701 phosphorylation (192). In contrast, however, Yamaoka and colleagues reported that *H. pylori* induced detectable STAT1 Tyr701 phosphorylation within 2 hours of stimulation, as measured by immunoblotting analysis (191). This was found to occur via the *H. pylori* adhesin, OipA, through an unknown mechanism, whereas the *cag*PAI was not required (191). Furthermore, Yamaoka *et. al.* reported elevated levels of tyrosine phosphorylated STAT1 in antral biopsies from infected patients, although the specific cell type(s) was/were not identified. It is unclear whether STAT1 was tyrosine phosphorylated directly by *H. pylori* or indirectly via elevated levels of cytokines in the inflamed mucosa, although phosphorylation was shown to correlate with the expression of a functional *oipA* gene by the infecting strain (191). Although a number of methods were employed in this chapter to reproduce the authors' *in vitro* STAT1 data, co-culture of cells with either *H. pylori* strain 251 or G27, both of which possess a functional *oipA* gene (data not shown), failed to induce STAT1 Tyr701 phosphorylation at any point between 30 minutes and 8 hours after stimulation. The reason for this discrepancy is unclear, although experiments from the previous study employed MKN45 gastric epithelial cells that had detectable levels of constitutively tyrosine phosphorylated STAT1 (191), suggesting that IFN-γ signalling pathways were activated in these cells, even in the absence of *H. pylori* stimulation.

Despite the inability of *H. pylori* to tyrosine phosphorylate STAT1 in our hands, we found that *cag*PAI^{+} *H. pylori* bacteria was able to rapidly induce STAT1 Ser727 phosphorylation. It has been shown that Tyr701 phosphorylation is required for dimerisation and nuclear translocation of STAT1, whereas concomitant Ser727 activation is essential for complete transcriptional functionality, particularly in response to IFN-γ treatment (322). Interestingly, IFN-γ-stimulated *IRF1* expression was reduced by up to 80% in human fibroblasts expressing a form of STAT1 in which tyrosine, but not serine, residues are phosphorylated (322). In this way, IFN-γ-induced tyrosine phosphorylated STAT1 may become fully transcriptionally activated following *cag*PAI-dependent STAT1 Ser727 phosphorylation, thus enhancing the production of pro-inflammatory

cytokines. However, a precise role for STAT1 Ser727 phosphorylation, in the absence of Tyr701 phosphorylation, in inflammatory responses is unclear, although studies have indicated that it may be involved in apoptosis (340-341).

These data suggest that *H. pylori* alone is unable to directly induce the tyrosine phosphorylation, and therefore nuclear translocation, of transcriptionally active STAT1. In contrast, we found that *H. pylori* can induce the expression of *IRF1*, which is an important downstream transcription factor of IFN-γ signalling (306). Although a previous study found this to occur in a *cag*PAI-dependent manner (191), we have extended this finding by demonstrating that *IRF1* expression was dependent on the recognition of *cag*PAI$^+$ *H. pylori* bacteria by NOD1 (Figure 3.10 and 3.11). Given that the IFN-γ-dependent *IRF1* expression that we observed required both STAT1 Tyr701 and Ser727 phosphorylation, it is likely that *cag*PAI-induced *IRF1* expression is likely to occur via another signalling pathway. Indeed, NF-κB was found to strongly and rapidly stimulate IRF1 production in response to TNF-α treatment (324). Based on the evidence presented here, NOD1-dependent NF-κB activation in response to *H. pylori* would appear to be a novel alternative mechanism.

A previous study demonstrated that IRF1 could bind the *NOD1* promoter and upregulate its expression (298). Given our findings that *cag*PAI$^+$ *H. pylori* bacteria upregulated *IRF1* transcription via NOD1, this suggests the existence of a positive inflammatory feedback mechanism between NOD1 and IRF1 in the context of *H. pylori* infection. Certainly, we found that expression of both *NOD1* and *IRF1* was elevated in gastric biopsies of patients infected with *H. pylori*, although this did not correlate with the *cag* status of the isolates, which may be due to small sample sizes. There was, however, a significant correlation between *NOD1*, *IRF1* and *IFNG* expression and gastritis severity, as was the case for *CXCL8* and *CXCL10* expression. In addition to the known requirement for NOD1 in the development of Th1 responses during *H. pylori* infection (292), another study found IRF1 to be essential in a similar manner (342). IRF1 null mice were more heavily colonised with *H. pylori* and failed to induce either Th1 or antibody responses during infection (342). Accordingly, these mice also failed to develop characteristic gastritis that is

associated with Th1 responses during *H. pylori* infection (342). Furthermore, IRF1 expression in $CD4^+$ T cells was shown to correlate with the production of Th1 cytokines, such as IFN-γ (343), which was not secreted by $CD4^+$ T cells isolated from $IRF1^{-/-}$ mice following *H. pylori* infection (342). These data highlight the importance of IRF1 in both epithelial and immune cell responses to *H. pylori* infection.

Finally, although we found that severe gastritis was induced only in patients infected with *H. pylori*, it was difficult to correlate gastritis severity with $CagA^+$ *H. pylori* isolates due to low sampling numbers. $CagA^+$ strains, however, were more often detected in patients with severe gastritis, than in those with normal or moderate pathology. Many studies have verified an association between infection with *cag*PAI^+ *H. pylori* and the development of severe disease, such as gastric cancer (Reviewed by (344)). Nevertheless, as only 25 % of patients with antral tumours in the present study had concurrent *H. pylori* infection, it is difficult to draw too many conclusions in this regard. Furthermore, there was some variation in the details of the pathologist reports regarding differences in tumour histology between patients, which made it difficult to accurately group tumour types and corresponding responses. Regardless, although there were no significant increases in *IFNG* or *CXCL10* expression in tumour compared to non-tumour tissue, statistically significant increases were detected in *CXCL8*, *IRF1* and *NOD1* expression (Figure 3.13; $p<0.05$). Elevated *CXCL8* expression in gastric cancer is thought to promote tumour neo-vascularisation (345-346). In contrast, elevated *IRF1* and *NOD1* expression is an interesting finding that has not been reported previously. IRF1 is generally regarded as an anti-tumour factor that mediates apoptosis of cancer cells (Reviewed by (347)) and although a loss of heterozygosity in the *IRF1* chromosomal region has been reported in gastric cancer (348), a functional phenotype has yet to be established in this context. Increased *NOD1* expression in tumour tissue is another intriguing finding, particularly as a concurrent *H. pylori* infection was absent in most cases. Increases in *IRF1* and *NOD1* expression suggest possible IFN-γ-dependent induction of this pathway and although *IFNG* is not upregulated in these cases of gastric cancer, there may be increased IFN-γ production from nearby immune cells.

The ability of gastric epithelial cells to recruit immune effectors to the site of *H. pylori* infection is paramount to the development of inflammation. We and others have demonstrated that NOD1-dependent recognition of *cag*PAI$^+$ bacteria is essential for the initiation of innate and eventually adaptive immune responses to the pathogen (24, 161, 293, 310, 317), and NOD1 appears to facilitate the induction of a skewed Th1 phenotype during *H. pylori* infection (292). The Th1 phenotype is characterised by the infiltration of IFN-γ-producing CD4$^+$ T cells (92, 235-237, 327) and these pathogen specific lymphocytes, in addition to IFN-γ, are necessary for the development of gastritis in the host and for the control of *H. pylori* infection (240). The production of large quantities of IFN-γ further regulated cell specific host responses and in this way, we have shown that IFN-γ treatment of *H. pylori*-infected gastric epithelial cells upregulates the expression of chemokines that are responsible for the recruitment of immune cells into the gastric mucosa. These effects were found to be mediated by *cag*PAI$^+$ *H. pylori* bacteria only, suggesting a role for NOD1 in this pathway. Furthermore, NOD1-dependent recognition of *cag*PAI$^+$ bacteria was found to induce *IRF1* expression, which itself is known to upregulate NOD1. Indeed, we and others have shown increased *NOD1* expression (299) during *H. pylori* infection and like *IFNG* and *IRF1*, the expression of this pathogen recognition molecule correlates with disease severity. These data demonstrate for the first time, a potential positive feedback mechanism between the key molecule involved in gastric epithelial cell recognition of *H. pylori*, and the primary cytokine that drives chronic inflammatory responses to this pathogen.

CHAPTER 4. Final Discussion

The pathogen recognition molecule, NOD1, is a critical component of the innate immune system that is able to recognise and respond to both extracellular and invading pathogens. NOD1 is expressed ubiquitously in most cell types and in this way, its responsiveness is not limited to mucosal surfaces such as epithelial cells. Indeed, recent studies have found a crucial role for NOD1 in macrophage (349) and dendritic cell (350) responses to invading pathogens, indicating its importance in effective myeloid cell function. While NOD1 is vital for the initiation of innate inflammatory responses and pathogen-specific immunity, studies have identified that allelic variation within the *NOD1* gene is associated with aberrant inflammatory signalling and in some instances, an increased risk of severe disease (351-353). These reports recapitulate the fine balance that exists between the establishment of effective immune responses and potentially pathogenic non-specific inflammation.

Host responses to the gastric pathogen, *H. pylori*, are largely dependent on gastric epithelial cell recognition via NOD1 (24, 161). In particular, the virulent *cag*PAI^{+} isolates possess a T4SS that translocates bacterial effectors into the host cell (21, 24-26). In this way, peptidoglycan is sensed via the intracellular NOD1 (140), which rapidly initiates a signalling cascade that culminates in the activation of NF-κB and the expression of pro-inflammatory chemokines (24) that recruit immune effectors to the gastric mucosa. While gastric epithelial cells are largely refractory to TLR stimulation via *H. pylori* products, such as LPS (24, 140, 151, 177) and flagella (174, 181-183), cells of myeloid lineage seem to be more responsive. Indeed, the TLR adaptor molecule, MyD88, is critical for the development of adaptive immune responses to *H. pylori*. Specifically, macrophage recognition of live *H. pylori* and its LPS is thought to occur via TLR2 and TLR4 respectively (169, 172), whereas recent reports suggest that TLR8 and TLR9 may be important for dendritic cell recognition of *H. pylori* RNA and DNA respectively (354). In this way, there appears to be a dual requirement by the host for NOD1 in the initial

recognition of *H. pylori* by gastric epithelial cells, and later for TLR recognition by macrophages and dendritic cells.

The aims of this study were to investigate the ability of NOD1 to activate additional signalling pathways in gastric epithelial cells following recognition of *cag*PAI$^+$ *H. pylori*, which may enhance pro-inflammatory responses. Furthermore, the literature suggests that IFN-γ, a crucial cytokine that drives adaptive immune responses to *H. pylori*, is capable of up-regulating NOD1 expression (298). Indeed, NOD1 protein levels are elevated in the gastric mucosa of *H. pylori*-infected patients (299). Therefore, we examined the ability of IFN-γ to enhance epithelial responsiveness to *cag*PAI$^+$ bacteria via NOD1. This was expanded upon to determine whether there is a correlation between the expression of IFN-γ- and NOD1- responsive genes and disease severity. In this way, the possibility of cross-talk between IFN-γ and NOD1 signalling pathways was addressed in order to determine the existence of a positive regulatory loop that may exacerbate chronic inflammatory responses to *cag*PAI$^+$ *H. pylori*.

While NOD1 recognition of *cag*PAI$^+$ *H. pylori* was found to rapidly activate NF-κB, a number of additional signalling responses were consistently shown to be induced in gastric epithelial cells in a *cag*PAI-dependent manner, although a specific mechanism of action was unclear. In this way, we evaluated the role of NOD1 in the *cag*PAI-dependent activation of signalling responses other than the canonical NF-κB pathway. Indeed, previous reports demonstrated that *H. pylori* activated p38 and JNK MAPKs in a *cag*PAI-dependent manner, yet further studies were not conducted to ascertain the specific mechanism(s) through which this occurs. Investigation of host responses to the invasive pathogens *L. monocytogenes* (355) and *S. flexneri* (356) revealed that NOD1 induced the activation of JNK and p38 MAPKs respectively. Accordingly, using a stably transfected NOD1 knock-down gastric epithelial cell line, we demonstrated that NOD1-dependent recognition of *cag*PAI$^+$ *H. pylori* was essential for the rapid activation of p38 and ERK MAPKs, which in turn were required for the activation of AP-1. In this way, we have shown that dual activation of the transcription factors, NF-κB and AP-1, occurs via a NOD1-dependent mechanism and is essential for the production of pro-inflammatory

chemokines. In contrast, we determined that NOD1 was dispensable for JNK activation by *H. pylori*, whilst verifying the findings of Girardin and colleagues showing NOD1-dependent JNK activation in response to *S. flexneri* (356). These results validated our co-culture model and highlighted that *cag*PAI^{+} *H. pylori* were capable of inducing signalling cascades in epithelial cells that were independent of NOD1 and CagA. Indeed, our collaborators found that characteristic CagA-induced host cytoskeletal re-arrangements, termed the "hummingbird" phenotype, were also dependent on JNK phosphorylation. In this way, we have shown that the hummingbird phenotype occurs independently of NOD1. This work was furthered to demonstrate that *cag*PAI-dependent JNK phosphorylation occurred via the activation of host Src kinases upon contact of the *H. pylori cag*PAI-encoded T4SS with the host cell.

A report published during the course of these studies elegantly demonstrated that *cag*PAI-encoded CagL protein localised to the tip of the Type-IV pilus and bound the $\alpha_5\beta_1$ integrin receptor on gastric epithelial cells via an arginine-glycine-aspartate (RGD) motif (285). This interaction was suggested to be essential for the translocation of CagA into the host cell and the subsequent activation of host Src kinases (285). In contrast, another group reported that the Type-IV pilus binds integrin β1 with greater affinity via the *H. pylori* CagA and CagY proteins in an RGD-independent mechanism (357), finding no role for the CagL RGD motif in either CagA translocation or IL-8 production during *H. pylori* stimulation (357). While the reason for these discrepancies are unclear, subsequent studies have supported a role for CagL in the activation of host signalling pathways, suggesting that it mimics many of the functions of fibronectin via its regulation of not only FAK and Src, but also the epidermal growth factor receptor (EGFR) and related proteins (358). Furthermore, CagL binding was shown to induce the dissociation of the metalloenzyme, ADAM17, from the integrin $\alpha_5\beta_1$ receptor during *H. pylori* stimulation (359). This resulted in the activation of ADAM17 and the NF-κB-dependent transcriptional repression of H,K-adenosine triphosphatase (ATPase) (359), the enzyme which is responsible for gastric acid secretion. Indeed, hypochlorhydria is a common symptom during the acute phase of *H. pylori* infection (145) and in this regard, CagL-

induced aberrant host cell signalling is postulated to be another significant contributing factor in the pathogenesis of *H. pylori* infection.

Host Src kinases are responsible for the tyrosine phosphorylation of multiple CagA EPIYA motifs once it enters the cell (25-26, 42). CagA phosphorylation of EPIYA-C or -D motifs facilitate binding to and phosphorylation of the host phosphatase, SHP-2, which induces a number of aberrant signalling pathways within the cell. In this way, CagA/SHP-2 signalling has recently been implicated in the manipulation of STAT3 signalling pathways in the host cell. Specifically, CagA was reported to bind to the cytoplasmic domain of the host gp130 receptor, whereby it induces Jak2 phosphorylation and subsequent STAT3 activation (360). Whilst investigators agree that CagA is capable of STAT3 activation, there is less consensus regarding the role that CagA phosphorylation plays. In particular, STAT3 activation has been reported to occur both dependently (361) and independently (362) of CagA phophorylation, with a recent study suggesting that phosphorylated CagA actually inhibits STAT3 activation (360). A possible mechanism for this was postulated to involve SHP-2, which negatively regulates STAT3 by directly de-phosphorylating tyrosine residues on the gp-130 receptor (363-364), or indirectly in this case via the activation of ERK MAPK, which can deactivate STAT3 (360). The investigators found that *H. pylori* strains encoding wild-type CagA, which can be tyrosine phosphorylated, preferentially activate the SHP-2/ERK pathway in epithelial cells, whereas strains possessing a mutated CagA protein, which cannot be tyrosine phosphorylated, instead activate STAT3. During stimulation with a $CagA^+$ isolate, CagA is constantly phosphorylated and de-phosphorylated within the cell by host kinases (45, 47). This process is likely to balance SHP-2/ERK and STAT3 activation, thereby creating a degree of signalling equilibrium. Gastric biopsies from patients infected with $CagA^+$ *H. pylori* had markedly enhanced STAT3 phosphorylation, as compared to basal levels detected in individuals infected with $CagA^-$ strains (291). This was confirmed in the Mongolian gerbil model, with STAT3 phosphorylation detected in gastric epithelial cells and inflammatory infiltrates of Mongolian gerbils infected with $CagA^+$ *H. pylori* only (362). STAT3 phosphorylation is elevated in many malignancies, including gastric cancer (291, 365) and is a poor prognostic marker for disease outcome

(366-367), something that is attributable to its potent anti-apoptotic, pro-proliferative, angiogenic and metastatic effects (188). The ability of CagA to induce STAT3 signalling in epithelial cells may be an important contributing factor towards the elevated risk of developing gastric cancer in patients infected with $CagA^+$ isolates (31, 33, 36, 38).

In our studies we noted that compared to wild-type bacteria, isogenic *H. pylori* 251 and G27 *ΔcagA* mutants, which possess a functional T4SS, induced markedly higher IP-10 and MIG production from cells co-stimulated with IFN-γ. Indeed, similar trends were also noted for the production of RANTES, MCP-1, Mip-1α and Mip-1β chemokines. These data indicate that CagA has an inhibitory effect on IFN-γ signalling pathways in gastric epithelial cells. SHP-2 is known to negatively regulate IFN-γ responses via the direct de-phosphorylation of STAT1 (368) and given that CagA activates SHP-2 in the host cell, we hypothesised that IFN-γ-induced STAT1 phosphorylation was being reversed by activated SHP-2 in cells stimulated with *cag*PAI^+ *H. pylori*. There was, however, no difference in the levels of STAT1 phosphorylation between cells co-stimulated with either IFN-γ and wild-type bacteria or IFN-γ and *H. pylori ΔcagA* mutants. Furthermore, STAT1 Tyr701 phosphorylation was actually enhanced in these cells as compared to those stimulated with IFN-γ alone. Intriguingly, a recent report found that STAT3 is capable of negatively regulating STAT1-dependent IP-10 and MIG production in IFN-α-stimulated cells (369). While STAT3 did not inhibit STAT1 phosphorylation or nuclear translocation, it prevented STAT1 complexes from binding the promoters of a number of pro-inflammatory genes (369). In the context of recent literature on CagA/STAT3 signalling, these findings suggest that CagA is able to negatively regulate IFN-induced responses of gastric epithelial cells via STAT3 activation (Figure 4.1). This appears to be the case *in vivo*, given that STAT3 is hyper-activated in $CagA^+$ *H. pylori*-infected individuals (291, 362), yet immunohistochemistry and *in situ* hybridisation studies have revealed that both IP-10 and MIG are not produced by gastric epithelial cells during *H. pylori* infection (336), despite the fact that levels of IFN-γ are elevated in the gastric mucosa during infection.

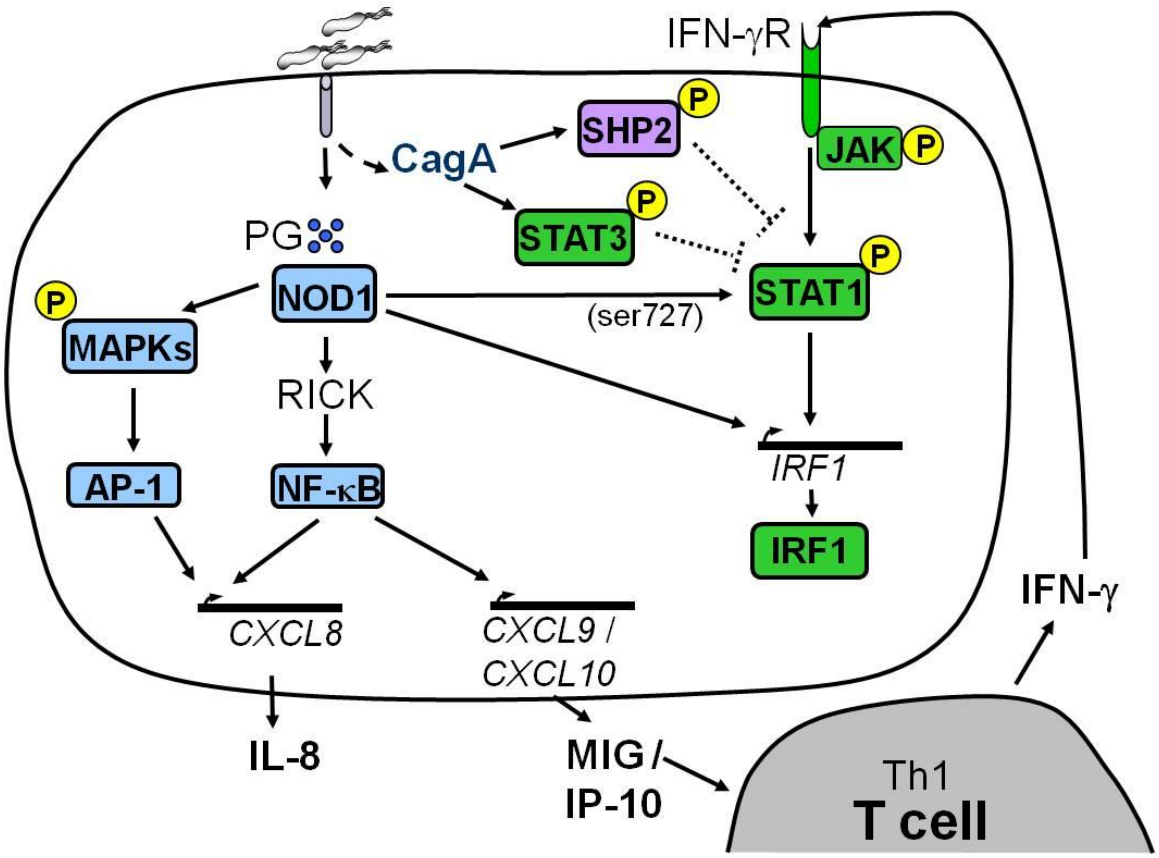


Figure 4.1 Overview of NOD1- and IFN-γ-dependent signalling responses in gastric epithelial cells during *H. pylori* infection.

Interestingly, the inhibitory effects of CagA were also noted for NOD1-dependent *IRF1* expression, suggesting that STAT3 may also inhibit a component of this pathway. It is not likely that STAT1 is responsible for *IRF1* expression in this case, given that Tyr701 phosphorylation is required for STAT1 nuclear translocation and subsequent *IRF1* expression (322) and we have clearly demonstrated that *H. pylori* does not induce the phosphorylation of this residue. We did, however, demonstrate that *cag*PAI^{+} bacteria are capable of inducing STAT1 Ser727 phosphorylation, although CagA was not involved. Intriguingly, TLR activation has been shown to induce STAT1 Ser727 phosphorylation in macrophages and dendritic cells, which occurs via activation of p38 MAPK (370-371). In this study, we have demonstrated that NOD1-dependent recognition of *cag*PAI^{+} *H. pylori* is essential for p38 MAPK activation upon stimulation of gastric epithelial cells. Therefore, we hypothesise that the *cag*PAI-dependent STAT1 Ser727 phosphorylation

observed occurs in a NOD1-dependent manner. Further studies are warranted to determine if this is indeed the case and to assess the significance of STAT Ser727 phosphorylation in the absence of concomitant tyrosine phosphorylation during *H. pylori* stimulation.

Numerous studies have reported the enhanced capacity of *cag*PAI^{+} *H. pylori* to induce gastritis (30-38), which correlates with augmented pro-inflammatory cytokine production and immune infiltrates in the gastric mucosa (200, 202). Due to the small sample size in our study, it was difficult to draw definitive conclusions between the *cag*PAI and disease severity in *H. pylori*-infected patients. We were, however, able to show a significant association between disease severity and the expression of pro-inflammatory molecules in the gastric mucosa. Intriguingly, while the expression of *IFNG*, *NOD1*, *IRF1*, *CXCL8*, and *CXCL10* were all elevated in *H. pylori*-infected individuals, there was no significant differences between patients infected with *cag*PAI^{+} or *cag*PAI^{-} isolates. While this may be an artefact of insufficient sample size, *cag*PAI^{-} *H. pylori* strains are capable of causing gastritis and severe disease, albeit less often than *cag*PAI^{+} strains (34). A recent study elucidated a mechanism whereby OMVs released by *cag*PAI^{-} strains are able to induce pro-inflammatory responses in gastric epithelial cells (312). These bacterial membrane blebs contain peptidoglycan and were shown to enter epithelial cells via membrane lipid rafts, where they were recognised in a NOD1-dependent manner (312). In this way, OMVs were shown to stimulate robust Th1 adaptive immune responses, similar to those induced by live *H. pylori*. Indeed, we have shown here that *H. pylori* OMVs isolated from *cag*PAI^{-} strains, rapidly activate MAPKs and AP-1 in gastric epithelial cells via NOD1, demonstrating that even *cag*PAI^{-} isolates are capable of initiating NOD1-dependent signalling cascades in epithelial cells, which are necessary to recruit inflammatory cells to the gastric mucosa. In this way, the ability of *cag*PAI^{-} *H. pylori* to induce expression of pro-inflammatory molecules in the gastric mucosa, such as *IFNG*, *NOD1*, *IRF1*, *CXCL8*, and *CXCL10*, is plausible. While separate reports have shown a correlation between IL-8 production and *cag*PAI^{+} strains, this is the first study to assess the correlation between *IFNG*, *NOD1*, *IRF1* and *CXCL10* expression and the *cag*PAI in a human cohort, albeit small.

Finally, we have shown that the expression of *IRF1* and *NOD1* are significantly enhanced in gastric tumour specimens as compared to the surrounding healthy tissue. While gastric cancer studies have reported mutations in the chromosomal region containing the *IRF1* gene, these are generally associated with loss of function (348). Indeed, IRF1 is a potent anti-tumour factor, due to its ability to induce growth arrest, apoptosis and inhibit cell transformation (372). Therefore, our finding of elevated *IRF1* expression in gastric cancer is curious and warrants further investigation. In particular, control of *IRF1* expression in tumours has been demonstrated to occur via mechanisms other than gene deletion or loss of protein function. These include aberrant signalling responses that lead to IRF1 inactivation, such as mRNA splicing aberrations (373) or inhibitory factors that directly bind IRF1 and inhibit function (374). The enhanced *NOD1* expression in tumour tissue is another intriguing finding, given that in most cases of gastric cancer analysed, patients were not infected with *H. pylori*, suggesting that up-regulation occurs via another mechanism, although we did not detect enhanced IFN-γ expression in tumour tissue. A functional role for NOD1 in gastric cancer is unclear, although a G796A SNP in the *NOD1* gene was recently shown to be associated with aberrant NOD1 signalling in patients suffering from Sarcoidosis (353). This allele was also reportedly associated with an elevated risk of antral atrophy and intestinal metaplasia in *H. pylori*-infected individuals (352). While this allele was also shown to correlate with the development of duodenal ulcer (351), another study found no association between the *NOD1* G796A SNP and gastritis or gastric ulcer (299). Although the role of this specific allele in disease development is controversial, we have shown for the first time in these studies that *NOD1* expression is associated with disease severity and gastric cancer, which indicates that NOD1 signalling may in fact be a critical factor in the potentiation of inflammatory responses.

These studies have demonstrated for the first time that NOD1 is capable of activating pro-inflammatory signalling cascades in gastric epithelial cells other than the canonical NF-κB pathway. In this way, NOD1-dependent recognition of *cag*PAI^{+} *H. pylori* or OMVs from *cag*PAI^{-} strains is able to rapidly induce the production of pro-inflammatory chemokines, which are essential for the recruitment of inflammatory cells to the gastric

mucosa. Our findings re-emphasise the importance of NOD1-dependent gastric epithelial responses and have furthered previous studies in NOD1 knock-out mice that demonstrated the role of NOD1 in the development of Th1-type adaptive immune responses to *H. pylori*. The recruitment of IFN-γ-secreting $CD4^+$ T cells to the gastric mucosa is a critical component of *H. pylori*-induced gastritis and we were able to show an association between IFN-γ, NOD1 and gastritis severity. Furthermore, NOD1-dependent recognition of *cag*PAI^+ *H. pylori* augmented epithelial cell responsiveness to IFN-γ, suggesting that both NOD1 and IFN-γ responses continuously upregulate pro-inflammatory signalling during infection. *H. pylori* infection is unusual in that it is seldom eradicated without therapeutic intervention, suggesting that NOD1 may be in a state of perpetual activation that facilitates constant inflammatory cell migration into the gastric mucosa. In this way we have demonstrated the importance of NOD1 in the induction and also maintenance of pro-inflammatory responses during *H. pylori* infection.

4.1 Future Directions

We have demonstrated a number of novel findings in this study that warrant further investigation. In particular, we have shown that *cag*PAI$^+$ *H. pylori* induce NOD1-dependent MAPK phosphorylation, which is critical for subsequent AP-1 activation and pro-inflammatory cytokine production. In particular, NOD1 was required for the rapid activation of p38 MAPK, which has been reported to induce STAT1 Ser727 phosphorylation following TLR stimulation. While we were able to show that *cag*PAI$^+$ *H. pylori* induced the phosphorylation of this residue in gastric epithelial cells, it is of interest to confirm that NOD1 is indeed responsible. Furthermore, evaluation of the significance of serine phosphorylated STAT1 without concomitant tyrosine phosphorylation may reveal additional signalling pathways not previously characterised in the context of *H. pylori* infection or other inflammatory responses.

Another noteworthy and unexpected finding was that CagA actually inhibited IFN-γ-dependent responses in gastric epithelial cells. Recent reports have demonstrated that CagA is capable of activating STAT3 in gastric epithelial cells. These findings suggest that STAT3 may inhibit IFN-γ-dependent responsiveness of cells, by inhibiting STAT1 binding to DNA. While this hypothesis supports the results of this study, validation of the CagA-dependent inhibitory effects of STAT3 on IFN-γ signalling is necessary. Indeed, this raises the possibility that although CagA is associated with the development of severe disease during *H. pylori* infection, it may act to dampen pathogen specific immune responses, thereby promoting the persistence of *H. pylori* in the stomach.

Finally, we have shown for the first time that *IRF1* and *NOD1* expression is significantly upregulated in gastric cancer, although the precise mechanism is unclear. Further work is necessary to investigate the functionality of IRF1 and NOD1 in gastric cancer and to ascertain whether enhanced expression correlates with common SNPs in their respective genes. Whilst requiring further investigation, enhanced *IRF1* and *NOD1* expression in gastric cancer is an exciting finding that may reveal novel mechanisms involved in the development and progression of gastric cancer.

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Appendices

Appendix 1. Virulence factors and host cell signalling in H. pylori recognition

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Role of virulence factors and host cell signaling in the recognition of *Helicobacter pylori* and the generation of immune responses

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Helicobacter pylori colonizes a large proportion of the world's population, with infection invariably leading to chronic, lifelong gastritis. While the infection often persists undiagnosed and without causing severe pathology, there are a number of host, bacterial and environmental factors that can influence whether infection provokes a mild inflammatory response or results in significant morbidity. Intriguingly, the most virulent *H. pylori* strains appear to deliberately induce the epithelial signaling cascades responsible for activating the innate immune system. While the reason for this remains unclear, the resulting adaptive immune responses are largely ineffective in clearing the bacterium once infection has become established and, as a result, inflammation likely causes more damage to the host itself.

Review
Future Microbiology

This article focuses on the initial cellular signaling events following host contact with *Helicobacter pylori* and how these events control the development of innate, and subsequently adaptive, immune responses to the bacterium. Specifically, we will discuss how bacterial virulence factors induce host signaling pathways and affect cellular integrity. Finally, we will examine interactions between *H. pylori* and the adaptive immune system, and discuss why the pathogen is able to persist.

Colonization & adherence

H. pylori is a spiral-shaped Gram-negative pathogen that colonizes the harsh environment of the stomach. Although there is limited knowledge regarding the initial colonization of individuals, the infection is generally acquired during childhood [1]. Transmission of *H. pylori* is thought to occur primarily through person-to-person contact via the oral–oral, gastric–oral and/or possibly fecal–oral routes, with sanitation and hygiene practices influencing the incidence of infection [2]. Developing countries tend to be associated with a higher prevalence of *H. pylori* infections than developed countries, where the incidence of infection is steadily declining [3]. There is, however, substantial variation in the carriage rates of *H. pylori* within developed nations [3], which is partially attributed to differences in hygiene practices, exposure to antimicrobials and ethnic/genetic predisposition to infection [2].

Once in the stomach, *H. pylori* produces large quantities of urease, which hydrolyzes urea to produce carbon dioxide and ammonia, thus neutralizing the surrounding pH and protecting the bacteria from the harsh acidic environment [4]. This is a crucial step in the initial stages of colonization that protects *H. pylori* whilst it traverses from the highly acidic lumen to the more neutral gastric mucosa, where the bacteria interact with the host epithelium. In addition to this, *H. pylori* possess unipolar flagella, which are critical both for the penetration of the mucus layer and for persistence in the ephemeral gastric mucosa. Both urease- [5–7] and flagella-deficient [8,9] *H. pylori* mutants are unable to colonize the stomach, highlighting how well this pathogen has adapted to such a specialized niche.

H. pylori produces numerous outer membrane proteins, many of which are adhesins belonging to the *H. pylori* outer membrane porins (Hop) family [10,11]. There is some variation in the distribution of adhesins amongst different strains, and a remarkable capacity to regulate expression of the genes encoding them, which can be altered to varying degrees in response to environmental cues [12–16]. Some of the better studied adhesins are the adherence-associated lipoproteins (AlpAB), sialic acid-binding adhesin (SabA), blood group antigen-binding adhesin A (BabA) and OipA (which will be discussed later).

Keywords

■ *Helicobacter pylori* ■ immunity ■ inflammation ■ signaling ■ virulence factor

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AlpAB

The *alpAB* operon encodes two homologues, which are possessed by virtually all isolates, and both AlpA and AlpB have been shown to be essential for adherence of *H. pylori* to epithelial cells [17–19] and for colonization of guinea pigs [20] and mice [21]. Interestingly, differential cytokine production was observed in gastric epithelial cells stimulated with isogenic *H. pylori* Δ*alpAB* mutants generated on different strain backgrounds [21]. Thus, certain strains may require these adhesins, not only for adherence and colonization, but also for the initiation of proinflammatory signaling cascades [21].

SabA & BabA

Epithelial cells express fucosylated ABO blood group antigens and sialyl-Lewis x and a (sLe^x and sLe^a) antigens, which are recognized by BabA and SabA, respectively [22–24]. SabA appears to be one of the most variable adhesins, with expression regulated by stomach acidity and inversely correlating with low pH [16]. Indeed there are a number of poly T/CT tracts within the *sabA* promoter that can turn the gene 'on' or 'off' [11,24,25]. A role for SabA in immune responses and disease outcome has also been reported, with studies finding that SabA can bind and activate neutrophils [26], and that SabA expression is associated with gastric cancer [16].

The BabA-encoding *babA2* gene is found in 28–62% of *H. pylori* isolates [10,27,28]. Interestingly, there is often greater homology between the *babA2* gene and Hop paralogues (*babB* and *babC*) within a genome than there is between *babA* orthologues amongst different strains, suggesting that *babA* may continually undergo DNA shuffling to produce new variants [29], differing substantially in their ability to bind fucosylated blood group antigens [30]. Recently, BabA expression was shown to be lost in *H. pylori* isolates recovered following long-term infection of rhesus macaques, gerbils and mice [14,31]. Indeed, the inflammation induced in the early stages of infection is known to upregulate the expression of sialylated mucosal antigens, whilst simultaneously reducing the levels of fucosylated antigens [32]. A switch in adhesin expression may be a mechanism through which *H. pylori* adapts to the changing gastric mucosa as disease progresses.

Virulence factors

H. pylori possesses a number of virulence factors that enhance disease by either directly causing localized cell damage, or by exacerbating the host immune response. There is increasing evidence, however, that despite the well-characterized role of these factors in disease, many may also serve to attenuate host immune responses. Regardless, the presence of certain bacterial factors is associated with severe disease (Table 1).

cag pathogenicity island

The *cag* pathogenicity island (*cag*PAI) is an approximately 40-kb region of DNA comprising 31 genes, and is found in some *H. pylori* strains [25,33,34]. Many of these genes are required for the assembly of a type IV secretion system (T4SS), which secretes the bacterial effectors, CagA and peptidoglycan, into the host cytoplasm [35–37]. It is estimated that approximately 60% of *H. pylori* isolates from Western countries, and virtually all East Asian isolates, possess the *cag*PAI [38]. The presence of the *cag*PAI is the most reliable bacterial indicator of disease outcome, strongly correlating with increased bacterial density [39,40], inflammation and, as a result, severe disease, such as peptic ulcer, atrophic gastritis and gastric cancer [41].

Cytotoxin-associated gene A

The cytotoxin-associated gene A (*CagA*) is located within the *cag*PAI, and encodes an approximately 120-kDa protein, which is translocated into host cells by the T4SS. Upon entry, CagA multimerizes [42], and is subsequently tyrosine phosphorylated by host Src and Abl kinases at multiple residues, known as EPIYA motifs [36,37,43]. CagA phosphorylation facilitates its interaction with a number of cytoplasmic host proteins, thus inducing aberrant signaling events and influencing pathways that affect proliferation, chemokine production, cytoskeletal rearrangement and epithelial integrity [44]. The EPIYA motifs are encoded within the 3′ region of the *cagA* gene, which is highly variable and enables the classification of Eastern and Western isolates based on the flanking DNA sequences. There are four distinct EPIYA variants, EPIYA-A, -B, -C and -D, and representative Western isolates generally possess a copy of EPIYA-A, -B and -C, while Eastern isolates possess EPIYA-A, -B and -D [45]. EPIYA-A and -B bind and activate the host C-terminal Src kinase (Csk), which inhibits phosphorylation of the CagA protein and, thus, negatively regulates CagA-induced signaling [46–48]. Phosphorylated EPIYA-C from Western and EPIYA-D from East Asian isolates bind and activate a host cytoplasmic phosphatase, SHP-2 [45,49], which is largely responsible for the disruption of signaling pathways. The flanking sequence of EPIYA-D binds SHP-2 with greater affinity than that of EPIYA-C

[45–46] and, as such, East Asian *cagA*-positive isolates correlate more strongly with severe disease than Western *cagA*-positive isolates [50–54]. Certain Western strains, however, may possess a *cagA* gene with multiple copies of the EPIYA-C segment, and these isolates are generally more virulent than those with only one [45,50,55]. Regardless, CagA with a single copy of the EPIYA-D motif is considered to be the variant with the greatest potential for inducing aberrant epithelial cell signaling [45].

CagA has been consistently shown to induce cytoskeletal rearrangements *in vitro* [56–58], interfering with epithelial tight-junction proteins, such as zonular occludin-1 (ZO-1) and the junction adhesion molecule (JAM), thus resulting in a loss of barrier function [59]. Epithelial cells transiently expressing CagA were shown to lose polarity, acquire the ability to degrade the basement membrane, and to invade the extracellular matrix [60]. In addition, *cag*PAI-positive *H. pylori* upregulates host expression of matrix metalloproteinases (MMPs) [61–63], which can further degrade the extracellular matrix and are often associated with malignant metastasis of a number of cancers [64]. While CagA-negative *H. pylori* strains are capable of inducing host cell motility [56], this occurs without concomitant MMP production by epithelial cells. Furthermore, CagA has been reported to promote proliferative and antiapoptotic gene regulation via activation of serum response elements [65], growth factor receptors and signaling molecules [66–68]. Indeed, studies have found elevated proliferation scores in the stomach of CagA-positive infected patients, compared with individuals infected with CagA-negative *H. pylori* [69,70]. Interestingly, CagA-negative isolates were found to induce higher levels of apoptosis, whereas CagA-positive strains did not [69,70]. Therefore, the uncontrolled cellular growth induced by CagA-positive *H. pylori*, in combination with a disruption in epithelial integrity and elevated expression of MMPs, may potentiate precancerous changes in the stomach, and may explain the elevated incidence of gastric adenocarcinoma in CagA-positive *H. pylori*-infected individuals [41].

Table 1. Association of *Helicobacter pylori* virulence factors with host responses and disease outcome.

Virulence factor	Colonization/cell damage	Inflammation/immunity	Disease outcome
*cag*PAI	Increased bacterial density	Increased production of cytokines and antimicrobials Infiltration of immune cells	Gastritis Peptic ulcer Gastric cancer
CagA	Disruption of epithelial junctions Epithelial motility and scattering	Cytokine production? Upregulation of oncogenic proteins	Atrophic gastritis Gastric cancer
Urease	Colonization Cellular injury (ammonia) Disruption of tight junctions	Activation and recruitment of monocytes and neutrophils Sequestration of IgA antibodies Inhibition of phagocytosis	Gastric damage
VacA	Colonization? Epithelial permeability Epithelial erosion and necrosis Vacuolation Apoptosis	Disruption of antigen processing Inhibition of T-cell proliferation	Ulceration
OipA	Adherence to epithelial cells Colonization?	Cytokine production?	Dysplasia Gastric cancer Duodenal ulcer
Hp-NAP	Adherence to host proteins Resistance to iron oxidation	Activation and recruitment of monocytes, dendritic cells and neutrophils Increased monocyte and neutrophil survival Cytokine production Polarization of Th1 immune responses	Gastric cancer? Peptic ulcer?
DupA	–	Cytokine production?	Duodenal ulcer? Decreased gastric cancer
Hsp60	Adherence to epithelial cells	Cytokine production	Mucosa-associated lymphoid tissue Lymphoma Gastric cancer (diffuse type)

Vacuolating cytotoxin A

The effects of the Vacuolating cytotoxin A (VacA) were initially attributed to CagA, until disruption of the *cagA* gene was found to have no effect on the vacuolating activity of *H. pylori* on host cells [71]. Subsequently, the *vacA* gene was identified, and its disruption was shown to ablate these cytotoxic effects [72]. Early confusion regarding the function of CagA may be attributed to the strong correlation between the presence of the most active forms of VacA and the presence of the *cag*PAI, which is associated with an increased risk of severe disease [39,54,73–75].

The *vacA* gene encodes a 140-kDa autotransporter pre-protoxin, which is proteolytically cleaved to produce the secreted, approximately 88-kDa, mature VacA toxin [76,77], which is activated in the extracellular milieu by an acidic or alkaline pH [78,79]. Whilst each *H. pylori* isolate possesses a copy of the *vacA* gene, not all strains secrete cytolytically active toxin [80], and allelic variation within *vacA* amongst different strains markedly affects toxin potency [72,74]. Polymorphic variation in the *vacA* gene enables classification based on three distinct variable regions: signal (s1a, s1b and s2), intermediate (i1 and i2) and

middle (m1 and m2). Allelic variants tend to cluster into specific subsets, such as s1/i1/m1 and s2/i2/m2, with a *vacA* s1/i1/m1 type being associated with more severe disease [81]. s2 alleles encode an altered signal peptide that prevents toxin secretion and, therefore, renders VacA nontoxic [74]. VacA m2 variants are able to act on fewer types of epithelial cell lines than m1 variants, owing to altered receptor-binding capabilities [82]. m2 variants, however, retain their ability to bind primary gastric epithelial cells and induce cytotoxicity [82], indicating that there may not be a difference in clinical outcome between infections with VacA m1 or a VacA m2 strains. The intermediate region was described only recently, and shown to be responsible for the cytotoxic activity of VacA. As such, the i1 allele is thought to be the best indicator of disease outcome, correlating strongly with the development of peptic ulcer, duodenal ulcer and gastric cancer [81,83].

VacA has been reported to bind a number of different host cell receptors, including receptor protein tyrosine phosphatase (RPTP), α and β [79,84,85], the EGF receptor (EGFR) [86] and lipid raft-associated receptors [87,88], such as sphingomyelin [89]. Interestingly, mice deficient in RPTPβ were found to be resistant to the formation of gastric ulcers following oral administration of purified VacA [90]. It appears that VacA may bind a number of receptors within lipid rafts, which results in endocytosis of the toxin into the host cell [87–89]. Intoxicated cells typically contain characteristic vacuoles, which are fusions of late endosomal and early lysosomal compartments [91]. In addition, VacA reduces the mitochondrial membrane potential and induces the release of cytochrome C, which results in apoptosis [92]. The precise mechanism of this is unclear, but has been suggested to occur via the VacA-induced activation of proapoptotic molecules, Bax and Bak [93]. VacA can also disrupt essential cellular functions, such as protein degradation [94] and the processing of extracellular ligands in antigen-presenting cells [95]. Furthermore, several reports have demonstrated that VacA directly inhibits both the activation and proliferation of human B cells, as well as both $CD4^+$ and $CD8^+$ T cells [96–99]. Interestingly, primary murine T cells were found to be resistant to the inhibitory effects of VacA, an effect thought to be partially attributable to reduced toxin affinity to these cells [100]. Finally, VacA has been shown to increase the permeability of polarized epithelial cell monolayers to low-molecular-weight molecules and ions [101], which may be a mechanism employed by *H. pylori* to acquire nutrients in the harsh gastric environment.

CagA versus VacA antagonism

Interestingly, CagA and VacA appear to exert antagonistic effects on each other. First, VacA-induced vacuole formation and apoptosis were inhibited in epithelial cells [102,103] by phosphorylated CagA, which indirectly interferes with the trafficking of VacA to intracellular compartments [104]. At later time points, unphosphorylated CagA was shown to upregulate host antiapoptotic factors [104]. Similarly, VacA inhibits CagA-induced epithelial scattering via inhibition of CagA-dependent mitogen-activated protein kinase (MAPK) activation [102,105]. It is possible that *H. pylori* may act to counter some of the effects of one virulence factor with those of another so as to minimize host damage once infection is established. It has also been hypothesized, however, that early epithelial cell damage caused by VacA assists *H. pylori*, particularly *cag*PAI-positive strains, in colonizing the host [106]. Salama *et al.* found that, while *H. pylori* Δ*vacA* mutants are not attenuated *in vivo* once infection is established, they have a reduced capacity to initially colonize the host [107]. This study, however, was performed using the mouse-adapted SS1 strain, which lacks a functional *cag*PAI [108]. Furthermore, experiments employing piglet and Mongolian gerbil disease models were unable to demonstrate a measurable effect of VacA on colonization by *cag*PAI-positive *H. pylori* or associated gastritis [109,110].

Outer membrane inflammatory protein

Outer membrane inflammatory protein (OipA) has been shown to act as an adhesin that facilitates bacterial attachment to the host epithelium [111]. Indeed, *oipA* mutants display significantly reduced adherence to epithelial cells *in vitro* [111]. By contrast, while an isogenic *H. pylori* Δ*oipA* mutant strain failed to colonize Mongolian gerbils [112], another study reported that a similar mutant was able to colonize guinea pigs to the same extent as the parental strain [20]. The *oipA* gene is found in all strains, although isolates possess a variant that has either been switched on or off via slip-strand mispairing [16], thereby implying that *oipA* expression is not required by all isolates to efficiently colonize the host. There is a strong correlation, however, between the presence of an on *oipA* gene and other virulence factors, such as CagA and the more potent VacA variant [111], which may explain why the presence of a functional *oipA* gene in *H. pylori* isolates also correlates with high bacterial density, neutrophil infiltration, severe gastritis and gastric cancer [16,113]. In addition, Yamaoka and colleagues reported a role for

OipA in proinflammatory cytokine production [15,16,113–116], although other groups have been unable to replicate this finding [111,112,117–119]. However, it does appear that a functional *oipA* gene enhances the ability of *cag*PAI-positive strains to stimulate gastric epithelial cells [10]. Elucidation of the cognate host receptor for OipA may offer insights into its exact role in colonization and mechanism of action on the host cell.

H. pylori neutrophil-activating protein

H. pylori neutrophil-activating protein (Hp-NAP) is a highly immunogenic, approximately 150-kDa protein that acts as a potent chemoattractant for monocytes and neutrophils [120,121], and promotes the adhesion of neutrophils to endothelial cells [121]. In addition, Hp-NAP stimulates neutrophils to release reactive oxygen species (ROS) [122], and induces the production of proinflammatory cytokines by immune cells [120,121]. Hp-NAP can promote the survival of monocytes by stimulating the production of IL-1β, which was proposed to act on the cell to upregulate the expression of antiapoptotic genes [123]. In this way, Hp-NAP-stimulated monocytes were also shown to prolong the survival of neutrophils in a coculture assay, although Hp-NAP had no direct effect on neutrophil survival [123]. It has also been reported that Hp-NAP can act as an adhesin to facilitate bacterial binding to mucin and other host proteins [124,125], which may assist *H. pylori* persistence in the stomach. These reports highlight how *H. pylori* can directly recruit immune cells to the site of infection and facilitate their persistence, thereby exacerbating inflammation. Furthermore, a recent study reported that the levels of serum Hp-NAP-specific antibodies were significantly higher amongst patients with gastric cancer and peptic ulcer, compared with asymptomatic individuals or those with chronic gastritis [126]. This suggests that elevated expression of Hp-NAP by certain *H. pylori* strains may increase the risk of developing severe disease, possibly via the exacerbation of inflammatory responses.

Duodenal ulcer promoting gene A

Duodenal ulcer promoting gene A (*dupA*) is a novel gene identified in some *H. pylori* strains, which was shown to be significantly associated with the development of duodenal ulceration, whilst negatively correlating with the incidence of gastric cancer [127]. Lu *et al.* found that *dupA* was involved in IL-8 production, both *in vitro* and *in vivo*, and it downregulated the expression of host oncogenes while upregulating the expression of the p53 tumor-suppressor gene, which may explain the reported inverse relationship with gastric cancer development [127]. Although some studies have confirmed the proposed association with duodenal ulcer [128], gastric cancer [129] or premalignant lesions [130], a number of studies have failed to show an association between the prevalence of *dupA* amongst *H. pylori* strains and the clinical outcome of infection, when sampling cohorts from a diverse range of geographical locations and ethnic groups [131–134]. In addition, these studies found that *dupA* was not involved in IL-8 production. Interestingly, *dupA* encodes an ATPase and is functionally homologous to the *H. pylori virB4* gene, which is involved in the assembly of the T4SS and, as such, studies have suggested that *dupA* may also be involved in the assembly of a novel secretion apparatus [127]. Apart from *dupA*, however, no other genes with homology to genes encoding T4SS components have been identified in *H. pylori* thus far [135].

H. pylori heat-shock protein 60

H. pylori heat-shock protein 60 (Hsp60) is a potent immunostimulatory antigen that has been reported to be involved in the adherence of *H. pylori* to epithelial cells [136] and in the induction of robust cytokine responses in gastric epithelial cells [137,138], monocytes [139–141] and macrophages [142]. The host immune system produces antibodies directed against *H. pylori* Hsp60 [143,144] and antigenic similarity between this protein and the human Hsp60 variant has been suggested to be associated with autoimmune diseases, such as B-cell mucosa-associated lymphoid tissue lymphoma [145,146]. A recent report has found an association between anti-Hsp60 antibodies and the development of diffuse-type gastric cancer [147].

Outer membrane vesicles

It has been reported that *cag*PAI-negative *H. pylori* strains are still capable of causing gastritis and severe disease, albeit at a lower frequency than *cag*PAI-positive strains [148]. As *cag*PAI-negative bacteria do not possess a T4SS, it is likely that they may drive the recruitment and activation of immune cells via other mechanism(s). One such mechanism involves outer membrane vesicles (OMVs), which are released during growth *in vitro* and *in vivo* by all Gram-negative organisms [149], including *H. pylori* [150]. Indeed, *H. pylori* OMVs have been shown to contain cell wall components and biologically active VacA, which is capable of inducing vacuolation [150–152]. These vesicles inhibit cellular proliferation at low doses

and induce growth cycle arrest and apoptosis at higher doses, an effect found to be independent of VacA [151,153]. Interestingly, *H. pylori* OMVs isolated from *cag*PAI-negative strains stimulated the secretion of IL-8 from gastric epithelial cells at amounts significantly higher than those induced by OMVs from *cag*PAI-positive strains. The reason for this, however, is unclear [151]. Recently, the mechanism whereby *H. pylori* OMVs induce IL-8 production in gastric epithelial cells was elucidated [154]. It was shown that these OMVs enter epithelial cells via membrane lipid rafts, and are 'sensed' by the innate immune system, resulting in proinflammatory cytokine responses [154]. Given that approximately only 2% of *H. pylori* are in contact with the gastric epithelium at any one time [155], OMVs may represent one means whereby the bacteria sustains inflammatory responses without needing to remain intimately associated with host cells.

Innate immunity

Colonization of the gastric mucosa by *H. pylori* results in the initiation of a robust innate immune response and the production of proinflammatory molecules that are involved in the direct killing of *H. pylori* or the recruitment of immune cells to the site of infection [156]. The innate signaling events that are induced early during infection are the driving force behind the development of adaptive immune responses to *H. pylori*, thus influencing disease severity and the likelihood of complications, such as ulceration and carcinogenesis.

Innate immune recognition of *H. pylori*

*cag*PAI-positive *H. pylori* are potent activators of the transcription factors nuclear factor (NF)-κB and activating protein 1 (AP-1), within gastric epithelial cells. These signaling cascades culminate in the secretion of proinflammatory cytokines, such as IL-8 [157,158], growth-regulated oncogene-α (Gro-α) [159], monocyte chemotactic protein-1 [160] and RANTES [161], which recruit immune cells to the site of infection. This mechanism of cytokine production was shown to be *cag*PAI dependent. We propose that this is primarily induced in epithelial cells via T4SS-dependent activation of the host cytosolic nucleotide oligomerization domain 1 (NOD1) molecule (Table 2).

Nucleotide oligomerization domain 1

Nucleotide oligomerization domain 1 senses a specific conserved motif of Gram-negative bacterial peptidoglycan [162,163] and, upon recognition, NOD1 associates with the serine-threonine kinase, RICK [164–166], which, in turn, activates TGF-β-associated kinase [167]. These events result in the activation of IκB kinase and degradation of IκBs [164,165,167,168], thus allowing the translocation of NF-κB from the cytoplasm to the nucleus and the transcription of proinflammatory molecules. With respect to *H. pylori*, studies have demonstrated that *cag*PAI-positive bacteria are able to induce IκBα degradation, which results in the release and nuclear translocation of NF-κB [169,170].

Nucleotide oligomerization domain 1 has been shown to activate NF-κB following recognition of peptidoglycan, which is translocated into host epithelial cells via the T4SS of *cag*PAI-positive bacteria [35,171–173]. Preliminary data from our laboratory suggests that RICK is also involved in this process [171]. In addition to activating the NF-κB pathway, *cag*PAI-positive *H. pylori* are potent inducers of host MAPKs [174], which can activate the transcription factor, AP-1. Interestingly, p38 and c-Jun N-terminal kinase (JNK) MAPKs are activated only by *cag*PAI-positive *H. pylori* [171,175,176], although the precise pathway is unclear. Recent work from our group found that, in addition to activating NF-κB, NOD1 is also capable of activating p38 and extracellular signal-related kinase (ERK) MAPKs, and is essential for AP-1 activation during *H. pylori* infection [171]. *cag*PAI-dependent JNK phosphorylation, however, does not require NOD1 [171] and, instead, is activated downstream of host Src kinases [177]. These data, together with the decrease in proinflammatory cytokine and Th1 immune responses in NOD1-knockout mice during *H. pylori* challenge [35,178], suggest that NOD1 may play a critical role in driving innate and adaptive immune responses to infection by *cag*PAI-positive *H. pylori* strains. By contrast, Hirata *et al.* reported that, in epithelial cells, *H. pylori* activated NF-κB independently of either NOD1 or RICK and, instead, induced signaling via the critical Toll-like receptor (TLR) adaptor molecule, MyD88 [179]. The reason for this discrepancy is unclear, as epithelial cell lines are relatively nonresponsive to TLR activation by live *H. pylori* bacteria, owing to defective receptor signaling [180].

Aside from the importance of the T4SS for NF-κB responses to *H. pylori*, there are conflicting reports regarding the ability of *H. pylori* virulence factors to induce the expression of proinflammatory cytokines by epithelial cells. For example, while some groups have shown that CagA can induce NF-κB activation [118,181],

Table 2. Role of pathogen recognition molecules in detecting *Helicobacter pylori*.

Host receptor	Ligand	*H. pylori* factor	Cell type	Cell-specific response
NOD1	Gram-negative bacterial peptidoglycan	Peptidoglycan	Epithelial cells	NF-κB and AP-1 activation Cytokine production Antimicrobials Polarization of Th1 immune responses
			Dendritic cells	IL-17 production
TLR2	Multiple bacterial lipoproteins and glycolipids	*H. pylori* lipopolysaccharide?	Epithelial cells	NF-κB activation Cytokine production
		Hp-NAP	Monocytes	NF-κB activation Cytokine production
			Dendritic cells	Cytokine production Th1 polarization
TLR4	Bacterial lipopolysaccharide	*H. pylori* lipopolysaccharide?	Macrophages?	Cytokine production
TLR5	Bacterial flagella	Nonresponsive to *H. pylori* flagella	Nonresponsive	Nonresponsive
TLR8/TLR13	ssRNA?	*H. pylori* RNA?	Dendritic cells?	No data
TLR9	Bacterial DNA	*H. pylori* DNA	Dendritic cells	Cytokine production
RIG-I	Viral RNA	*H. pylori* RNA	Epithelial cells	IFN-β Others?

NOD: Nucleotide oligomerization domain; RIG: Retinoic acid-inducible gene; TLR: Toll-like receptor.

this is thought to occur after 24 h of coculture, which is much later than NOD1-induced NF-κB activation, which occurs within 1 h of infection [35,171]. Lamb *et al.*, however, recently reported that CagA was essential for *H. pylori*-induced NF-κB activation within 1 h of infection, and for subsequent IL-8 production *in vitro* [182]. The reason for this inconsistency is unclear; however, CagA is generally considered to be dispensable for NF-κB-induced cytokine production in response to *H. pylori* [35,65,158,183–185]. Similarly, some researchers have reported that CagA can activate ERK MAPK [68] and AP-1 [186,187]; however, other groups have been unable to reproduce this finding [65,171]. Discrepancies in the reported functions of CagA may highlight strain-specific variations in the virulence and function(s) of the *cagA* gene amongst different *H. pylori* isolates, or even differences in the expression levels of host molecules within epithelial cell lines.

TLR signaling

Toll-like receptors are a group of important pathogen-recognition molecules that trigger innate immune responses upon sensing differing microbial components [188]. TLR2 recognizes a range of molecules, including lipoproteins from Gram-negative organisms [189,190], whereas TLR4 generally recognizes lipopolysaccharide (LPS) from these organisms [191,192], and both TLRs are capable of inducing robust proinflammatory responses upon stimulation. *H. pylori* LPS, however, is substantially less endotoxic and immunogenic than that of other Gram-negative pathogens, such as *Escherichia coli* [193–196]. Indeed, detailed studies of *H. pylori* LPS have found that the structure and modification of the lipid A component differs considerably from the traditionally potent variants expressed by other pathogens [197]. *H. pylori* LPS undergoes less phosphorylation and acetylation than LPS of other Gram-negative pathogens [198,199] and, furthermore, the repeating units of the LPS O-side chains mimic Lewis^x and Lewis^y blood group antigens [200,201] that are expressed on the host epithelium. *H. pylori* host mimicry is thought to play a role in immune evasion by the bacteria.

The structural differences of *H. pylori* LPS may also explain its ability to activate TLR2 in gastric epithelial cells [202–205]. There are, however, a number of contradictory studies that have reported *H. pylori* LPS to signal via the conventional TLR4 receptor [206–210]. Inconsistencies in the literature may be attributable to the varying expression patterns of TLRs and their respective coreceptors in different gastric epithelial cell lines that are commonly used to study *H. pylori* [180,203,204,207,211]. It is also important to note that many studies have relied on artificial TLR transfection and overexpression systems. Furthermore, strain-specific variation in LPS structure, in addition to the purity and quantity of LPS used (nanograms vs milligrams), were cited as possible explanations for the varying results [210]. Regardless, *H. pylori* LPS is a weak activator of epithelial cell signaling, and live bacteria are still capable of inducing robust proinflammatory responses, even in

the absence of TLR2 and TLR4 signaling in cells [35,163,170,171,212]. Despite having minimal effects on gastric epithelial cells, some studies have reported that *H. pylori* LPS can be recognized via TLR4 in monocytes and macrophages [207,210], whereas TLR2 is thought to be responsible for macrophage recognition of the whole *H. pylori* bacteria [210]. Indeed, Hp-NAP, which is probably released upon bacterial lysis [124], was shown to stimulate TLR2-transfected HEK293 cells [120] and, furthermore, the treatment of murine splenocyte preparations with Hp-NAP was shown to induce robust cytokine responses [213].

H. pylori infection has been reported to upregulate TLR2 and TLR4 expression *in vivo* in epithelial cells and infiltrating immune cells [207,209,214], and both receptors are thought to be involved in the generation of adaptive immune responses to *H. pylori*. Rad *et al.* reported that both TLR2 and TLR4 were required for dendritic cell (DC) cytokine production in response to stimulation with *H. pylori* lysates, whereas only TLR4 was required for the recognition of *E. coli* lysates [215]. Interestingly, TLR2 and TLR4 were found to be dispensable for the recognition of live *H. pylori* in experiments using TLR2/4 double-knockout bone marrow-derived DCs (BMDCs). By contrast, TLR2/4/9-knockout BMDC cytokine production was significantly reduced, with investigators finding that TLR9 can recognize *H. pylori* DNA [215]. While these results suggest that *H. pylori* DNA is a potent inducer of DC responses, cytokine production was not completely abolished in TLR2/4/9-knockout cells, whereas DCs lacking MyD88 were virtually nonresponsive to *H. pylori* stimulation [215]. Similar experiments performed using macrophages found that TLR2, TLR4 and MyD88 were all essential for complete cytokines responses to *H. pylori*, whereas TLR9 was dispensable [216]. These data highlight the different mechanisms employed by immune cells, such as macrophages and DCs, to respond to *H. pylori*. Regardless, MyD88 is undoubtedly a critical mediator of adaptive immunity, as MyD88-knockout mice have increased bacterial colonization and impaired immune responses to *H. pylori* infection [217]. Interestingly, DCs were shown to recognize *H. pylori* RNA in a MyD88-dependent manner, with TLR8 or TLR13 considered possible candidates [215].

Another key TLR involved in host defense at mucosal surfaces is TLR5, which responds to bacterial flagella [218]. This receptor is widely expressed across various gastric epithelial cell lines [180,203,219]. *H. pylori* flagellin, however, is poorly immunogenic [203,219,220], and lacks the consensus amino acid motifs that are critical for recognition by TLR5 [221]. Indeed, flagellin-responsive epithelial cell lines are not responsive to purified *H. pylori* flagella [219,220]. Furthermore, isogenic *H. pylori* mutants, lacking FlaA and/or FlaB proteins, retained their ability to induce IL-8 production by gastric epithelial cells [219], whereas transfection of a dominant-negative TLR5 receptor into epithelial cells only partially diminished epithelial responses to *H. pylori* [203]. As mentioned previously, *H. pylori* flagella mutants are unable to colonize the stomach, suggesting that *H. pylori* may have evolved to express immunologically inert flagella in order to efficiently colonize the host without inducing excessive inflammation. Interestingly, this characteristic appears to have been conserved across other *Helicobacter* species and members of the campylobacterales [222].

T4SS-induced epithelial signaling

A number of recent studies have reported that the T4SS of *H. pylori* is able to activate host signaling cascades in the absence of NOD1-dependent peptidoglycan recognition, or via the 'sensing' of other known pathogen-associated molecular patterns. Kwok *et al.* found that the *H. pylori* CagL protein, which localizes to the tip of the type IV pilus, binds to and activates the integrin $\alpha_5\beta_1$ receptor on gastric epithelial cells via an arginie–glycine–aspartate (RGD) motif [223]. This interaction was shown to be required for the translocation and phosphorylation of CagA in the host cell, in addition to the activation of host FAK and Src kinases during infection [223]. By contrast, another group reported that the T4SS binds integrin β_1 with greater affinity via the *H. pylori* CagA and CagY proteins, and this occurs via an RGD-independent mechanism [224]. Furthermore, this group found no role for the RGD motif of CagL in either CagA translocation or IL-8 production during *H. pylori* infection [224]. While the reason for these discrepancies are unclear, subsequent studies have supported a role for CagL in the stimulation of host signaling pathways, reporting that it mimics many of the functions of fibronectin via its regulation, not only of FAK and Src, but also the EGFR and related proteins [225]. Furthermore, CagL binding was shown to induce the dissociation of the metalloenzyme, ADAM17, from the integrin $\alpha_5\beta_1$ receptor during *H. pylori* infection [226]. This resulted in the activation of ADAM17 and the NF-κB-dependent transcriptional repression of

H,K-adenosine triphosphatase (ATPase) [226], the enzyme which is responsible for gastric acid secretion. Indeed, hypochlorhydria is a common symptom during the acute phase of *H. pylori* infection [156] and, in this regard, CagL-induced aberrant host cell signaling is postulated to be another significant contributing factor in the pathogenesis of *H. pylori* infection.

Innate immune responses to *H. pylori*

Secretion of proinflammatory factors

Studies have consistently shown elevated levels of IL-1β, IL-2, IL-6, IL-8, Gro-α and TNF-α in the gastric mucosa of *H. pylori*-infected individuals [227–231]. IL-1, IL-8 and Gro-α levels, in particular, are generally higher in patients infected with *cag*PAI-positive strains [231,232]. Many of these chemokines/cytokines have overlapping functions, which influence apoptosis and proliferation and are involved in the recruitment, activation and differentiation of lymphocytes, neutrophils, macrophages and DCs [156].

Antimicrobial molecules

The expression of a number of host antimicrobial compounds is upregulated in the gastric mucosa of *H. pylori*-infected individuals. These include the neutrophil-derived α-defensins 1, 2 and 3 [233,234] and the epithelial-derived peptides, LL37/hCAP18 [235], and human β-defensins (hBDs). hBDs are potent antimicrobial peptides, and both hBD2 and hBD3 have been demonstrated to exert potent microbicidal action against *H. pylori* [236–238]. *H. pylori* was shown to upregulate hBD2, hBD4 and LL37 expression in a *cag*PAI-dependent manner [172,235,236,239–241], whereas hBD1 and hBD3 expression was found to be upregulated independently of *cag*PAI [172,239,242,243]. Specifically, hBD2 expression is induced via NOD1-dependent NF-κB activation [172,239], while p38 was shown to be essential for hBD4 expression [241]. Transactivation of the EGFR, and subsequent ERK MAPK phosphorylation was found to be responsible for *H. pylori*-dependent hBD3 [172,239] and, possibly, hBD1 expression [242]. Grubman *et al.* extended these findings to demonstrate that the culture supernatants of gastric epithelial cells stimulated with *cag*PAI-positive bacteria exerted potent antimicrobial action against *H. pylori* [172]. This activity was primarily associated with NOD1-dependent hBD2 production [172]. Despite this, however, there is no correlation between bacterial density and the concentration of hBD2 in the gastric juice of infected individuals [234], suggesting that *H. pylori* is able to successfully avoid antimicrobial-mediated killing during infection. Interestingly, studies have found that hBD2 is chemotactic for DCs and lymphocytes *in vitro* [244,245]. By contrast, another study found that all hBDs (1–4) were chemotactic for macrophages and mast cells, but not DCs and lymphocytes [246]. Regardless, β-defensins have not yet been shown to play a role in the recruitment of immune cells during *H. pylori* infection.

Infiltration of immune cells

The interaction of *H. pylori* with the epithelium results in the activation and rapid recruitment of monocytes and neutrophils to the site of infection [193,247–252]. This is partially attributed to the direct chemotactic activity of virulence factors, such as urease [249] and Hp-NAP [121,122], which are expressed by all *H. pylori* isolates. As mentioned previously, T4SS effector translocation into gastric epithelial cells by *cag*PAI-positive *H. pylori* results in the secretion of chemokines [35–37] and the further recruitment of immune cells. As a result, *cag*PAI-positive *H. pylori* strains are generally associated with more severe gastritis than infection with *cag*PAI-negative isolates [41]. Neutrophils, macrophages and DCs that are recruited to the gastric mucosa perpetuate the inflammatory cycle by sampling antigens and secreting additional proinflammatory cytokines. It is important to note that inflammatory cell infiltration and corresponding gastritis is minimal in *H. pylori*-infected severe-combined immunodeficient mice [253], and adoptive transfer of $CD4^+$ T cells is capable of restoring these responses [254]. These data indicate that the recruitment of inflammatory mediators to the site of infection is a largely T-cell-mediated event.

Phagocytosis of *H. pylori* by infiltrating immune cells is thought to be a largely ineffective process owing to a number of innovative evasion strategies employed by the bacteria [255]. First, *H. pylori* are not efficiently opsonized by host antibodies. This has been attributed to the harsh acidic environment of the stomach [256,257], and to the actions of urease [258], both of which have been shown to interfere with the process. *cag*PAI-positive *H. pylori* can also actively inhibit their uptake into macrophages, and those that are engulfed are able to prevent phagosome maturation, instead inducing the formation of megasomes that fail to clear the bacteria [259–261]. By contrast, *cag*PAI-negative bacteria are rapidly engulfed and killed by these cells [259].

H. pylori has also evolved sophisticated mechanisms of neutralizing/avoiding ROS and nitric oxide (NO). Macrophages produce large amounts

of inducible NO synthase (iNOS) during *H. pylori* infection [262], and this enzyme converts arginine into NO. *H. pylori* is able to inhibit this process via the arginase, RocF, which rapidly sequesters and hydrolyzes arginine into urea before it can be converted into NO by iNOS [263]. In addition, phagocytosed *H. pylori* are able to disrupt the cellular targeting of NADPH oxidase to neutrophil phagosomes, resulting in the accumulation of superoxide anions in the extracellular milieu [264]. While these superoxide anions can react with NO to produce highly toxic peroxynitrite, this is detoxified by an alkylhydroperoxide reductase, encoded by the *H. pylori ahpC* gene [265]. As such, sustained levels of ROS during infection are likely to be more detrimental to the host than the bacteria, and the associated DNA damage that results is postulated to be another risk factor in the development of cancer [266,267]. Despite the plethora of evasion strategies employed by *H. pylori* to minimize the effectiveness of macrophages and neutrophils, proximity is another key factor. Although *H. pylori* has been demonstrated to infiltrate the lamina propria [268], it resides predominantly in the lumen of the stomach, and phagocytes do not readily traverse this epithelial layer as in other mucosal sites.

Disease-associated polymorphisms

A number of studies have investigated the contribution of host genetic polymorphisms to the development of *H. pylori*-induced morbidity. Certain proinflammatory polymorphisms in the IL-1 gene cluster, encoding IL-1α, IL-1β and their receptor, IL-1ra, have been demonstrated to influence disease outcome [269]. IL-1β polymorphisms were shown to be associated with a significantly increased risk of developing precancerous abnormalities [270] and intestinal and diffuse-type (non-cardia) gastric cancer in individuals with *H. pylori* infection [270,271]. An even stronger correlation was noted if the patient was infected with a *cagA*-positive or VacA s1/m1 strain [272]. IL-1β is a potent inhibitor of gastric acid secretion [273], and hypochlorhydria is a hallmark of noncardia gastric cancer [269]. Indeed, transgenic mice expressing human IL-1β in the stomach were shown to spontaneously develop gastritis and carcinomas, an effect that was exacerbated by concomitant *Helicobacter felis* infection [274]. Proinflammatory polymorphisms in the genes encoding TNF-α, IL-10 and TLR4 have also all been linked to an elevated risk of noncardia gastric cancer [271,275,276]. In addition, a common polymorphism in *IL-8* gene promoter, which results in enhanced mucosal IL-8 production, has been linked to increased risk of severe gastritis and premalignant changes [277]. An increased risk of gastric cancer, however, was only noted in Asian populations [269]. Finally, a polymorphism in the gene encoding NOD1 has been linked to an elevated risk of duodenal ulcer and more severe gastritis [278,279].

Adaptive immunity

Infection with *H. pylori* results in the development of vigorous humoral and cell-mediated adaptive immune responses. Nevertheless, in most cases, these appear to be relatively ineffective in eliminating the pathogen from the gastric mucosa [280]. Results from immunization studies in mice have revealed that the immune response generated during chronic infection differs markedly from responses induced by vaccination [280]. This indicates that early events in the course of a dynamic *H. pylori* infection may actively skew the generation of an effective immune response, thus resulting in a failure to eradicate the pathogen.

As mentioned previously, certain *H. pylori* virulence factors can either directly or indirectly, via the stimulation of epithelial cells, recruit neutrophils, macrophages and DCs to the gastric mucosa. These cells perpetuate nonspecific inflammatory responses, whilst sampling and processing antigens to present to pathogen-specific lymphocytes. The cytokines secreted by antigen-presenting cells during lymphocyte priming polarize T-helper (Th) cells into specific functional classes, such as Th1, Th2 and Th17, which secrete particular cytokine profiles and have different roles in *H. pylori* infection [280,281].

Th1 versus Th2 responses to *H. pylori*

Th1 $CD4^+$ lymphocytes produce a cytokine profile that includes IFN-γ, TNF-α and IL-2, and is associated with cell-mediated immunity, which is important in the protection against intracellular parasites and viral infections [282]. Th2 cells produce the cytokines IL-4, IL-5 and IL-13, and are associated with antibody-driven humoral immunity [282]. Early studies investigating the immune responses in *H. pylori*-infected individuals reported a predominantly Th1 phenotype, characterized by the gastric infiltration of $CD4^+$ Th cells [283,284] and the production of large quantities of IFN-γ and IL-12 [285–288]. While there are conflicting reports linking the number of IFN-γ-secreting cells with the severity of gastritis [286,289], infusion of IFN-γ into mice has been shown to induce precancerous lesions, even without concomitant *H. pylori* infection [290]. Similarly, pretreatment of mice with IL-4 prevented the development

of gastritis in mice infected with *H. felis* [291]. Paradoxically, *in vivo* neutralization of IFN-γ was shown to alleviate *H. felis*-associated gastritis in mice, without affecting vaccine-induced protection [292], and studies employing IFN-γ-knockout mice demonstrated that immunized animals were capable of inducing a protective immune response to *H. pylori* infection [288,293]. By contrast, however, other groups have reported that IFN-γ is indispensable for vaccine-induced protection [294,295]. Differences in the strain of *H. pylori* used, and the gender of the infected mice, were cited as possible explanations for discrepancies between these results [295]. Regardless, this variation in the literature suggests that the Th1-mediated IFN-γ-driven immune response may not be the sole mechanism of controlling *H. pylori* infection. Indeed, the Th1/Th2 model is limited in some regards, owing to the identification of additional Th subsets [296], particularly Th17, which has emerged as an important mediator of autoimmune inflammatory disorders [297] and immune responses to extracellular bacteria [298].

Th17 cells

Recent studies have focused on the role of Th17 cells during *H. pylori* infection, which secrete large amounts of IL-17 that can act on a variety of target cells to upregulate the production of proinflammatory molecules [299]. While the role of Th17 during *H. pylori* infection is still unclear, IL-17 cytokine levels are elevated during *H. pylori* infection [300,301]. In addition, Th17 is thought to be involved in the generation of immunity following vaccination, as protection was shown to be associated with elevated levels of IL-17 in the gastric mucosa of *H. pylori*-infected mice [302] and a rapid rise in the infiltration of $CD4^+$ $IL\text{-}17^+$ T-cells in *H. felis*-infected animals [303]. Protection was reversed if mice were administered a neutralizing anti-IL-17 antibody, which correlated with a reduction in gastritis and infiltrating neutrophils [303]. As such, the absence of robust Th17 responses during the acute phase of infection may be a determining factor in the failure to naturally clear infection. Regardless, Khamri *et al.* reported that human monocyte-derived DCs cocultured with *H. pylori* are capable of inducing IL-17 production by T cells *in vitro*, and that the *H. pylori* T4SS and, to a lesser extent, CagA, were required for this response [304]. These DCs also stimulated the production of IFN-γ by T-cells; however, this was found to occur independently of the *H. pylori* T4SS. While Kao *et al.* confirmed the ability of DCs to induce IL-17 production by T cells, they found this to be independent of VacA or CagA [305]. This group used murine BMDCs, however, so some discrepancies in host responses between mice and humans are likely.

Tregs

Another confounding factor influencing the efficacy of a sustained *H. pylori*-specific immune response is the failure to successfully clear infection. While *H. pylori*-treated DCs stimulate IFN-γ production by naive T cells, prolonged exposure of DCs to the bacterium was shown to result in impaired cytokine production and an inability to induce sufficient T-cell responses [306]. Furthermore, memory T cells isolated from *H. pylori*-infected patients had impaired proliferation and IFN-γ-secreting potential when rechallenged with *H. pylori* [307,308], despite retaining the ability to respond to stimulation with other antigens. This was shown to be mediated by the presence of Tregs, the numbers of which are elevated in the gastric mucosa of *H. pylori*-infected individuals [309–311]. These cells indirectly facilitate the persistence of *H. pylori* in the stomach, by suppressing excessive inflammatory responses in an attempt to protect the host [308,310]. Indeed, Treg depletion was shown to reduce *H. pylori* colonization of the stomach [305], and a reduction in Treg numbers correlates with an elevated incidence of peptic ulcer disease [311]. Similarly, there is a negative correlation between gastritis and gastric expression of the Treg marker, Foxp3 [310,312].

Levels of IL-10 and TGF-β are elevated in the gastric mucosa during *H. pylori* infection [313,314], and these cytokines are known to be important mediators of Treg function [315–317]. Interestingly, *H. pylori*-stimulated DCs produced more IL-10 and TGF-β than DCs stimulated with *E. coli* [305,306], and elevated production of these cytokines correlated with the ability of DCs to induce Tregs [305]. It appears that *H. pylori*, more so than other pathogens, is capable of stimulating the expression of cytokines that promote Treg function. Tregs may be a means by which *H. pylori* establishes pathogen/host equilibrium during persistent infection, thereby minimizing excessive damage to the host. This strategy is probably successful, considering that the proportion of infections that result in severe disease, such as peptic ulcer and gastric adenocarcinoma, are 15 and 0.5–2%, respectively [318].

Antibodies

From acute infection studies, it has been shown that *H. pylori* infection elicits a strong antibody response in the host, with detectable serum IgM antibodies present 4 weeks after infection [319].

IgA- and IgM-secreting cells can be found in the gastric mucosa [320] and IgA antibodies are detectable in gastric juice [143]. The role of *H. pylori*-specific antibodies in host immunity or clearance is questionable, however, as IgA-deficient patients do not experience enhanced gastritis or an increased susceptibility to disease [321]. Akhiani *et al.* even found that IgA-knockout mice had reduced *H. pylori* colonization [322], suggesting that IgA antibodies may actually facilitate bacterial persistence, possibly through immune evasion [323,324]. Furthermore, both IgA- and antibody-deficient mice achieved the same levels of vaccine-induced protective immunity as wild-type animals [325–328].

Results from the numerous *Helicobacter* immunization studies suggest that cell-mediated, rather than humoral immune responses, are the most efficacious in controlling *H. pylori* infection [329]. However, it is noteworthy that no vaccine has achieved sterilizing immunity against *H. pylori* and, as such, no specific polarized immune response or component can be discounted when considering how infection is best controlled. It is also important to note that almost all of the vaccination studies to date have been performed in mice of C57BL/6 background, which are more prone to a default Th1 response and, thus, are more likely to rely on cell-mediated immunity to fight infection.

In addition, there are differences in antibody responses induced by live *Helicobacter* and those induced by immunization. Interesting comparisons were made in a study comparing the antibody profiles of mice infected with *H. felis* and mice immunized with bacterial sonicate [330]. *H. felis* infection resulted in the proliferation of large numbers of IgA- and IgG-secreting cells, whereas immunization resulted primarily in the production of IgG1 antibodies, which is indicative of a Th2 response [330]. In addition, IgG antibodies were identified in the gastric mucosa of immunized mice, implying a specific localized response in these animals that was absent in unimmunized mice [330]. Reduced IgA production in immunized mice compared with *H. felis*-infected mice has been reported previously [331]. Furthermore, pups suckling from immunized mice displayed passive immunity to *H. pylori*, which was mediated exclusively by IgG antibodies in the mother's milk, although protection was not sustained after weaning [332]. These results suggest that *H. pylori* infection skews immune responses to encourage the production of a relatively inefficient ratio of antibody isotypes, which fail to clear the pathogen.

Conclusion

H. pylori colonizes the gastric mucosa early during childhood and, without therapeutic intervention, persists for the life of the host. While infection invariably results in chronic gastritis, a number of contributing pathogen, host and environmental factors influence the severity and likelihood of disease. *H. pylori* possess numerous virulence factors, such as the T4SS, CagA, VacA, Hp-NAP and adhesins, which are involved in colonization, host cell damage and the induction or modulation of immune responses. Gastric epithelial cells respond more strongly to *cag*PAI-positive *H. pylori* isolates, which are able to inject pathogenic factors into the host cell cytoplasm via the T4SS. These factors either directly cause cell damage or induce innate and eventually, adaptive immune responses. This is characterized primarily by a polarized proinflammatory Th1 response, which is specific, yet ineffective in clearing *H. pylori* and, as such, recent studies suggest that a Th17-skewed response may be more useful.

Furthermore, and although controversial, we suggest that antibodies may play an important role in the regulation of *H. pylori* infection. Owing to the limited lifespan of animals, to date, most studies have focused on acute, rather than chronic, immune responses in naive animals. Aggressive adaptive responses that are rapidly induced in response to *H. pylori* are undoubtedly dampened over time, which is probably an attempt by the host to adapt to infection and minimize self damage. This is an important consideration when assessing the most effective response(s) by which the immune system can clear infection. In particular, an immune response generated by vaccination of naive animals that is effective in preventing/reducing colonization is likely to differ to the response required to clear a chronic infection. As such, cell-mediated and humoral responses are likely to be important in the control of infection at various stages of disease.

Future perspective

H. pylori has evolved over time to colonize the highly specific gastric niche, developing strategies to effectively avoid immune clearance. Infection, therefore, is unusual, in that it persists for the life of the host, despite inducing potent proinflammatory responses. In addition to understanding the mechanisms of initial pathogen colonization and subsequent recognition by the host, assessing the immune responses in a well-established chronic *H. pylori* infection should also be a focal point of research. In particular, it

is clear that *H. pylori* actively skews host immune responses to facilitate its persistence in the gastric mucosa, which results in a dampening of pathogen-specific responses over time.

While *in vitro* coculture assays provide useful insights into direct pathogen–host interactions on a cellular level, these systems are largely one dimensional, in the sense that they do not adequately portray the complex associations between *H. pylori* and components of the innate and adaptive immune system. Furthermore, studies utilizing current animal models tend to focus on the early immune responses of naive hosts to *H. pylori* infection, rather than on acute-to-chronic infection transition, and the associated differences in host/pathogen responses.

In the coming years, there is likely to be a greater emphasis on the evolution of chronic *H. pylori* infection throughout the life of the host. In particular, transcriptome and proteomic analysis will provide valuable *in vivo* data on the constantly evolving interactions between a host and pathogen, assessing initial colonization and the later stages once infection is well established. These experiments will help clarify how *H. pylori* is able to regulate overactive immune responses, and should provide an insight into the most efficacious responses for clearing the pathogen. This information may help stratify patients according to a pathogen/host profile in order to personalize treatment strategies. Finally, there is much knowledge to be gained from understanding the potential benefits of *H. pylori*-induced immune protection against the development of extragastric autoimmune disorders, such as asthma, lupus and inflammatory bowel disease.

Executive summary

Colonization & adherence

- The *Helicobacter pylori* genome encodes numerous outer membrane proteins involved in adhesion to the gastric epithelium.
- Adhesin expression can vary substantially in response to environmental cues throughout the various stages of disease.

Virulence factors

- The *cag* pathogenicity island (*cag*PAI) is a major virulence factor, encoding a type IV secretion system (T4SS), which secretes bacterial effectors, such as CagA and peptidoglycan, into the host cell.
- *cag*PAI and CagA are associated with an increased risk of severe gastritis and gastric cancer.
- CagA induces aberrant signaling within the host cell, which can promote precancerous changes in the stomach.
- A number of virulence factors can directly or indirectly induce host cell damage via apoptosis or the recruitment and modulation of immune mediators.

Innate Immunity

Innate immune recognition of *H. pylori*

- The cytosolic host protein, NOD1, recognizes peptidoglycan, which is translocated into the cell via the T4SS of *cag*PAI-positive bacteria, and this induces the activation of proinflammatory signaling pathways.
- The importance of Toll-like receptors for recognition is controversial and appears to be cell-type specific, as *H. pylori* express atypical lipoprotein and flagella.
- CagL facilitates T4SS binding to $\alpha_5\beta_1$ integrin on epithelial cells and activates signaling pathways.

Innate immune responses to *H. pylori*

- *H. pylori* infection stimulates proinflammatory cytokine production, which is involved in the recruitment of immune cells.
- *H. pylori* has developed an efficient mechanism of avoiding both phagocytic clearance and toxic reactive oxygen species, produced in large quantities by the host during infection.

Adaptive immunity

Th1 versus Th2 responses to *H. pylori*

- *H. pylori* infection induces a skewed Th1 immune response, characterized by IFN-γ secretion and the infiltration of $CD4^+$ lymphocytes into the gastric mucosa.
- IFN-γ increases gastritis, whereas IL-4 decreases gastritis. However, the precise roles of Th1 and Th2 responses in vaccine-induced protection are controversial.

Th17 cells

- Recent studies have revealed Th17 responses are important for the control of *H. pylori* and the development of protective immunity.

Tregs

- Tregs control excessive inflammation and prevent bacterial clearance by dampening host responses to *H. pylori*.

Antibodies

- The role of antibodies in the control/clearance of *H. pylori* infection is controversial – many studies have excluded a significant role. However, vaccination induces a different antibody isotype profile and expression pattern to those induced during *H. pylori* infection, suggesting that the pathogen skews host immune responses to favor persistence in the stomach.

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- ▪ of interest
- ▪▪ of considerable interest

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Appendix 2. NOD1 directs H. pylori killing by antimicrobial peptides

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The innate immune molecule, NOD1, regulates direct killing of *Helicobacter pylori* by antimicrobial peptides

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Summary

The cytosolic innate immune molecule, NOD1, recognizes peptidoglycan (PG) delivered to epithelial cells via the *Helicobacter pylori cag* pathogenicity island (*cag*PAI), and has been implicated in host defence against *cag*PAI$^+$ *H. pylori* bacteria. To further clarify the role of NOD1 in host defence, we investigated NOD1-dependent regulation of human β-defensins (*DEFB*s) in two epithelial cell lines. Our findings identify that NOD1 activation, via either *cag*PAI$^+$ bacteria or internalized PG, was required for *DEFB4* and *DEFB103* expression in HEK293 cells. To investigate cell type-specific induction of *DEFB4* and *DEFB103*, we generated stable *NOD1* 'knockdown' (KD) and control AGS cells. Reporter gene assay and RT-PCR analyses revealed that only *DEFB4* was induced in an NOD1-/*cag*PAI-dependent fashion in AGS cells. Moreover, culture supernatants from AGS control, but not AGS *NOD1* KD cells, stimulated with *cag*PAI$^+$ *H. pylori*, significantly reduced *H. pylori* bacterial numbers. siRNA studies confirmed that human β-defensin 2 (hBD-2), but not hBD-3, contributes to the antimicrobial activity of AGS cell supernatants against *H. pylori*. This study demonstrates, for the first time, the involvement of NOD1 and hBD-2 in direct killing of *H. pylori* bacteria by epithelial cells and confirms the importance of NOD1 in host defence mechanisms against *cag*PAI$^+$ *H. pylori* infection.

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Introduction

A puzzling aspect of the interactions between *Helicobacter pylori* and the gastric mucosa is the ability of this pathogen to persist despite the strong induction of inflammatory immune responses. Little is known about the host factors that limit bacterial loads or the bacterial factors involved in immune evasion. It has long been known that *H. pylori* strains harbouring a Type IV secretion system (T4SS), encoded by the *cag* pathogenicity island (*cag*PAI), induce greater levels of nuclear factor-κB (NF-κB) activation and interleukin-8 (IL-8) secretion than *cag*PAI lacking strains (Crabtree *et al.*, 1999). However, the early innate immune recognition events leading to extensive inflammation have, until recently, remained elusive. Three pathogen recognition molecules (PRMs) of the extracellular Toll-like Receptor (TLR) family, TLR 2, 4 and 5, have been implicated in recognition of *H. pylori* (Kawahara *et al.*, 2001; Smith *et al.*, 2003). TLR recognition of conserved microbial pathogen-associated molecular patterns (PAMPs) initiates pro-inflammatory signaling cascades that culminate in NF-κB activation and IL-8 production (Akira and Takeda, 2004). However, TLR2 and 4 are either absent or non-signaling in epithelial cell lines, such as HEK293 and AGS gastric epithelial cells (Smith *et al.*, 2003), which respond specifically to *cag*PAI$^+$ *H. pylori* strains (Backert *et al.*, 2004; Viala *et al.*, 2004; Bauer *et al.*, 2005). Furthermore, *H. pylori* bacteria lacking flagella, a TLR5 ligand, were still able to induce pro-inflammatory signaling in these cells (Lee *et al.*, 2003). Thus, although TLR2 or other PRMs may be involved in epithelial cell signaling to *H. pylori* (Hirata *et al.*, 2006), it appears that other PRMs play a predominant role in the

recognition of *cag*PAI⁺ *H. pylori*. Indeed, we previously reported that an intracellular PRM, NOD1, responded specifically to cell wall peptidoglycan (PG) delivered into gastric epithelial cells by the T4SS of *cag*PAI⁺ *H. pylori* strains (Viala *et al.*, 2004). NOD1 recognition of *H. pylori* PG was shown to result in NF-κB activation and subsequent IL-8 production in epithelial cells (Viala *et al.*, 2004).

Pathogen recognition molecules are thought to directly inhibit microbial replication through the regulation of innate immune effectors. Cationic antimicrobial peptides of the β-defensin (hBD) family are one type of effectors that are secreted by epithelial cells. Although *DEFB1*, the gene encoding hBD-1, appears to be constitutively expressed (McCray and Bentley, 1997), studies have demonstrated the upregulation of *DEFB4* and *DEFB103*, the hBD-2 and hBD-3 genes respectively, in response to stimulation with either pro-inflammatory cytokines (IL-1α, IL-1β), pathogenic bacteria (*Salmonella enterica, Enterococcus faecalis*, enteroinvasive *Escherichia coli*, *H. pylori*) or their products (O'Neil *et al.*, 2000; George *et al.*, 2003; Uehara *et al.*, 2003).

Studies demonstrated that direct contact of live *H. pylori* with the epithelium was absolutely required for *DEFB4* activation (Wada *et al.*, 1999; O'Neil *et al.*, 2000). NF-κB activation was determined to be a critical step in hBD-2 peptide production in response to stimulation with *cag*PAI⁺ *H. pylori* strains (Wada *et al.*, 2001). Consistent with the reported role of NOD1 in recognition of *cag*PAI⁺ *H. pylori* (Viala *et al.*, 2004), this PRM was shown to upregulate *DEFB4*, but not *DEFB103*, expression in response to *cag*PAI⁺ *H. pylori* in AGS cells (Boughan *et al.*, 2006). Despite these advances, the direct role of β-defensins in host defence against *H. pylori* remains to be proven. While several studies have examined the antimicrobial activity of purified recombinant hBD-2 (rhBD-2) against *H. pylori* (Hamanaka *et al.*, 2001; George *et al.*, 2003), no direct evidence for the link between induction of pro-inflammatory gene expression by *H. pylori* and a host antimicrobial response has been found so far. In order to provide a broader understanding of the induction of antimicrobial peptide responses to *H. pylori*, we utilized two epithelial cell line models, AGS and HEK293. Moreover, stable NOD1 'knockdown' (KD) cells in the AGS cell line were generated and used to not only examine NOD1-mediated regulation of β-defensin expression, but also antimicrobial production in response to *H. pylori* stimulation. This is the first study to provide direct evidence of the antimicrobial properties against *H. pylori*, of supernatants of AGS cell stimulated with *H. pylori*, and the first to show that this process occurs in a manner dependent on NOD1 and the *cag*PAI. In addition, this study is the first to demonstrate that hBD-2 contributes, at least in part, to direct killing of *H. pylori* by AGS cell supernatants. Identification of additional antimicrobial factor(s) contained in these AGS cell supernatants may aid in determining other host factors responsible for limiting bacterial loads during *H. pylori* infection *in vivo*.

Results

NOD1 regulates DEFB4 and DEFB103 promoter activation in response to H. pylori *and PG in HEK293 cells*

In order to obtain a broader understanding of β-defensin regulation in epithelial cells, we employed two cell lines, AGS and HEK293. These cell lines are known to provide useful models of the interactions between *H. pylori* and the gastric mucosa (Bauer *et al.*, 2005), and have been shown to respond to *cag*PAI⁺ *H. pylori* in a NOD1-dependent manner (Viala *et al.*, 2004). β-defensin expression in these cell lines was examined by RT-PCR (Fig. 1A). *DEFB1* was constitutively expressed by AGS cells, whereas stimulation with the *cag*PAI⁺ *H. pylori* 251 strain for 8 h induced *DEFB4* expression in these cells (Fig. 1A). Low level constitutive *DEFB103* expression was further upregulated in response to stimulation with *H. pylori* 251. *DEFB1* was constitutively present in HEK293 cells, and was further upregulated in response to stimulation with *H. pylori*. By contrast, endogenous *DEFB4* and *DEFB103* expression in HEK293 cells was undetectable even after co-culture with *H. pylori* for 8 h (Fig. 1A). To determine the role of the *cag*PAI in β-defensin regulation, HEK293 cells, transfected with hBD-2 and hBD-3 reporter constructs, were stimulated for 8 h with *H. pylori* 251 or isogenic *cagA, cagM* or *cagPAI* mutant bacteria. Stimulation of HEK293 cells with *H. pylori* 251 induced 2.6 and 1.8- fold activation of hBD-2 and hBD-3 reporter activity respectively ($P = 0.029$; Fig. 1B and C). Stimulation of HEK293 cells with isogenic *cag* mutant strains of *H. pylori* failed to induce significant hBD-2 and hBD-3 reporter activity (P = not significant; ns; Fig. 1B and C). Similar results were observed using another *cag*PAI⁺ *H. pylori* isolate (strain 26695; data not shown). Interestingly, it appears that at least in HEK293 cells, CagA can mediate hBD reporter activation (Fig. 1B and C). This may be a downstream response to NF-κB activation mediated by CagA, via either transforming growth factor-β-activated kinase 1 (TAK1) or mitogen-activated protein kinases (MAPKs; Allison *et al.*, 2009; Lamb *et al.*, 2009).

Previous studies have shown that internalization of PG into HEK293 cells results in induction of NOD1 signaling (Girardin *et al.*, 2003; Viala *et al.*, 2004). It was therefore hypothesized that internalization of PG should result in the upregulation of β-defensin expression. To test this hypothesis, PG preparations from *H. pylori* 26695 and another Gram-negative pathogen shown to have NOD1

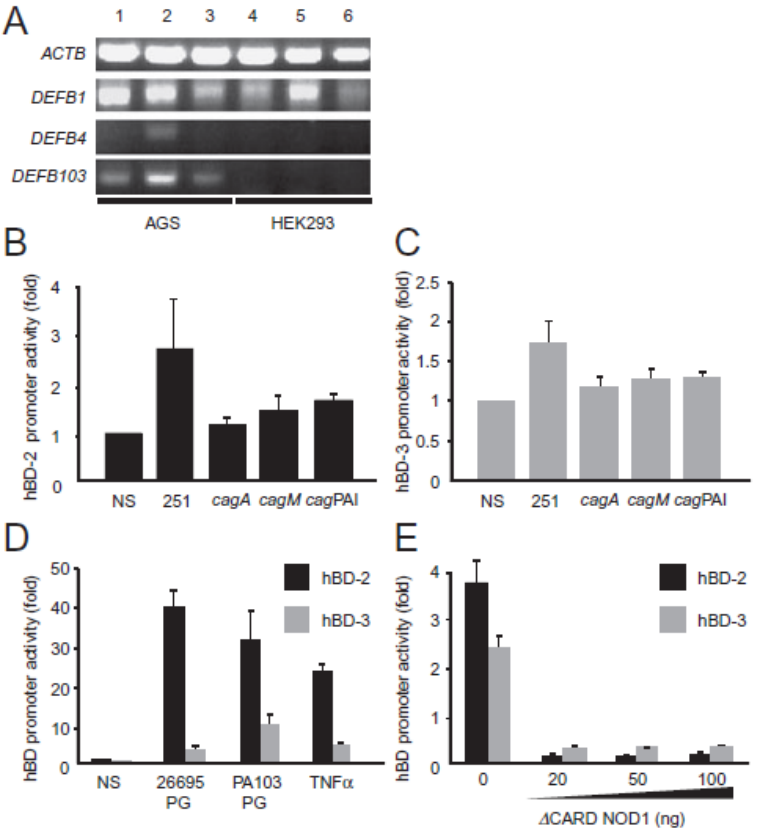


Fig. 1. NOD1 regulates *DEFB4* and *DEFB103* expression in response to *H. pylori* and PG in HEK293 cells.
A. β-defensin mRNA expression in epithelial AGS (lanes 1–3) and HEK293 (lanes 4–6) cell lines. Cells were either left untreated (lanes 1 and 4) or stimulated for 8 h with *H. pylori* 251 (lanes 2 and 5) or TNFα (lanes 3 and 6). β-defensin expression was measured relative to standardized *ACTB* expression.
B and C. HEK293 cells were transfected with hBD-2 (B) or hBD-3 (C) reporter constructs and stimulated with *H. pylori* 251 or isogenic *cagA*, *cagM* or *cag*PAI mutants for 8 h. Reporter activity was determined by normalization to *Renilla* activity. Data are expressed as fold increases in firefly luciferase activity, compared with non-stimulated cells. Error bars indicate the SEM determinations of samples in triplicate, representative of three independent experiments.
D. HEK293 cells that were transfected with hBD-2 or hBD-3 luciferase reporter constructs were stimulated for 8 h with TNFα, or purified PG from *H. pylori* 26695 or *P. aeruginosa* strain 103 (PA103). Error bars indicate the SEM determinations of values representative of three experiments conducted in triplicate.
E. HEK293 cells transfected with hBD-2 or hBD-3 reporter constructs were co-transfected with varying concentrations of ΔCARD NOD1 and stimulated with *H. pylori* 251 for 8 h. Error bars indicate the SEM determinations of values representative of three experiments conducted in triplicate.

activity, *Pseudomonas aeruginosa* (Travassos *et al.*, 2005), were intracellularly delivered into HEK293 cells that had been transfected with hBD-2 or hBD-3 reporter constructs. *H. pylori* and *P. aeruginosa* PG induced high hBD-2 reporter activity (40 and 32 fold respectively), and, to a lesser extent, hBD-3 reporter activity (4 and 12 fold respectively) in these cells (Fig. 1D).

To confirm that NOD1 recognition of PG regulates β-defensin expression, HEK293 cells were co-transfected with hBD-2 or hBD-3 reporter constructs and increasing concentrations of a construct encoding a NOD1 molecule without a caspase activation and recruitment domain (CARD). This construct is known to produce a dominant negative effect on NOD1 signaling (Viala *et al.*, 2004; Travassos *et al.*, 2005). Stimulation with *H. pylori* 251 or intracellular delivery of 26695 PG induced both hBD-2 and hBD-3 reporter activity, which was abrogated by co-transfection with 20–100 ng of ΔCARD NOD1 (Fig. 1E and data not shown, respectively). In contrast to a previous study in AGS cells (Boughan *et al.*, 2006), which found a role for NOD1 in *DEFB4* expression only, our data suggest that NOD1 mediates both *DEFB4* and *DEFB103* expression in HEK293 cells in response to *H. pylori* and PG.

NOD1 KD AGS clones express reduced levels of NOD1 mRNA and exhibit impaired pro-inflammatory responses to H. pylori

In order to further investigate the role of NOD1 in β-defensin regulation in AGS cells, we generated stable KD cell lines for the *NOD1* gene. Potential NOD1 KD clones ($n = 5$) and AGS control clones ($n = 5$), in which an irrelevant gene (enhanced green fluorescent protein, EGFP) was 'knocked down', were initially screened for IL-8 production in response to *cag*PAI$^+$ *H. pylori* (data not shown). A potential NOD1 KD clone and an AGS control clone that responded appropriately to stimulation with *H. pylori* were selected based on this analysis, and examined further by RT-PCR for *NOD1* expression and for expression of *CXCL8*, which encodes the pro-inflammatory cytokine IL-8, which is regulated by NF-κB (Fig. 2A). When compared with the AGS control clone, reduced levels of both *NOD1* and *CXCL8* expression were observed in the NOD1 KD AGS cells (Fig. 2A).

In order to assess the effect of NOD1 knockdown on AGS cell responses to *H. pylori*, cells were stimulated with *H. pylori* 251 or the isogenic *cag*PAI mutant for 24 h prior

to mRNA extraction and qRT-PCR analysis. *NOD1* transcription was upregulated 1.8-fold in AGS control cells in response to stimulation with *H. pylori* 251 and, to a lesser extent, by the *cag*PAI mutant ($P = 0.028$; Fig. 2B). AGS NOD1 KD cells displayed reduced basal *NOD1* expression levels compared with AGS control cells, and did not significantly upregulate *NOD1* expression in response to either *H. pylori* strain tested (Fig. 2B).

To further characterize the responses of AGS NOD1 KD cells to *H. pylori,* AGS control and NOD1 KD cells were transfected with an NF-κB reporter construct, and stimulated for 4 h with *H. pylori* 251 or 251 *cag*PAI. The NF-κB promoter in AGS control cells was induced over 25-fold by stimulation with *H. pylori* 251 compared with non-stimulated AGS control cells (Fig. 2C). Conversely, knockdown of NOD1 resulted in only 10-fold induction of NF-κB reporter activity after incubation with *H. pylori* 251, compared with non-stimulated AGS NOD1 KD cells ($P = 0.032$; Fig. 2C). This 10-fold NF-κB reporter induction may be attributed to an NOD1-independent CagA-dependent response (Brandt *et al.*, 2005). Stimulation with *H. pylori* 251 *cag*PAI did not activate the NF-κB promoter in either cell clone tested (Fig. 2C). NF-κB binding to NF-κB promoter sequences, assessed by electrophoretic mobility shift assay (EMSA), was strongly induced in AGS control cells after 2 h of co-culture with *H. pylori* 251 (Fig. 2D). NF-κB binding was severely impaired in AGS NOD1 KD cells after 2 h of incubation with *H. pylori* 251 (Fig. 2D). These results demonstrate that epithelial cell responses to *cag*PAI$^+$ *H. pylori* are impaired in AGS NOD1 KD cells.

Recognition of *H. pylori* PG by NOD1 initiates a signaling pathway that culminates with the production of pro-inflammatory cytokines, such as IL-8 (Viala *et al.*, 2004). To further characterize cell responses in AGS NOD1 KD cells, we verified that knockdown of NOD1 results in reduced IL-8 production in response to *H. pylori*. To achieve this, the levels of IL-8 secretion by AGS cells were assessed by enzyme-linked immunosorbent assay (ELISA), following co-incubation of AGS cells with *H. pylori* 251 or isogenic mutant strains for 24 h. AGS control cells produced high levels of IL-8 upon stimulation with *H. pylori* 251 (Fig. 2E). Stimulation of the cells with *H. pylori cagA, cagM* or *cag*PAI isogenic mutant bacteria resulted in significantly reduced responses ($P < 0.0001$; Fig. 2E). Knockdown of NOD1 also resulted in considerably decreased IL-8 secretion in cells stimulated with *H. pylori* 251 and isogenic mutant strains ($P < 0.0001$; Fig. 2E). These data demonstrate that the NOD1 signaling pathway is significantly impaired in AGS NOD1 KD cells.

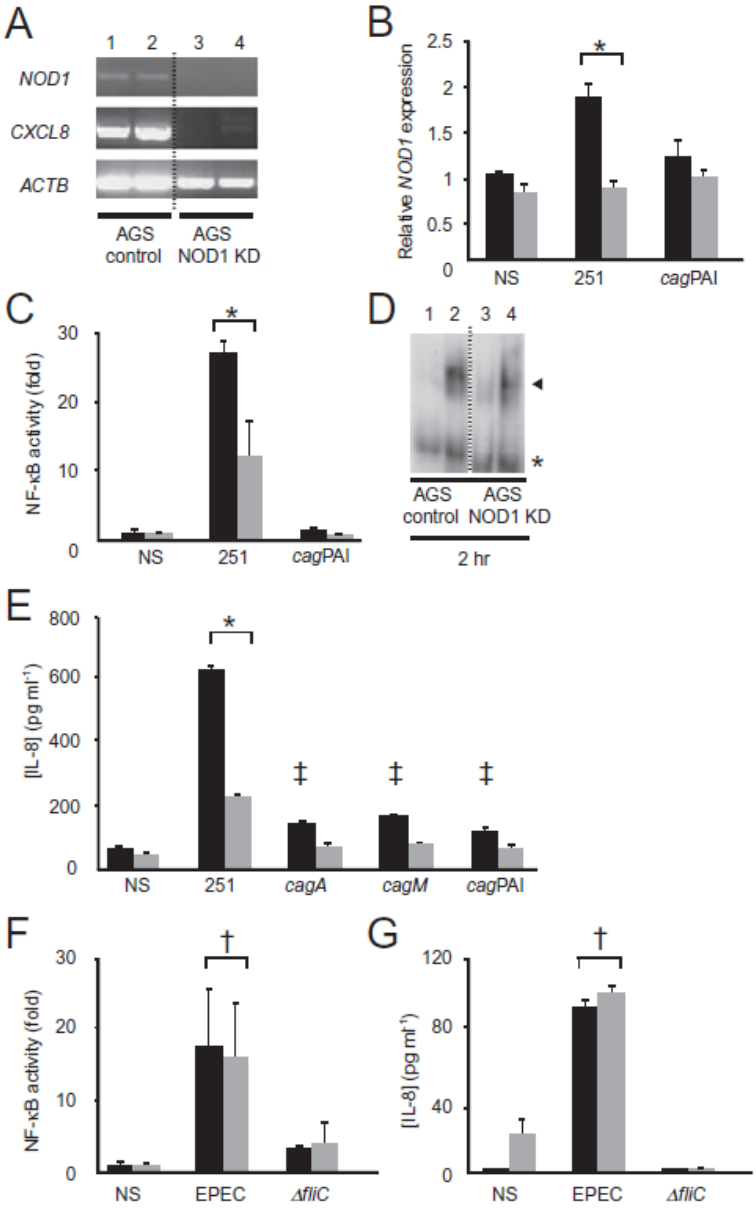
Knockdown of NOD1 has no effect on an NOD1-independent signaling pathway

The specificity of the knockdown of NOD1 in AGS cells was investigated by assessing the ability of the cells to signal via an NOD1-independent epithelial cell signaling pathway. The TLR5 signaling pathway was chosen for this purpose as AGS cells endogenously express functional TLR5, the PRM for bacterial flagellin (Gewirtz *et al.*, 2004). AGS cells were stimulated with enteropathogenic *E. coli* (EPEC E2348/69), which induces pro-inflammatory responses in epithelial cells primarily via EPEC flagellin signaling through TLR5 (Zhou *et al.*, 2003; Badea *et al.*, 2009). AGS control and AGS NOD1 KD cells were also stimulated with an isogenic flagellin mutant, EPEC Δ*fliC*, which lacks the flagellar filament protein (Badea *et al.*, 2009). AGS control and NOD1 KD cells exhibited similar levels of NF-κB activation (P = ns; Fig. 2F) and secreted equivalent levels of IL-8 (P = ns; Fig. 2G) in response to co-incubation with wild-type EPEC. Stimulation of both cell types with EPEC Δ*fliC* considerably decreased NF-κB promoter activation and abrogated IL-8 production (Fig. 2F and G). These data demonstrate that although the NOD1 signaling pathway has been disrupted, TLR signaling is intact in the AGS NOD1 KD cells.

NOD1 regulates DEFB4, but not DEFB103, promoter activation and gene expression in AGS cells

AGS NOD1 KD cells were used to determine the role of NOD1 in the regulation of β-defensin expression in AGS cells. To achieve this, AGS control and AGS NOD1 KD cells were transfected with hBD-2 or hBD-3 reporter constructs, containing the promoter regions of *DEFB4* or *DEFB103*, respectively. The *DEFB4* promoter was activated via NOD1 in a manner dependent on an intact *cag*PAI, 8 h after stimulation with *H. pylori* ($P = 0.012$; Fig. 3A). *DEFB103* promoter activity was induced in response to co-culture with *H. pylori* to similar extents, irrespective of the *H. pylori* strain or AGS cell clone used (P = ns; Fig. 3B).

To confirm the respective roles of NOD1 in *DEFB4* and *DEFB103* expression induced by *H. pylori*, RT-PCR and qRT-PCR were performed on AGS control and NOD1 KD cells that were stimulated with *H. pylori* 251, or the *cag*PAI mutant for 24 h. As a control, expression of *DEFB1*, which encodes hBD-1, was also analyzed by qRT-PCR, as this defensin is known to be constitutively produced by epithelial cells (McCray and Bentley, 1997). As expected, *DEFB1* expression was not upregulated by either AGS cell clone in response to stimulation with *H. pylori* (data not shown). In contrast, constitutive expression of *DEFB4* was not observed by RT-PCR in either cell clone analyzed (Fig. 3C). Moreover, *DEFB4* mRNA expression was induced in AGS control cells in response to *H. pylori* 251, whereas expression levels were drastically impaired in AGS NOD1 KD cells (Fig. 3C). These findings were confirmed by qRT-PCR analysis, which demonstrated



increased *DEFB4* expression in AGS control cells that were stimulated with *H. pylori* 251 (Fig. 3D), but not in cells stimulated with the *cag*PAI mutant, nor in AGS NOD1 KD cells co-incubated with either parental *H. pylori* or *cag*PAI mutant bacteria ($P < 0.0001$; Fig. 3D). Thus NOD1 is involved in both hBD-2 reporter activity and *DEFB4* gene expression in response to *H. pylori*. As hBD-3 reporter activity was differentially regulated to hBD-2 reporter activity in AGS cells, we determined whether this was also the case at the transcriptional level.

Low level constitutive expression of *DEFB103* was observed by RT-PCR in both AGS cell clones, and induction of *DEFB103* expression in response to *H. pylori* was not mediated by NOD1 and did not involve *cag*PAI-dependent signaling (Fig. 3C). These results demonstrate that NOD1 mediates *DEFB4* expression in AGS cells, as knockdown of NOD1 results in diminished *DEFB4* expression in response to stimulation with *H. pylori*. Conversely, as reported previously (Boughan *et al.*, 2006), *DEFB103* expression in response to

Fig. 2. NOD1 KD in AGS cells results in reduced *NOD1* expression and IL-8 production in response to *H. pylori*, but has no effect on TLR5-dependent signaling induced by EPEC.
A. AGS control and AGS NOD1 KD cells were left untreated or co-cultured with *H. pylori* 251 for 6 h. *NOD1* and *CXCL8* mRNA expression was evaluated by RT-PCR, and normalized relative to *ACTB* expression. Cells were either left untreated (lanes 1, 3) or stimulated with *H. pylori* 251 (lanes 2, 4).
B. *NOD1* mRNA expression in AGS control (dark shading) and AGS NOD1 KD (light shading) cells in response to stimulation for 24 h with parental *H. pylori* 251 or isogenic mutant strains was assessed by qRT-PCR. As a control (NS), cells were left untreated. *NOD1* gene expression was normalized to *ACTB* expression. Error bars indicate SEM determinations of three separate experiments performed in triplicate. $^{*}P = 0.028$.
C. NF-κB reporter activity in AGS control (dark shading) and AGS NOD1 KD (light shading) cells either stimulated with *H. pylori* 251 parental or *cag*PAI mutant strains for 4 h, or left untreated (NS). Error bars indicate the SEM determinations of values representative of three experiments conducted in triplicate. $^{*}P = 0.032$.
D. EMSA analysis of NF-κB binding from AGS control and NOD1 KD cells stimulated with *H. pylori* 251 bacteria (lanes 2, 4) for 2 h, or left untreated (lanes 1, 3). Nuclear extracts were prepared and incubated with radioactively labeled probes. Data are representative of three separate experiments. The symbol '◀' indicates specific binding to the probe, whereas the asterisk '*' indicates non-specific binding.
E. IL-8 secretion in culture supernatants of AGS control (dark shading) and AGS NOD1 KD (light shading) cells stimulated for 24 h with either parental *H. pylori* 251 or isogenic mutant strains. Data are expressed as the mean + SEM of IL-8 concentrations representative of four experiments conducted in triplicate. $^{*}P < 0.0001$; $^{\ddagger}P < 0.0001$ compared with AGS control cells stimulated with *H. pylori* 251.
F. NF-κB reporter activity in AGS control (dark shading) and AGS NOD1 KD (light shading) cells stimulated with wild-type EPEC or EPEC Δ*fliC* for 4 h, or left untreated (NS). Error bars indicate the SEM determinations of values representative of three experiments conducted in triplicate. $^{\dagger}P$ = ns.
G. IL-8 secretion in AGS control (dark shading) and AGS NOD1 KD (light shading) cells stimulated for 24 h with either wild-type EPEC or Δ*fliC* isogenic mutant bacteria, or left untreated (NS). Data correspond to the mean + SEM of IL-8 concentrations representative of three experiments conducted in triplicate. $^{\dagger}P$ = ns.

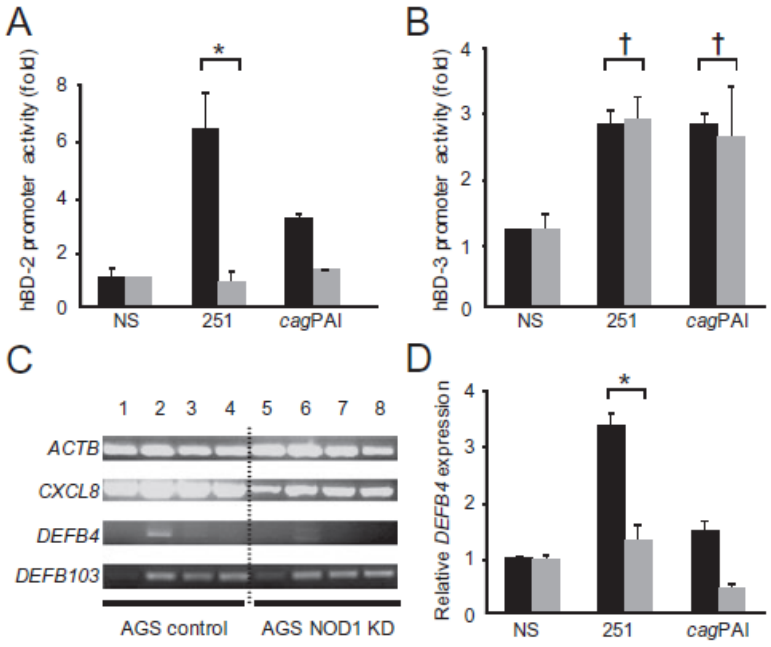


Fig. 3. *DEFB4*, but not *DEFB103*, expression is NOD1-/*cag*PAI-dependent in response to *H. pylori* in AGS cells.
A and B. AGS control (dark shading) and AGS NOD1 KD (light shading) cells were transiently transfected with luciferase reporter constructs for hBD-2 (A) or hBD-3 (B). Cells were stimulated with parental *H. pylori* 251 or *H. pylori* 251 *cag*PAI mutant strains for 8 h, or left untreated (NS). Error bars indicate the SEM determinations of values representative of three experiments conducted in triplicate. $^{*}P = 0.012$; $^{\dagger}P$ = ns.
C. *DEFB4*, *DEFB103* and *CXCL8* mRNA expression in AGS control and AGS NOD1 KD cells in response to stimulation for 24 h with parental *H. pylori* 251 or isogenic mutant strains was assessed by RT-PCR. Cells were left untreated (lanes 1,5), or stimulated with *H. pylori* 251 (lanes 2,6), 251 *cagA* (lanes 3,7) or 251 *cag*PAI (lanes 4,8). *NOD1* gene expression was measured relative to standardized *ACTB* expression.
D. Expression of *DEFB4* mRNA in AGS control (dark shading) and AGS NOD1 KD (light shading) cells stimulated with *H. pylori* 251 or 251 *cag*PAI bacteria was quantified by qRT-PCR. As a control, cells were left untreated (NS). Levels of expression of *DEFB2* were normalized to *ACTB* expression levels. Data are expressed as fold activation compared with non-stimulated AGS control cells. Each reaction was performed in triplicate. $^{*}P < 0.0001$.

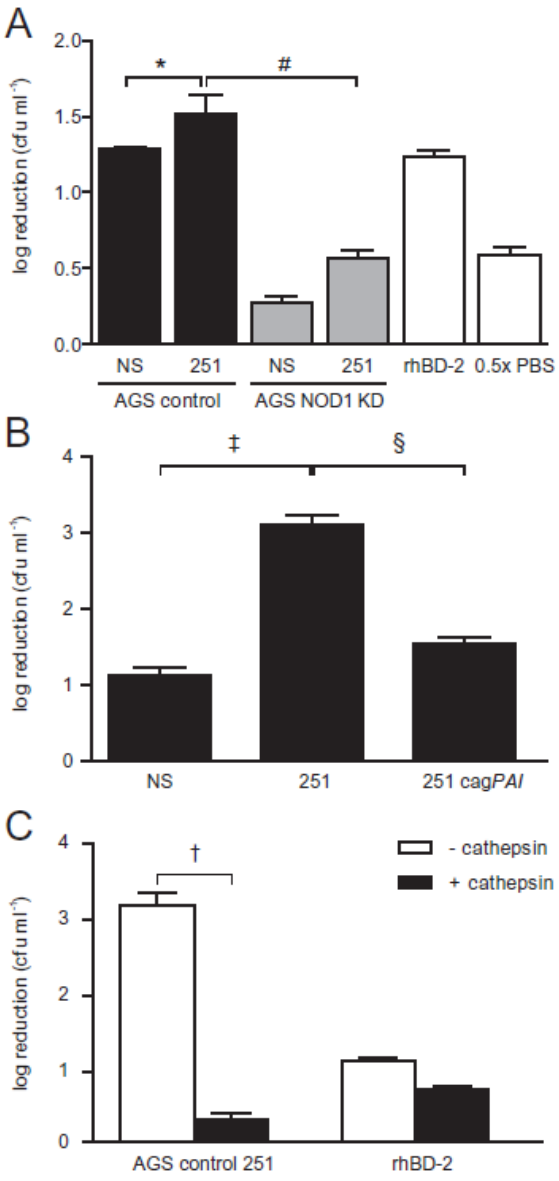


Fig. 4. Killing of *H. pylori* by *H. pylori*-stimulated AGS cell supernatants is *cag*PAI-/NOD1-dependent.
A and B. Culture supernatants collected from AGS control and AGS NOD1 KD cells, which had been stimulated for 8 h with either *H. pylori* 251 or *H. pylori* 251 *cag*PAI, or left untreated (NS), were investigated for their antimicrobial activity against *H. pylori* 251. The supernatants were lyophilized, resuspended in 0.5× PBS and diluted to (A) 2 or (B) 20 mg ml^{-1} total protein. The antimicrobial activities of these supernatants were compared with those of rhBD-2 or 0.5× PBS (positive and negative controls respectively). Viable counts were performed at 0 and 4 h after incubation with the supernatants, and the data expressed as log reduction over 4 h (cfu ml^{-1}). Error bars represent the standard error of four replicates. The experiments were performed three times (A) or twice (B), with similar results. $^{*}P = 0.021$; $^{\#}P = 0.032$, $^{\S}P = 0.034$; $^{\ddagger}P = 0.021$.
C. rhBD-2 (10^{-6} M) and supernatants collected from AGS control cells stimulated with *H. pylori* 251 (20 mg ml^{-1} total protein) were treated with 100 ng cathepsin L prior to antimicrobial assays. $^{\dagger}P = 0.021$.

H. pylori was found to occur independently of the presence of NOD1 and the *cag*PAI in AGS cells.

Antimicrobial activity of H. pylori*-treated AGS culture supernatants*

Viala and colleagues detected higher bacterial loads in Nod1$^{-/-}$ mice infected with *cag*PAI^{+} *H. pylori* than in wild-type mice, which suggests that NOD1 may regulate the production of effectors that interfere with the efficiency of *H. pylori* colonization (Viala *et al.*, 2004). Furthermore, several reports have demonstrated killing of *H. pylori* by rhBD-2 and rhBD-3 (Hamanaka *et al.*, 2001; George *et al.*, 2003). In order to determine whether NOD1 regulates secretion of molecules that are antimicrobial for *H. pylori*, AGS control and NOD1 KD cells were stimulated with *H. pylori* 251 or *cag*PAI bacteria, and cell culture supernatants were collected after 8 h (Fig. 4A). The positive control, rhBD-2 (10^{-6} M), reduced viable *H. pylori* 251 by 0.67–1 log after 4 h of co-incubation. Supernatants of AGS control cells that had been stimulated with *H. pylori* 251 exhibited maximal bactericidal activity (2–3 log; $P = 0.021$; Figs 4A-C). This activity was significantly reduced in non-stimulated control cells ($P = 0.021$; Fig. 4A, B), and totally abrogated in both *H. pylori* 251-stimulated and non-stimulated AGS NOD1 KD cells ($P = 0.032$; Fig. 4A). The supernatants of AGS control cells stimulated with *H. pylori* 251 *cag*PAI possessed significantly decreased antimicrobial activity in comparison to supernatants of cells stimulated with parental *H. pylori* 251 ($P = 0.034$; Fig. 4B). Thus NOD1 signaling mediates the secretion of antimicrobial effectors by AGS cells in response to stimulation with *H. pylori* in a *cag*PAI-dependent manner.

In order to determine whether β-defensins may contribute to the antimicrobial effect of *H. pylori* 251-stimulated AGS control cell supernatants, supernatants were treated with cathepsin L for 1 h prior to incubation with *H. pylori* 251. Cathepsin L has been shown to selectively degrade hBD-2 and hBD-3 after 1 h of incubation (Taggart *et al.*, 2003). Cathepsin L treatment significantly impaired the antimicrobial activity of 251-stimulated AGS control cell supernatants ($P = 0.021$; Fig. 4C). These results, together with the reported *cag*PAI-/NOD1-dependence of *DEFB4* expression and bacterial killing in AGS cells (Figs 3C, D and 4A, B), suggest that hBD-2 is likely to contribute to the microbicidal properties of the *H. pylori*-stimulated AGS cell supernatants. To confirm this observation, we used RNA

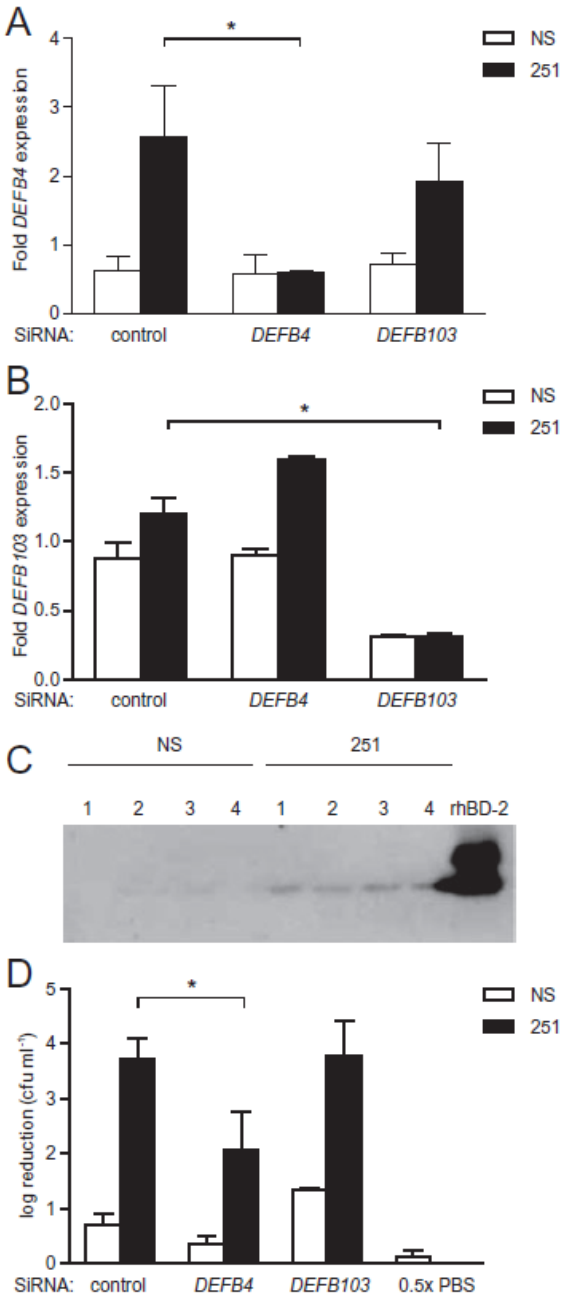


Fig. 5. Killing of *H. pylori* by *H. pylori*-stimulated AGS cell supernatants is hBD-2, but not hBD-3 dependent.
A and B. *DEFB4* (A) and *DEFB103* (B) expression in AGS cells that were transfected with siRNA directed to *DEFB4* or *DEFB103* and stimulated for 8 h with *H. pylori* 251 was assessed by qRT-PCR. Cells were transfected with unrelated siRNA as a negative control. Levels of expression of target genes were normalized to *ACTB* expression levels. Data are expressed as fold activation compared with non-stimulated AGS control cells. Each reaction was performed in triplicate. Error bars indicate the SEM determinations of four independent experiments for *DEFB4* and three independent experiments for *DEFB103*. $^*P < 0.05$.
C. hBD-2 production in AGS cells was assessed by Western blotting. Cells were transfected with siRNA directed to *DEFB4* (Lane 4), *DEFB103* (Lane 3), an unrelated gene (Lane 2), or not transfected (Lane 1) and stimulated or not for 8 h with *H. pylori* 251.
D. Culture supernatants collected from AGS control cells, which had been transfected with *DEFB4* siRNA, *DEFB3* siRNA or an unrelated siRNA and either stimulated for 8 h with *H. pylori* 251 or left untreated (NS), were investigated for their antimicrobial activity against *H. pylori* 251. The supernatants were lyophilized, resuspended in 0.5× PBS and diluted to 20 mg ml^{-1} total protein. The antimicrobial activities of these supernatants were compared with those of 0.5× PBS. Viable counts were performed at 0, and 4 h after incubation with the supernatants, and the data expressed as log reduction (cfu ml^{-1}) over 4 h. Error bars represent the standard error of three independent experiments. $P < 0.0001$.

silencing. Thus, AGS control cells were transfected with siRNA directed to either *DEFB4* mRNA, *DEFB103* mRNA or an unrelated mRNA. Decreased gene expression levels following siRNA transfection were verified by qRT-PCR. Transfection with siRNA to *DEFB4*, but not *DEFB103*, significantly reduced the expression levels of *DEFB4* in AGS cells stimulated with *H. pylori* 251 (Fig. 5A). Similarly, *DEFB103* expression was significantly reduced in cells transfected with *DEFB103* siRNA (Fig. 5B). Although increased levels of hBD-2 protein were detected by Western blotting of the supernatants of cells that had been stimulated with *H. pylori* 251 as compared with non-stimulated cells, no differences in hBD-2 production were observed between cells transfected with the varying siRNA constructs (Fig. 5C). This is most likely because the levels of hBD-2 synthesis in AGS cells were at the lower limit of detection for the assay (10 pg). Most importantly, the antimicrobial activity of *H. pylori*-stimulated AGS cell supernatants against *H. pylori* 251 was significantly reduced in cells transfected with *DEFB4*, but not in samples from *DEFB103* siRNA-treated cells (Fig. 5D). These results indicate that hBD-2 is the key antimicrobial peptide responsible for killing of *H. pylori* bacteria by AGS cell supernatants.

Discussion

We have previously shown that the gastric epithelium can respond to *H. pylori* through interactions between NOD1 and PG delivered by the *H. pylori* T4SS, encoded by the *cag*PAI (Viala *et al.*, 2004). Recognition of *H. pylori* PG activates signaling cascades that culminate in the secretion of pro-inflammatory mediators, including chemokines (Viala *et al.*, 2004) and antimicrobial peptides (Boughan *et al.*, 2006). Boughan and colleagues reported that NOD1-mediated hBD-2, but not hBD-3, reporter activity in AGS cells transiently transfected with NOD1 siRNA, and

demonstrated that a functional T4SS was necessary for this process (Boughan *et al.*, 2006). The present study has demonstrated differential cell-type specific *DEFB4* and *DEFB103* regulation in two epithelial cell lines, HEK293 and AGS. Furthermore, using isogenic *cag*PAI mutant bacteria and AGS cells stably expressing NOD1 siRNA, we were able, for the first time, to demonstrate NOD1-/*cag*PAI-dependent regulation of hBD-2 gene expression at the transcriptional level. The key and most novel finding of this study, however, was the first demonstration of a direct hBD-2-dependent antimicrobial effect of gastric epithelial cells on *H. pylori*. We established that this effect was mediated by a NOD1-/*cag*PAI-dependent mechanism, thereby further implicating NOD1 as an important regulator of host defence responses to *H. pylori*.

It has previously been demonstrated that expression of the inducible β-defensins, hBD-2 and hBD-3, is upregulated in epithelial cells through activation of TLR, NOD and/or MAPK pathways (Birchler *et al.*, 2001; Ogushi *et al.*, 2001; Krisanaprakornkit *et al.*, 2002; Moon *et al.*, 2002; Macredmond *et al.*, 2005; Boughan *et al.*, 2006; Voss *et al.*, 2006; Wehkamp *et al.*, 2006). Given that defensin responses appear to be cell line- and stimulus-dependent (Uehara *et al.*, 2007), the present study utilized two *NOD1*-expressing epithelial cell lines, HEK293 and AGS, to investigate the role of this PRM in regulating β-defensin expression in response to *H. pylori*. We have determined that, as for defensin responses to other bacterial pathogens and their products (Vora *et al.*, 2004), β-defensin regulation in response to *H. pylori* is cell-line dependent. In HEK293 cells, regulation of both hBD-2 and hBD-3 by live *H. pylori* bacteria seems to be a NOD1-/*cag*PAI-dependent process (Fig. 1B, C and E). Indeed, internalization of NOD1-reactive PG molecules from *H. pylori* and *P. aeruginosa* was sufficient to drive hBD-2 and, to a lesser extent, hBD-3, reporter activity in HEK293 cells (Fig. 1D). These findings differ to those for β-defensin expression in AGS cells, as reported both previously (Boughan *et al.*, 2006), and in the current work, thus further highlighting the contribution of host factors in responses to *H. pylori* (Bauer *et al.*, 2005).

The *DEFB4* promoter region contains the consensus sequences for three NF-κB sites, whereas only one activating protein-1 (AP-1) site is present in this region (Wada *et al.*, 1999). It is therefore often concluded that NF-κB activation is central to the induction of *DEFB4* expression in host cells (Wada *et al.*, 2001; Voss *et al.*, 2006; Wehkamp *et al.*, 2006). This is indeed supported by the present data for NOD1 and NF-κB-dependent induction of this defensin in AGS cells (Fig. 3). Nevertheless, there is some preliminary evidence, at least from the *DEFB4* reporter studies in AGS cells (Fig. 3A), that *H. pylori* may also induce *DEFB4* expression via a *cag*PAI-independent pathway. One mechanism by which this may occur is via AP-1 activation by MAPKs. Indeed, we have recently shown that after 1 h of stimulation, *H. pylori* can activate MAPK extracellular regulated kinase (ERK), via NOD1 in a manner dependent on the *cag*PAI (Allison *et al.*, 2009). Furthermore, given that the *DEFB103* promoter contains several AP-1 response elements, but is devoid of any NF-κB sites (Jia *et al.*, 2001), it is also possible that ERK may be involved in the *cag*PAI-independent *DEFB103* expression observed here (Fig. 3B and C). Consistent with these suggestions, the ERK pathway was shown to be activated by bacteria lacking the *cag*PAI (Keates *et al.*, 1999; Wessler *et al.*, 2000). Also, pretreatment of AGS cells with the ERK inhibitor, U0126, significantly diminished *DEFB103* promoter induction in response to *H. pylori* (Boughan *et al.*, 2006).

Although there have been several reports showing defensin expression in response to *H. pylori*, the antimicrobial effects of defensins against *H. pylori* has previously only been observed indirectly. Supernatants from cells transfected with a *DEFB4* expression construct were found to possess antimicrobial activity against *H. pylori* (Uehara *et al.*, 2003). Several studies have also determined that purified rhBD-2 and rhBD-3 are microbicidal for *H. pylori* (Hamanaka *et al.*, 2001; George *et al.*, 2003). Furthermore, it was recently established that *H. pylori*-infected NOD1 knockout mice expressed lower levels of mRNA encoding a murine homologue of hBD-2 than wild-type animals (Boughan *et al.*, 2006). While these results imply that NOD1 may interfere with colonization of *H. pylori* by regulating the production of antimicrobial effectors, there is a lack of direct evidence for this phenomenon. The present study is the first to demonstrate that *H. pylori* can induce gastric epithelial cells to upregulate the endogenous production of soluble mediators with high antimicrobial activity against *H. pylori*. Moreover, we have shown, for the first time, that this process occurs in a NOD1-/*cag*PAI-dependent manner (Fig. 4A and B respectively). As *DEFB4* promoter activity and mRNA expression were inducible in *NOD1*-expressing AGS control cells by *cag*PAI$^+$ *H. pylori* (Fig. 3A and D), it was hypothesized that the antibacterial effect of the supernatants was dependent, at least in part, on the action of hBD-2. Moreover, supernatants demonstrated no antimicrobial activity in higher salt solutions (data not shown), which is consistent with the known salt-dependence of hBD-2 (Bals *et al.*, 1998), whereas hBD-3 is known to be salt-insensitive (Harder *et al.*, 2001). In order to determine whether β-defensins may contribute to the observed antimicrobial activity of the AGS cell supernatants, we treated cells with cathepsin L, which has been shown to degrade hBD-2 and hBD-3 in 60 and 30 min respectively (Taggart *et al.*, 2003). We established that treatment of *H. pylori* 251-stimulated AGS control cell supernatants with cathepsin L resulted in a 20% reduction in antimicrobial activity

(Fig. 4C), consistent with the findings of a previous study, which demonstrated that under identical conditions, cathepsin L treatment reduced the antimicrobial activity of hBD-2 against *P. aeruginosa* by 20% (Taggart *et al.*, 2003). These data suggested that hBD-2 may play a role in epithelial cell killing of *H. pylori*. Nevertheless, it is possible that cathepsin L treatment also resulted in the inactivation of other antimicrobial substances present in the supernatants. Thus, the roles of the individual β-defensins in bacterial killing were investigated using siRNA-mediated knockdown of β-defensin gene expression. Knockdown of *DEFB4* significantly reduced, but did not abrogate, the antimicrobial activity of AGS cell supernatants, whereas transfection of AGS cells with *DEFB103* siRNA had no effect on bacterial killing. This demonstrates a central role for hBD-2 in the antimicrobial defence of gastric epithelial cells against *H. pylori*.

Epithelial cells are known to secrete antimicrobial peptides other than hBD-2 and hBD-3 in response to *H. pylori* infection, including hBD-1 (O'Neil *et al.*, 2000), LL-37 (Hase *et al.*, 2003), adrenomedullin (Allaker and Kapas, 2003), Elafin, DUOX2, DMBT1 and siderocalin (Hornsby *et al.*, 2008). As *DEFB1* is constitutively expressed (Fig. 1A), this defensin is not likely to be a major contributor to the observed NOD1-/*cag*PAI-dependent antimicrobial activities of the *H. pylori*-stimulated AGS cell supernatants (Fig. 4A and B). In contrast, the remaining antimicrobial molecules above appear to be secreted in a *cag*PAI-dependent manner (Hase *et al.*, 2003; Hornsby *et al.*, 2008), and may therefore synergize with hBD-2 to contribute to the observed antimicrobial effect of the cell supernatants from AGS cells with a functional NOD1. We are currently investigating the identity of the additional factor(s) involved in NOD1-dependent killing of *H. pylori*. We propose that hBD-2, potentially together with one or more of these additional factors, may play an important role in host defence by significantly reducing bacterial loads during infection *in vivo* (Viala *et al.*, 2004).

Experimental procedures

Bacterial strains and culture conditions

Strains used throughout this study were: *H. pylori* 251, 251 *cagA* and 251 *cagM* (Viala *et al.*, 2004); 251 *cag*PAI (Kaparakis *et al.*, 2009); EPEC E2348/69 (Levine and Edelman, 1984); EPEC *ΔfliC* (Badea *et al.*, 2009).

Helicobacter pylori strain 251 and isogenic mutants were routinely cultured on horse blood agar (HBA; Oxoid, Hampshire, UK) or in brain heart infusion broth (BHI; Oxoid, Hampshire, UK) as described previously (Ferrero *et al.*, 1998). *H. pylori* used in co-culture assays was grown in BHI supplemented with 10% (v/v) FCS (Invitrogen, Auckland, New Zealand) and Skirrows selective supplement (155 μg l^{-1} polymixin B, 6.25 mg l^{-1} vancomycin, 3.125 mg l^{-1} trimethoprim, 1.25 mg l^{-1} amphotericin B; all from Sigma, MO, USA). *H. pylori* cultures were incubated for 18 h shaking at 140 r.p.m. at 37°C under microaerobic conditions (Viala *et al.*, 2004). Prior to the addition of *H. pylori* to epithelial cells, and bacteria were pelleted by centrifugation at 3220 *g* for 15 min, washed twice in sterile phosphate-buffered saline (PBS). Bacteria were added to cells at a multiplicity of infection (MOI) equal to 10 bacteria per epithelial cell. Viable counts of *H. pylori* were performed by serial dilution. Bacteria were plated onto HBA, incubated for 3–5 days at 37°C, and enumerated. *H. pylori* 26695 and *Pseudomonas aeruginosa* PA103 PG were purified using a previously described procedure (Girardin *et al.*, 2003; Viala *et al.*, 2004).

EPEC E2348/69 and EPEC *ΔfliC* were routinely cultured on Luria Bertani medium (Badea *et al.*, 2003). Cultures were incubated under aerobic conditions at 37°C, with shaking at 160 r.p.m., for 18 h. EPEC cultures used in co-culture assays were diluted 1:100 from overnight cultures, and further incubated aerobically at 37°C with shaking at 160 r.p.m., for 2 h. Prior to addition to epithelial cells, bacteria were prepared as for *H. pylori*. Viable counts were performed by serial dilution. Bacteria were plated onto Luria Bertani agar, and enumerated after overnight incubation at 37°C.

Cell culture

AGS control and AGS NOD1 KD cells were generated by integration of an expression vector containing a small interference RNA (siRNA) directed to either the gene encoding enhanced green fluorescent protein (EGFP) or the CARD of the *NOD1* gene respectively (J. Viala, data not shown). AGS cells were routinely cultured in RPMI 1640 cell culture media supplemented with 1.46 g l^{-1} L-glutamine (Invitrogen, Auckland, New Zealand). HEK293 cells were routinely cultured in DMEM cell culture media (Invitrogen, Auckland, New Zealand). Culture media was supplemented with 10% (v/v) fetal calf serum (FCS) and 50 units ml^{-1} penicillin (Invitrogen, Auckland, New Zealand), 50 μg ml^{-1} streptomycin (Invitrogen, Auckland, New Zealand) and 40 μg ml^{-1} gentamicin (Pharmacia, WA, Australia).

Cell transfection and stimulation

AGS or HEK293 cells were seeded in 96-well plates at 1×10^5 cells ml^{-1}, and incubated for 24 h at 37°C in 5% CO_2. The following day, cells were transfected and incubated for 24 h with 60 ng $well^{-1}$ of Igκ luciferase (Viala *et al.*, 2004), hBD-2 luciferase or hBD-3 luciferase reporter DNA constructs (Boughan *et al.*, 2006). The total amount of DNA to be transfected was kept constant at 230 ng $well^{-1}$ by the addition of pcDNA3 as appropriate (Viala *et al.*, 2004). Co-transfection with 50 ng $well^{-1}$ of dTK *Renilla* plasmid (Promega, WI, USA) was used to standardize for well-to-well variations in transfection efficiencies. Transfection was achieved with 4 μl FuGene reagent per μg total DNA (Roche, IN, USA). For dominant-negative studies, HEK293 cells were co-transfected with 20, 50 or 100 ng $well^{-1}$ of ΔCARD NOD1 DNA construct (Bertin *et al.*, 1999). For intracellular delivery of PG, HEK293 cells were co-transfected with 40 ng ml^{-1} 26695 or PA103 PG (Viala *et al.*, 2004). All plasmid DNA was prepared using the PureYield Plasmid Midiprep System (endotoxin free; Promega, WI, USA). For siRNA studies, AGS control and NOD1 KD cells, grown in 15 cm^2 tissue culture dishes (IWAKI, Japan), were transfected and incubated for 2 days with a mixture con-

taining two independent siRNA constructs (5 mM each, Qiagen, Victoria, Australia) directed to either *DEFB4*, *DEFB103* or an unrelated control siRNA in 75 μl of HiPerfect Transfection reagent (Qiagen, Victoria, Australia) and 1 ml FCS-free RPMI.

Twenty-four hours after transfection, cells in RPMI or DMEM not supplemented with antibiotics were stimulated with *H. pylori* strain 251, isogenic mutant *H. pylori* strains, EPEC E2348/69, EPEC Δ*fliC* (MOI = 10) or 50 ng ml^{-1} recombinant human TNFα (Chemicon, Victoria, Australia). Cell media were replaced with RPMI or DMEM, as appropriate, after 1 h of co-incubation. Cells were incubated for a further 3 or 7 h, as appropriate.

Luciferase assay

Following stimulation, the medium was removed and cells were lysed with 50 μl well^{-1} of Reporter Lysis Buffer (Promega, WI, USA). Sample aliquots (20 μl) and Luciferase assay substrate (30 μl) (Promega, WI, USA) or coelenterazine *Renilla* substrate (50 μl) (Synchem, Kassel, Germany) were added to a 96-well polystyrene plate (Uniplate, Whatman), and luminescence was measured using a FLUOstar Optima luminometer (BMG Labtech, Victoria, Australia). All samples were measured in triplicate.

Electrophoretic mobility shift assay

Nuclear extracts were prepared as previously described (Philpott *et al.*, 2002). Nuclear extracts (5 μg) were combined with binding buffer (10 mM Hepes, pH 7.8, 100 mM NaCl, 1 mM EDTA and 10% glycerol), 1 μg of poly-(dI-dC) and 0.5 ng of radiolabeled NF-κB consensus oligonucleotide (Promega, WI, USA). After 30 min incubation at room temperature, samples were electrophoresed using a 5% polyacrylamide gel in Tris-borate–EDTA buffer. Gels were dried before exposure to film.

RNA extraction

AGS control and AGS NOD1 KD cells that had been grown in 24-well plates (IWAKI, Japan) and stimulated with *H. pylori* 251 and isogenic mutant strains for 6 or 24 h, as appropriate, were lysed by incubation with 1 ml of TRIzol Reagent (Invitrogen, CA, USA). Cell lysates were vigorously mixed with 200 μl of chloroform and the mixture applied to Phase Lock Gel tubes (Eppendorf, Hamburg, Germany) and centrifuged at 12 000 *g* and 4°C for 5 min. RNA was precipitated from the supernatants using 1:1 volumes of isopropanol and overnight incubation at −20°C. RNA was pelleted by centrifugation at 12 000 *g* for 30 min at 4°C and washed in 75% (v/v) ethanol, prior to resuspension in diethylpyrocarbonate (DEPC)-treated H_2O. Contaminating DNA was removed from RNA samples by the addition of 10× RQ1 DNase Buffer (Promega, WI, USA), followed by 1:10 volumes of RQ1 RNase-free DNase (Promega, WI, USA) and incubation at 37°C for 1 h. RNA was subsequently purified using the RNeasy Mini Kit (Qiagen, Victoria, Australia) in accordance with the manufacturer's instructions. RNA was eluted in 30 μl of RNase-free H_2O. RNA concentrations and purity were determined using the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, DE, USA) and visualized using agarose gel electrophoresis.

Genomic DNA preparation

AGS control cell genomic DNA was prepared using the Masterpure DNA Purification Kit (Epicentre, WI, USA) according to the manufacturer's instructions using 0.5–3 × 10^6 total AGS cells. Purified DNA was resuspended in 50 μl of Tris-EDTA (TE) buffer (Epicentre, WI, USA). DNA concentrations were calculated using the Nano-drop ND-1000 spectrophotometer and verified by agarose gel electrophoresis.

Reverse transcription polymerase chain reaction (RT-PCR)

RNA (1 μg) was reverse transcribed using SuperScript III (Invitrogen, CA, USA), according to the manufacturer's instructions. cDNA (2 μl) was added to 200 μmol dNTPs (Promega, WI, USA), 10× PCR reaction buffer (Roche, Mannheim, Germany), 0.5 μl units *Taq* DNA polymerase (Roche, Mannheim, Germany) and 40 pmol of primers (Table S1). PCR was performed using the DNA Engine thermal cycler (MJ Research, Watertown, MA, USA). Samples were heat-denatured for 1 min at 94°C, then amplified with 40 cycles consisting of successive incubations at 94°C, 58°C and 72°C for 1 min each, followed by a final 10 min extension at 72°C. PCR products were routinely electrophoresed using a 2% (w/v) agarose gel in Tris-acetate EDTA (TAE) buffer (Amresco, OH, USA).

Quantitative RT-PCR (qRT-PCR)

Primers for the genes encoding NOD1 (*NOD1*), hBD-2 (*DEFB4*), hBD-3 (*DEFB103*) and β-actin (*ACTB*) (Table S2) were designed using the Primer Express primer design software. Each reaction mix consisted of 1.6 μl of 625 nM forward and reverse primers (Micromon, Victoria, Australia), 10 μl SYBR GREEN PCR Master Mix (Applied Biosystems, Warrington, UK) and 2.4 μl cDNA template (diluted 1:50) or genomic DNA standards. The optimal amounts of genomic DNA standards were determined to be 1.5 μg, 75 ng and 3.75 ng per reaction. Each reaction was made to a final volume of 20 μl with ultrapure distilled H_2O. Reactions were performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Victoria, Australia) using the following conditions: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and 60°C for 1 min. Cycle threshold (Ct) values were calculated as the lowest cycle number producing an exponential increase in PCR product amplification. Following PCR, a melting curve analysis was performed as follows: 95°C for 15 s, 60°C for 20 s, followed by slow heating at 0.03°C s^{-1} up to 95°C. The purity of amplicons was determined by visualization of a single peak in the melting curve. No peaks were observed in the samples without cDNA or in which reverse transcriptase had not been added. All PCR reactions were performed in triplicate in optical Thermo-Fast 96 detection plates (ABgene, Surrey, UK). To ensure standardization of amplification efficiencies for all primer sets, standard curves were constructed by plotting average Ct values against the logarithm of the concentrations of the genomic DNA standards. cDNA concentrations of the target genes (*DEFB4*, *DEFB103* and *NOD1*) for each test sample were determined from the standard curve and normalized to expression of *ACTB*.

Enzyme linked immunosorbent assay

The levels of IL-8 secreted by AGS control and AGS NOD1 KD cells in the culture supernatants were determined using the BD

OptEIA Human IL-8 ELISA kit (BD Biosciences Pharmingen, CA, USA) in accordance with manufacturer's specifications. Absorbances at 405 nm were measured and standard curves were constructed using the DeltaSOFT3 software to determine IL-8 concentrations in test samples.

Antimicrobial assay

Culture supernatants of AGS control and AGS NOD1 KD cells that had been co-cultured with *H. pylori* 251 or 251 *cag*PAI (MOI = 10) for a total of 8 h were lyophilized, and resuspended in 0.5× PBS. Bradford protein assays (Bio-Rad, CA, USA) were performed to determine supernatant protein concentrations and samples were diluted to 2 or 20 mg ml^{-1} total protein in 0.5× PBS. Supernatants were then dialyzed against 0.5× PBS with two buffer changes for 2 h at room temperature, followed by overnight dialysis at 4°C using 0.5–3 ml Slide-A-Lyzer 3500 MWCO Dialysis Cassettes (Pierce, IL, USA) or Spectra/Por 3 3500 MWCO Dialysis membranes (Fisher Scientific, NSW, Australia). Lyophilized and dialyzed supernatants were inoculated with approximately 10^7 *H. pylori* 251 per ml and incubated at 37°C for 4 h. Bacteria were enumerated by serial dilutions, performed at 0 and 4 h time points. PBS (0.5×) was used as a negative control, and 10^{-6} M rhBD-2 (Sigma) used as a positive control for antimicrobial assays. For cathepsin inhibition experiments, supernatants were incubated with 100 ng cathepsin L (Sigma) for 60 min at 25°C prior to the addition of *H. pylori* as described previously (Taggart *et al.*, 2003). The reaction was terminated after 60 min by the addition of 50 μM cathepsin L inhibitor (Calbiochem Darmstadt, Germany).

Western blotting

All SDS-PAGE reagents were purchased from Invitrogen (Victoria, Australia). Lyophilized and dialyzed supernatants (200 μg) were prepared in 4× NuPAGE LDS Sample Preparation Buffer and 10× NuPAGE Sample Reducing Agent and separated on 4–12% NuPAGE Bis-Tris gels in NuPAGE MES SDS Running Buffer. Proteins were transferred to nitrocellulose using the Mini Trans-Blot cell (Bio-Rad, NSW, Australia). Membranes were incubated with a biotinylated affinity purified goat anti-human BD-2 antibody (PeproTech, NJ, USA) at 4°C overnight, then strepdavidin-HRP at room temperature for an hour (1:1000; Chemicon). Antigen-antibody complexes were detected using Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare, NSW, Australia).

Statistical analysis

Antimicrobial efficiencies of cell culture supernatants were compared using the non-parametric Mann–Whitney *U*-test. Luciferase reporter activity, levels of gene expression by qRT-PCR and levels of IL-8 production were compared using the Student's *t*-test. *P*-values below 0.05 were considered significant.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. RT-PCR primer sequences.
Table S2. qRT-PCR primer sequences.

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Appendix 3. PAR1 and Helicobacter-induced Gastritis

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Protease-Activated Receptor-1 Down-regulates the Murine Inflammatory and Humoral Response to *Helicobacter pylori*

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BACKGROUND & AIMS: *Helicobacter pylori* infection results in a diversity of pathologies, from asymptomatic gastritis to adenocarcinoma. The reason for these diverse outcomes is multifactorial and includes host factors that regulate severity of *Helicobacter*-induced gastritis. Protease-activated receptors (PAR) are environmental sensors that can detect tissue damage and pathogens. Whereas PAR-2 has proinflammatory activity and PAR-1 can protect the gastric mucosa against chemical damage, neither has previously been examined for their potential roles in regulating *Helicobacter* pathogenesis. **METHODS:** PAR-$1^{-/-}$, PAR-$2^{-/-}$, and wild-type mice were infected with *H pylori* for up to 2 months then colonization levels determined by colony-forming assay, gastritis by histology, and serum antibody levels by enzyme-linked immunosorbent assay. Responsiveness of primary epithelial cells to PAR-1 activation was assessed by calcium mobilization assay. Primary epithelial cells, macrophages, and dendritic cells were cocultured with *H pylori* and nuclear factor (NF)-κB, and cytokine secretion was determined by enzyme-linked immunosorbent assay. **RESULTS:** Two months postinfection, *H pylori* levels were significantly reduced in PAR-$1^{-/-}$ and increased in PAR-$2^{-/-}$ mice. This effect on colonization was inversely correlated with inflammation severity. Infection of PAR-$1^{-/-}$ mice induced an increased serum antibody response. Primary epithelial cells were activated by a PAR-1-activating peptide. *H pylori* stimulation of primary epithelial cells, but not macrophages or dendritic cells, from PAR-$1^{-/-}$ mice induced increased levels of NF-κB and the proinflammatory cytokine macrophage-inflammatory protein (MIP)-2. PAR-1 also down-regulated MIP-2 secretion in response to *cag* pathogenicity island activity. **CONCLUSIONS: PAR-1 protects the host against severe *Helicobacter*-induced gastritis. This may be mediated by suppressing the production of proinflammatory cytokines such as MIP-2.**

Chronic infection with the human pathogen *Helicobacter pylori* can result in a range of conditions, including gastritis, peptic ulcer disease, mucosal associated lymphoid tissue lymphoma and, most seriously, gastric adenocarcinoma.[1] The reason why this infection produces such diverse sequelae is complex, multifactorial, and not completely understood. Whereas variations in bacterial virulence factors, eg, the cytotoxin-associated gene pathogenicity island (*cag*PAI) and *vacA*, as well as environmental factors are clearly very important,[2,3] it is becoming increasingly evident that host genetic factors also play a central role in host susceptibility to the more severe outcomes of *H pylori* infection. Investigations of host genes, initially focused on the possible association between polymorphisms of key cytokine genes and gastric cancer found that, depending on the ethnic group studied, a number of cytokine polymorphisms including interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-10 may increase susceptibility to *Helicobacter*-associated pathologies.[4,5]

Other genes can play important roles by indirectly impacting on bacterially driven inflammatory responses and sequelae. For example, individuals with short alleles for *MUC1*, the main mucin lining the gastric epithelium, have significantly increased incidences of *H pylori* infection and gastric cancer.[6,7] In mouse models, this mucin limits the ability of *H pylori* to colonize the gastric epithelium and bind to gastric epithelial cells.[8] Because adherence to epithelial cells is required for *cag*PAI to induce a cytokine-signaling cascade, host factors such as MUC1 that limit bacterial attachment are also likely to protect against more severe disease development.

Protease-activated receptors (PAR) are a family of G-protein-coupled receptors expressed on a wide range of cell types, including epithelial cells and leukocytes, and their activation triggers a diverse range of effects. For example, activation of PAR-2 by a cognate protease such as trypsin has proinflammatory activity in the gastrointestinal tract.[9] In contrast, PAR-1, predominantly activated by the serine protease thrombin, has been demon-

Abbreviations used in this paper: cagPAI, cytotoxin-associated gene pathogenicity island; MPO, myeloperoxidase; PAR, protease-activated receptors.

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strated to play a protective role in the gastric mucosa against ethanol-induced damage in rodents.[10] However, expression of PAR-1 in the human gastric mucosa is increased during gastritis and during *H pylori* infection.[11,12] It is thus unclear whether PAR-1 plays a protective role against damage resulting from *H pylori* infection or, alternatively, contributes to the pathology. Because no previous studies have assessed the potential role of these receptors in *Helicobacter* pathogenesis, we used knockout mouse models to explore the effects of PAR-1 and PAR-2 expression on the host inflammatory response to *H pylori* infection.

Materials and Methods

Bacterial Culture

H pylori SS1 (VacA$^+$, *cag*PAI dysfunctional),[13] 251 (*cag*PAI functional), and 251 *cagM* mutant (*cag*PAI dysfunctional)[14] strains were cultured in brain heart infusion broth (BHI; Oxoid, Basingstoke, UK) containing 5% horse serum (JRH Biosciences, Brooklyn, Victoria, Australia) and 0.02% Amphostat, under microaerophilic conditions for 24 hours at 37°C.

Infection of Mice

C57BL/6 PAR-1$^{-/-}$ mice[15] and 129/Sv PAR-2$^{-/-}$ mice[16] were kindly provided by Dr S. R. Coughlin (University of California, CA) and J. Morrison (Monash University, Melbourne, Australia), respectively. Specific pathogen-free PAR-1$^{-/-}$, PAR-2$^{-/-}$, and sibling littermate wild-type controls were bred within the Veterinary Science animal house, University of Melbourne. Infection experiments involved age-matched female mice and were performed under University of Melbourne Animal Ethics Committee approval (No. 05197). Mice were infected intragastrically once with 10^7 *H pylori* suspended in 0.1 mL BHI. *H pylori* infection levels within mouse stomachs were quantified by a colony-forming assay and severity of gastritis histologically, as previously described.[8]

Serum Antibody Enzyme-Linked Immunosorbent Assay

Sera were collected by cardiac puncture and anti-*Helicobacter* antibody levels determined by standard direct enzyme-linked immunosorbent assay (ELISA). Maxisorp immunoplates (Nunc, Roskilde, Denmark) were coated overnight with 50 μL of *H pylori* lysate (100 μg/mL) in bicarbonate buffer, pH 9.6. Wells were blocked with 1% (wt/vol) bovine serum albumin (BSA) in phosphate-buffered saline (PBS) (PBS-BSA) for 45 minutes at room temperature (RT). Sera were serially diluted 1:10 (immunoglobulin [Ig] A) or 1:100 (IgG) in PBS-BSA, and 50 μL was added to duplicate wells, before incubation at RT for 1 hour. After washing, 50 μL of horseradish peroxidase-conjugated goat anti-mouse IgG or IgA (Pierce, Rockford, IL; diluted 1:5000 and 1:10,000, respectively, in PBS-BSA) was added per well and incubated at RT for 45 minutes. Color was developed by addition of 3,3',5,5;-tetramethylbenzidine (TMB) (Invitrogen, Camarillo, CA), and the reaction was stopped by adding 50 μL of 1 mol/L H_2SO_4. Absorbance was read at 450 nm, and end point titers were calculated.

Primary Murine Gastric Epithelial Cell Culture Assays

To prepare primary gastric epithelial cell cultures, stomachs were removed from <4-week-old mice and prepared as previously described,[14] and then cells were cultured at 37°C and 5% CO_2 for 72 hours. Epithelial cells were either used for a calcium mobilization assay or cocultured with *H pylori*-SS1 (10^7/well) for 24 hours, before collection of supernatants for analysis of cytokine production. Nuclear factor (NF)-κB (p65) activity was quantified from cell pellets using an ActivELISA Kit (IMGENEX, San Diego, CA) as per manufacturer's protocol.

To confirm their epithelial nature, 72-hour gastric cell cultures were fixed with methanol (10 minutes), endogenous peroxidase activity removed with 3% H_2O_2, blocked with PBS-BSA (30 minutes), labeled with a 1:50 dilution of rabbit polyclonal anti-cytokeratin (Invitrogen, Carlsbad, CA; overnight at 4°C), and 1/100 FITC-conjugated swine anti-rabbit antibody (Dako, Glostrup, Denmark) added. Fluorescent epithelial cells were counted under fluorescence microscopy (Olympus BX60; Olympus Corp, Tokyo, Japan).

Reverse-Transcription Polymerase Chain Reaction

RNA (250 ng) harvested from primary epithelial cells using TRIZOL (Invitrogen) according to manufacturer's instructions was reverse transcribed into complementary DNA (cDNA) using the Quantitect Reverse Transcription kit (Qiagen, Hilden, Germany). Polymerase chain reaction (PCR) synthesis: 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 30 seconds, in a final volume of 20 μL containing 1 μL cDNA template, 10 μL 2X PCR Mastermix (GoTaq Green Mastermix; Promega, Madison, WI), and 0.2 μmol/L primers. For quantitative PCR, each reaction was performed in duplicate in 20 μL containing 1 μL cDNA, 0.2 μmol/L primers, and 10 μL 2X QuantiTect SYBR Green PCR Mastermix (QIAGEN) using an Mx3000P cycler, (Stratagene, La Jolla, CA). Cycling conditions: 1 cycle at 94°C for 5 minutes, 40 cycles at 94°C for 30 seconds, 40 cycles at 60°C for 30 seconds, and 40 cycles at 72°C for 30 seconds. Relative expression was determined by REST formula.[17] Primers for murine PAR-1 consisted of forward primer GTCTTCCCGCGTCCCTAT and reverse primer GGGGGACCAGTTCAAATGTA; murine PAR-2 consisted of forward primer TTGGAGGTATCACCCTTCTG and reverse primer AAGCCTGGTTCTACCGGAAC. Primers for murine β-actin were as previously described by Rad et al.[18]

Calcium Mobilization Assay for Measuring PAR-1 Activation

Cell suspensions (4×10^6/mL) were loaded with 1 μmol/L FURA-2AM (Invitrogen) in buffer containing 121 mmol/L NaCl, 5.4 mmol/L KCl, 0.8 μmol/L $MgCl_2.6H_20$, 25 mmol/L HEPES sodium salt, 1.8 mmol/L $CaCl_2$, 5.5 mmol/L glucose, 6 mmol/L $NaHCO_3$, and 0.1% BSA, pH 7.4, for 30 minutes at 37°C. Cells were then centrifuged (350*g* for 5 minutes), resuspended in buffer without FURA-2AM, and incubated (30 minutes at 37°C) to allow label hydrolysis. Labeled cells (2×10^6/mL in buffer without BSA) were dispensed into 96-well flat-bottom plates (OptiPlate; Packard, Biosciences, Mt. Waverley, Australia) at 100 μL/well. A FLUOstar OPTIMA (BMG Labtech, Offenburg, Germany) fluoro-spectrophotometer for automated injection of peptides measured fluorescence. Changes in the ratio of an emission reading (at 510 nm) produced by an excitation of 340 and 380 nm indicate calcium mobilization from intracellular stores into the cytoplasm. Baseline readings were collected for 10 seconds prior to injection of agonists (100 μmol/L TFLLR PAR-1 agonist peptide or FTLLR control peptide, from Mimotopes, Victoria, Australia).

H pylori *Stimulation of Macrophages and Dendritic Cells*

Bone marrow-derived dendritic cells were obtained as described previously.[19] Briefly, bone marrow cells were cultured in complete RPMI containing 5% supernatant from an Ag8653 myeloma cell line transfected with murine GM-CSF cDNA (kindly provided by Dr Anna Walduck) for 8 days at 37°C. Resulting cells were confirmed to be bone marrow-derived dendritic cells by measurement of major histocompatibility complex (MHC) II and CD11c expression by flow cytometry. Macrophages were prepared by incubating splenocytes on 100-mm tissue culture plates (Becton Dickinson Labware, Franklin Lakes, NJ) containing complete RPMI for 1 hour. After washing off nonadherent cells, loosely adherent macrophages were dislodged by pipetting, pelleted by centrifugation at 1000*g* for 10 minutes, then resuspended in complete RPMI. Macrophage and bone marrow-derived dendritic cells suspensions (10^5 cells/mL) in complete RPMI were stimulated in 24-well tissue culture plates with either live *H pylori* SS1 (10^7 cells/mL) or *H pylori* lysate (5 μg/mL) for 24 hours, centrifuged at 3000*g* for 10 minutes, supernatants collected, and secreted cytokines quantified by ELISA.

Cytokine ELISAs

Ninety-six-well Maxisorp plates (Nunc) were coated with either anti-mouse IL-10 (0.1 μg/well; Pharmingen, San Diego, CA), interferon (IFN)-γ (0.2 μg/well; Pharmingen), or macrophage-inflammatory protein (MIP)-2 (0.2 μg/mL; R&D Systems, Minneapolis, MN) overnight at 4°C, in bicarbonate buffer, pH 9.6. Plates were blocked with PBS-BSA (1 hour), prior to addition of samples in duplicate (100 μL/well; 3 hours). Bound cytokines were detected after 1 hour with either biotinylated anti-mouse IL-10 (0.1 μg/well; Pharmingen), IFN-γ (0.1 μg/well; Pharmingen), or MIP-2 (0.75 μg/well; R&D Systems), followed by 100 μL horseradish peroxidase-conjugated streptavidin (Pierce; 1:5000 in blocking buffer; 1 hour). Finally, color was developed using TMB substrate, as for antibody ELISAs, and cytokine concentrations determined against a standard curve of recombinant IL-10 (Pharmingen), IFN-γ (Pharmingen), or MIP-2 (R&D Systems).

Myeloperoxidase Activity

Myeloperoxidase (MPO) activity within gastric tissues was quantified using a modified method.[20,21] Briefly, gastric tissue (100–200 mg) was homogenized with 10 volumes of PBS, centrifuged at 1000*g*, and the pellet resuspended in PBS containing 0.5% (wt/vol) hexadecyltrimethylammonium bromide. It was then snap frozen in liquid nitrogen and thawed twice. Aliquots (38 μL) of homogenate were mixed with TMB substrate (Zymed) to 500 μL then, after 1 minute, 50 μL of mixture transferred into a 96-well flat-bottom plate containing 87.5 μL of 20 mmol/L sodium acetate (pH 3.0) to stop the reaction. One unit of enzyme activity caused a change in absorbance of 1.0/min at 690 nm and 37°C.

Statistical Analyses

Statistical analyses were performed using SPSS software (SPSS, Inc, Chicago, IL; version 16.0). For comparisons of histologic grading scores, data were compared by nonparametric Mann–Whitney analysis. For all other analyses, data were log transformed and compared by 1-way ANOVA with Dunnett post hoc analysis.

Results

Mice Deficient in PAR-1 Exhibit a Reduced Colonization by H pylori *That Is Associated With an Exacerbated Gastritis*

Wild-type and PAR-$1^{-/-}$ mice were infected with *H pylori*-SS1 for 1 day, 1 week, 1 month, and 2 months before removal of stomachs and quantification of bacterial burden by colony-forming assay. Whereas PAR-1 expression had no effect on colonization up to 1 month postinfection, there was a significant decline in *H pylori* colonization in PAR-$1^{-/-}$ mice, 2 months postinfection (Figure 1). This contrasted starkly with PAR-$2^{-/-}$ mice in which *H pylori* colonization actually increased after 2 months infection (Figure 2).

Reduced colonization in PAR-$1^{-/-}$ mice coincided with a marked increase in the severity of gastritis. Histologic examination revealed no significant effect of *H pylori* infection on inflammation in either wild-type or PAR-$1^{-/-}$ mice up to a month postinfection (data not shown). Two months postinfection, wild-type mice had still not

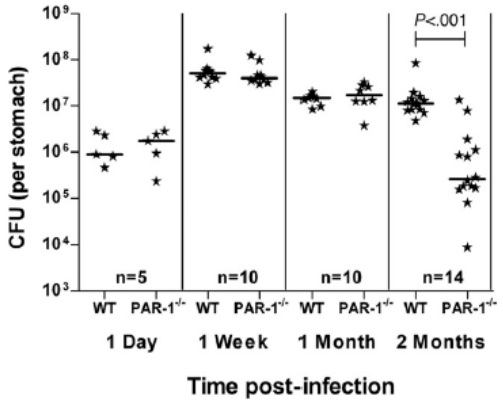

Figure 1. Chronically infected PAR-1$^{-/-}$ mice present with reduced *Helicobacter pylori* colonization levels. Bacterial colonization in PAR-1$^{-/-}$ and wild-type C57BL/6 (WT) mice was quantified by colony-forming assays at stated time points following infection with 10^7 *H pylori*-SS1. Individual mice are shown with group medians (*horizontal bar*). Data are pooled from 2 experiments (numbers of mice shown in each column). Two months postinfection, *H pylori* levels were significantly (>40-fold) lower in PAR-1$^{-/-}$ mice compared with WT controls (ANOVA; $P < .001$), demonstrating that PAR-1 deficiency was detrimental to *H pylori* colonization.

BASIC—ALIMENTARY TRACT

developed a significant inflammatory response compared with uninfected controls (Figure 3*A*, *C*, and *E*). In contrast, PAR-1$^{-/-}$ mice infected for 2 months with *H pylori*-SS1 developed significantly elevated levels of cell infiltrate, mucus metaplasia, and atrophy compared with infected wild-type mice (Figure 3*A*, *C*, and *E*). Representative images of the pathology in *H pylori*-infected PAR-1$^{-/-}$ mice are presented in Figure 4. Conversely, the increased colonization in PAR-2$^{-/-}$ mice 2 months postinfection was associated with a decrease in inflammation relative to infected wild-type controls, although this did not reach significance (Figure 3*B*, *D*, and *F*).

Whereas PAR-2 has previously been shown to possess proinflammatory activity, the observations involving PAR-1 were highly novel. We therefore proceeded to examine the responses of these PAR-1$^{-/-}$ mice and their derived cells.

H pylori *Infection of PAR-1-Deficient Mice Induces Elevated Serum Antibody Levels*

As a marker of the acquired immune response, we quantified *Helicobacter*-specific antibody levels in sera of *H pylori*-infected PAR-1$^{-/-}$ and wild-type mice. Serum levels of both anti-*H pylori* IgA and IgG were significantly increased in PAR-1$^{-/-}$ mice infected with *H pylori* for 2 months, compared with wild-type controls (Figure 5).

Primary Epithelial Cells Express PAR-1 and Are Responsive to PAR-1 Activation

Regulation of inflammation in response to *H pylori* infection can occur at several levels, with the main cell populations being epithelial cells that are directly exposed to the bacteria in vivo, as well as cells of the immune system such as CD4$^+$ T cells and macrophages. We theorized that an environmental protease receptor is likely to be functional at the site of infection, rather than at distal immune sites, therefore implicating epithelial cells as the lead candidate for the regulatory effects of PAR-1. Whereas murine immune cells are known to express PAR-1,[22,23] the expression and activity of this receptor in epithelial cells have not previously been studied.

After confirming that our primary gastric cultures were epithelial cells (purity wild-type = 95.3% ± 3.2% and PAR-1$^{-/-}$ = 94.5% ± 2.4%) and that only the wild-type cells expressed PAR-1 messenger RNA (mRNA) (Figure 6*A*), we examined whether these were responsive to activation via PAR-1. PAR-1 can be specifically and uniquely activated by a peptide (TFLLR) that interacts with a receptor-binding site, inducing an increase in cytosolic calcium (the standard readout for PAR-1 activation). A nonactivating peptide (FTLLR) was used as a negative control. When cultures of wild-type primary epithelial cells were stimulated with the PAR-1-activating peptide, there was an immediate and specific calcium mobilization response relative to the control peptide (Figure 6*B*). This demonstrates that gastric epithelial cells not only express PAR-1 but are responsive to activation via this receptor.

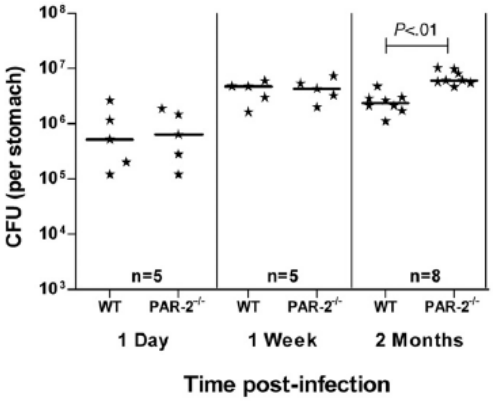

Figure 2. Chronically infected PAR-2$^{-/-}$ mice present with increased *Helicobacter pylori* colonization levels. Bacterial colonization in PAR-2$^{-/-}$ and wild-type 129/Sv (WT) mice was quantified by colony-forming assays at stated time points following infection with 10^7 *H pylori*-SS1. Individual mice are shown with group medians (*horizontal bar*). Numbers of mice are shown in each *column*. Two months postinfection, *H pylori* levels were significantly (3-fold) higher in PAR-2$^{-/-}$ mice compared with WT controls (ANOVA; $P < .001$), demonstrating that PAR-2 presence was detrimental to *H pylori* colonization.

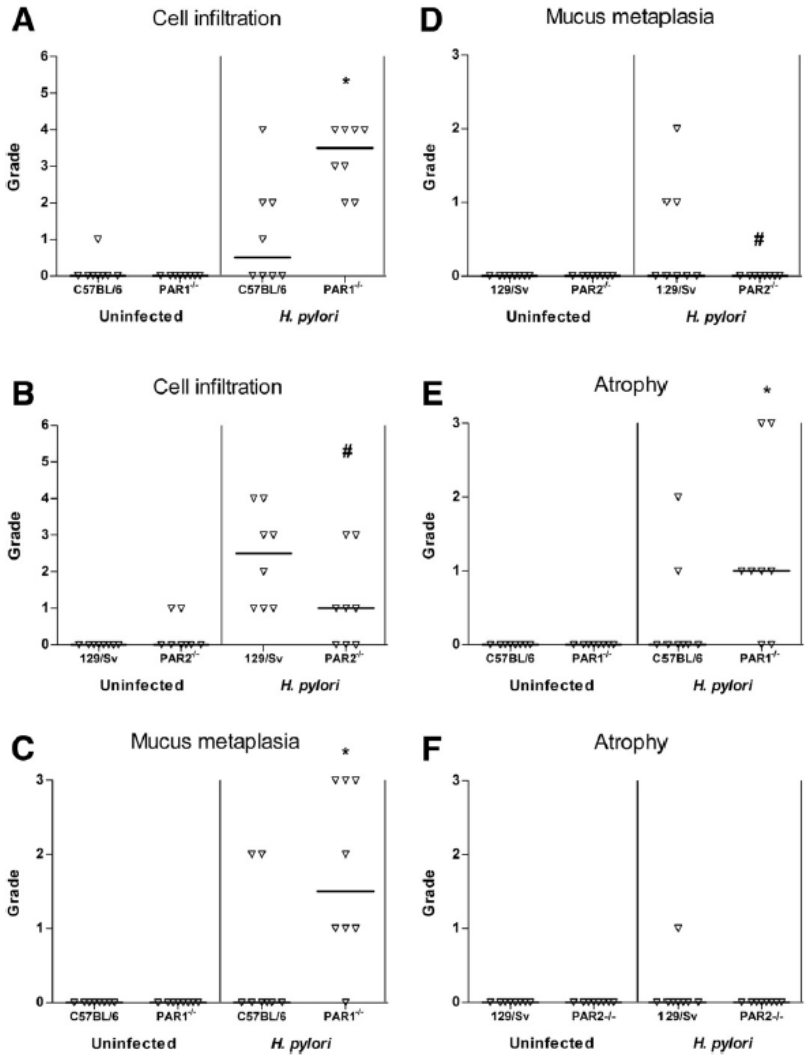

Figure 3. Chronic infection of PAR-1$^{-/-}$ mice with *Helicobacter pylori* induces increased severity of gastritis. Inflammation resulting from *H pylori* infection was assessed on blinded H&E-stained gastric sections from n = 8 PAR-1$^{-/-}$, PAR-2$^{-/-}$, and wild-type mice (C57BL/6 and 129/Sv, respectively), infected with *H pylori* SS1 for 2 months. Sections were graded for cell infiltration (0–6), mucus metaplasia (0–3), and atrophy (0–3). *Points* present scores for individual mice, and *horizontal bars* the group median values. Infected PAR-1$^{-/-}$ mice had significantly more severe gastritis (*greater than infected C57BL/6 wild-type control; $P < .05$, Mann–Whitney test), whereas infected PAR-2$^{-/-}$ mice had reduced gastritis, although this did not reach significance (#cell infiltration = 0.056, mucus metaplasia = 0.064, Mann–Whitney test, compared with infected 129/Sv wild-type control).

H pylori *Up-regulates PAR-1 Expression and Down-regulates PAR-2 Expression in Primary Gastric Epithelial Cells*

To examine whether *H pylori* infection can modulate PAR expression, we first quantified gastric PAR-1 expression in C57BL/6 mice, either uninfected (n = 4) or infected with *H pylori* for 2 months (n = 4). No difference in PAR-1 expression was detected between infected and uninfected mice by quantitative PCR (data not shown). Because PAR-1 is expressed by many cell populations, we theorized that background expression by other cells could be masking any effects of *H pylori* on PAR expres-

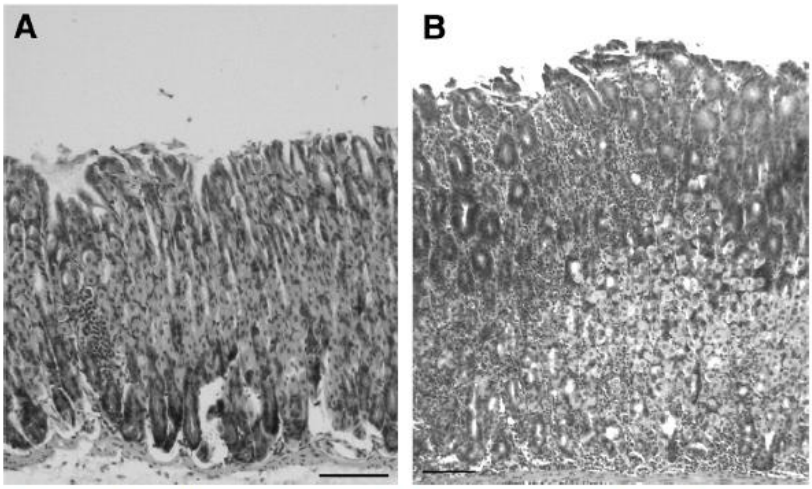

Figure 4. Histopathology in PAR-1$^{-/-}$ and PAR-1$^{+/+}$ mice chronically infected with *Helicobacter pylori*. Representative histologic sections presenting average inflammation from PAR-1$^{-/-}$ and wild-type mice infected with *H pylori*-SS1 for 2 months. (*A*) Typical mild inflammation in infected PAR-1$^{+/+}$ mice, with a low level cellular infiltrate (CI score = 1). (*B*) Typical inflammation in infected PAR-1$^{-/-}$ mice, presenting with moderate cell infiltration (score = 3), mild atrophy (score = 1), and thickened mucosa. An image showing average gastritis is representative, but some infected PAR-1$^{-/-}$ mice presented with severe atrophy and moderate/severe mucus metaplasia. *Scale bar*, 100 μm.

BASIC—ALIMENTARY TRACT

sion by epithelial cells. We therefore quantified the expression of PAR-1, as well as PAR-2, by isolated primary gastric epithelial cells. This revealed an inverse effect, with *H pylori* coculture up-regulating the expression of PAR-1 in these epithelial cells but down-regulating PAR-2 expression (Figure 6*C*).

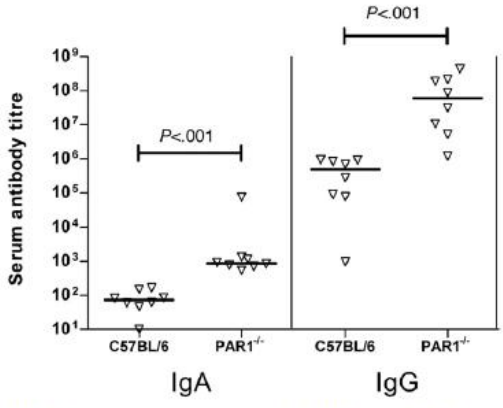

Figure 5. *Helicobacter pylori* infection of PAR-1$^{-/-}$ mice induces an elevated serum antibody response. Control C57BL/6 and PAR-1$^{-/-}$ mice (n = 8) were infected with *H pylori*-SS1 for 60 days, sera collected, and *H pylori*-specific antibodies quantified by ELISA. Data shown are end point titers (uninfected controls subtracted). Antibody levels induced by infection of PAR-1$^{-/-}$ mice were significantly greater than in wild-type controls (ANOVA).

H pylori *Coculture Increases Secretion of the Proinflammatory Cytokine MIP-2 by PAR-1$^{-/-}$ Gastric Epithelial Cells*

Coculture of human gastric epithelial cells with *H pylori* can stimulate IL-8 secretion via a mechanism involving PAR-2.[24] Because our data indicated that PAR-1 and PAR-2 have opposing effects, we speculated that the anti-inflammatory activity of PAR-1 may be mediated by down-regulation of the mouse IL-8 functional homologue MIP-2. IL-8 in humans and MIP-2 in mice are key cytokines involved in neutrophil chemotaxis. We therefore cocultured primary epithelial cells from PAR-1$^{-/-}$ and wild-type mice with *H pylori* and quantified the levels of secreted MIP-2. We also determined whether this coculture would induce differential secretion of the cytokines IL-10 and IFN-γ, previously associated with regulation of *Helicobacter*-induced gastritis and produced by epithelial cells.[25,26] No IL-10 or IFN-γ was detected in any cell cultures (Figure 7*A*). Whereas low levels of MIP-2 were secreted by wild-type cells stimulated with *H pylori*, significantly more MIP-2 was produced by stimulated PAR-1$^{-/-}$ epithelial cells (Figure 7*A*). Furthermore, PAR-1 regulation of MIP-2 production by epithelial cells appears mediated by reducing NF-κB activation because levels of this important transcription factor were elevated in PAR-1$^{-/-}$ epithelial cells following *H pylori* stimulation (Figure 7*B*).

H pylori appears to induce MIP-2 secretion by epithelial cells via 2 distinct mechanisms. One is *cag*PAI independent because MIP-2 was secreted by PAR-1$^{-/-}$ epithelial

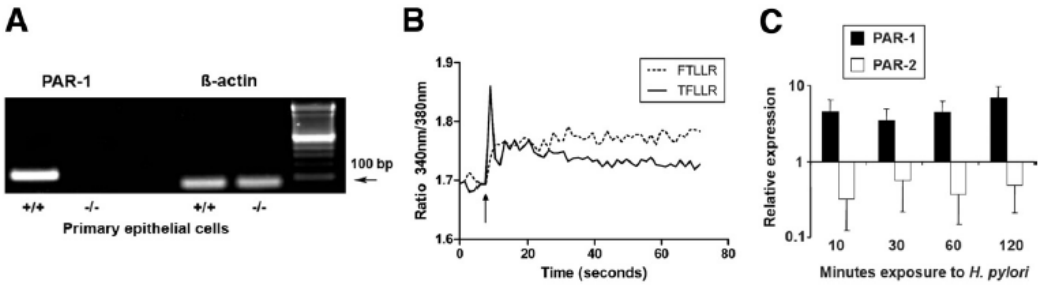

Figure 6. Primary epithelial cells express PAR-1 mRNA and can be activated via PAR-1. (*A*) RNA extracted from gastric primary epithelial cells from C57BL/6 wild-type (+/+) and PAR-1$^{-/-}$ mice (−/−) were reversed transcribed into cDNA then PCR reactions were performed using PAR-1 and β-actin primers. Wild-type primary epithelial cells expressed PAR-1 mRNA, which was absent from PAR-1$^{-/-}$ cells. (*B*) Primary epithelial cells from wild-type C57BL/6 mice were labeled with FURA-2AM, prior to in vitro stimulation with either a PAR-1-activating peptide (*TFLLR*) or a control inactive peptide (*FTLLR*). *Arrow* indicates time at which peptides were added. PAR-1 activation triggered release of stored calcium into the cytoplasm that was detected spectrophotometrically by comparing excitation wavelengths at 340 and 380 nm. The PAR-1-activating peptide induced calcium mobilization in primary epithelial cells, indicating responsiveness to activation via PAR-1. (*C*) Primary epithelial cells from wild-type C57BL/6 mice (n = 3) were cocultured with *H pylori* for 10, 30, 60, or 120 minutes. PAR-1 and PAR-2 expression were quantified by real-time PCR, and expression was calculated relative to unstimulated control and housekeeping gene (β-actin) using the REST formula.

cells stimulated with *H pylori*-SS1, which has a dysfunctional *cag*PAI[13] (Figure 7*A* and *C*). The second is mediated by *cag*PAI because MIP-2 was secreted by PAR-1$^{-/-}$ epithelial cells stimulated with *H pylori* strain 251 but not when stimulated with an isogenic 251 mutant strain that has a dysfunctional *cag*PAI (Figure 7*C*). Both of these mechanisms were suppressed in epithelial cells expressing PAR-1 (Figure 7). In contrast, macrophages and dendritic cells from PAR-1$^{-/-}$ and wild-type mice produced equivalent MIP-2 in response to stimulation with live *H pylori* or lysate (Figure 7*D* and *E*), suggesting that PAR-1-regulating activity is predominantly mediated via the epithelial cell.

Because MIP-2 is a potent chemokine for neutrophils, we compared the levels of myeloperoxidase (a neutrophil marker) in gastric tissue from infected mice. Whereas infection did not significantly affect MPO levels in gastric tissues of wild-type mice, *H pylori* infection significantly elevated this enzyme in PAR-1$^{-/-}$ mice (Figure 8) indicating a role for PAR-1 in reducing neutrophil infiltration into infected mucosa.

Discussion

The severity of inflammation arising because of *H pylori* infection is the key factor that drives progression to its associated pathologies. In this study, we show that expression of PAR-1, a serine protease receptor, downregulates the inflammatory response to *H pylori* infection in mice. This provides a novel demonstration that PAR-1 is an important host regulator of *Helicobacter*-driven gastritis. Most notably, at 2 months postinfection, mice deficient in this receptor developed an increased severity of inflammation, accompanied by a decrease in bacterial colonization and an increase in *H pylori*-specific serum antibodies.

A key observation made in this study was the increased production of the proinflammatory cytokine MIP-2 by PAR-1$^{-/-}$ epithelial cells, but not macrophages or dendritic cells, cocultured with live *H pylori*. This finding, plus our demonstrations (1) that epithelial cells respond to PAR-1-activating peptide and (2) that PAR-1 is upregulated by epithelial cells cocultured with *H pylori*, suggests that PAR-1 acts as a negative regulator of *Helicobacter*-driven inflammation, limiting the production of epithelial cell-derived proinflammatory cytokines. MIP-2 is the mouse functional homologue of human IL-8, and an important feature of these cytokines is their neutrophil chemotactic activity. Gastritis with infiltration of neutrophils (commonly referred to as active gastritis) is associated with more severe inflammation and increased susceptibility to the development of associated diseases in *H pylori*-infected humans. Hence, a host process that regulates neutrophil infiltration could have a profound effect on disease susceptibility. The demonstration of increased levels of the neutrophil enzyme MPO in the gastric mucosa of infected PAR-1$^{-/-}$ mice further supports our hypothesis that the increased severity observed in these mice is due to loss of regulation of the neutrophil chemokine MIP-2.

Neutrophils and macrophages attracted by MIP-2 into the gastric mucosa would become activated and in turn produce cytokines including IL-12 and IL-23 that help drive a proinflammatory T-cell response; *Helicobacter*-induced gastritis is largely driven by a T helper cell (Th)1 and/or Th17 type immune response.[27,28] Cytokines produced by T cells also promote antibody secretion. Hence, there is a logical process by which loss of PAR-1 expression resulting in increased MIP-2 production by epithelial cells can lead to increased neutrophil and macrophage

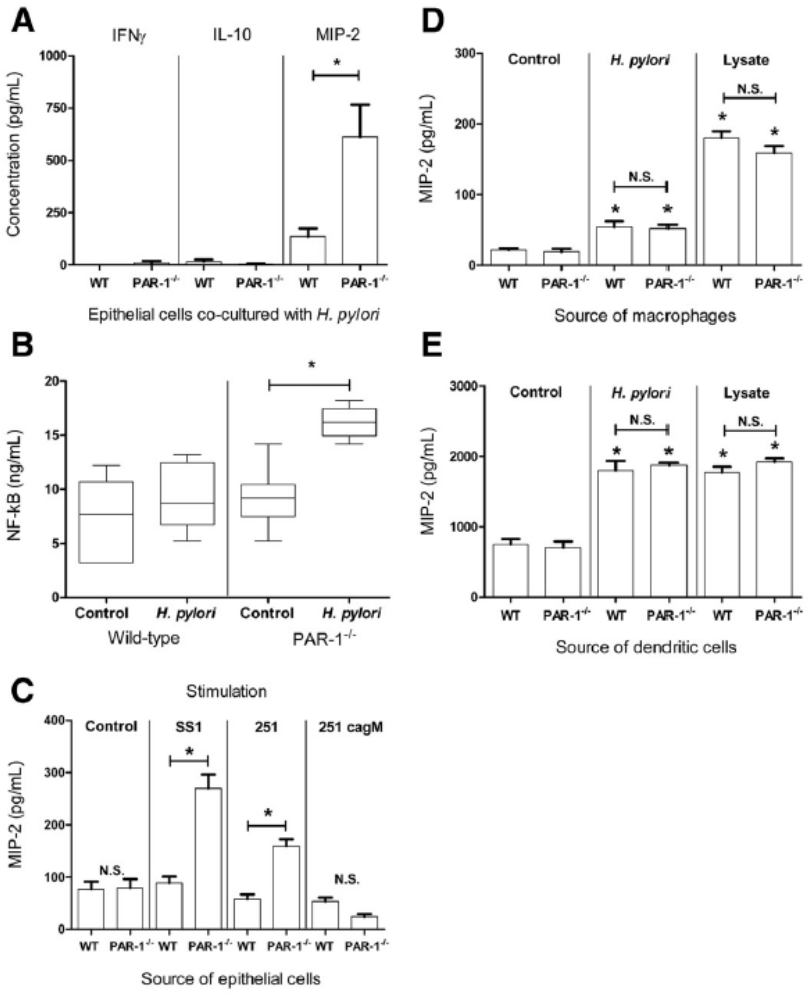

Figure 7. PAR-1$^{-/-}$ epithelial cells but not macrophages or dendritic cells secrete elevated MIP-2 in response to *H pylori*. Primary epithelial cells from individual PAR-1$^{-/-}$ or wild-type (WT) mice (n = 6) were cocultured for 1 day with *H pylori*-SS1 then supernatants and cells collected. (*A*) Cytokines in supernatants (unstimulated backgrounds subtracted) and (*B*) NF-κB in cell nuclear extracts were quantified by ELISA. Neither IFN-γ nor IL-10 were detectable in supernatants from either group. Whereas MIP-2 was detected in supernatants from both groups, PAR-1$^{-/-}$ epithelial cells produced significantly more MIP-2 than did wild-type controls (*$P < .05$; ANOVA). NF-κB levels were significantly increased in *H pylori*-stimulated PAR-1$^{-/-}$ epithelial cells (*$P < .05$; ANOVA). (*C*) PAR-1 down-regulates cytokine production induced by *cag*PAI; MIP-2 was secreted by PAR-1$^{-/-}$ primary epithelial cells stimulated with *H pylori* 251 but not by an isogenic *cagM*-deficient mutant (*251 cagM*) that has a dysfunctional *cag*PAI (*$P < .05$; ANOVA). (*D*) Macrophages and (*E*) dendritic cells from individual PAR-1$^{-/-}$ or wild-type mice (n = 6) were cultured either with no stimulation (*Control*), with live *H pylori*-SS1 (*H pylori*), or with 5 μg/mL *H pylori* lysate. Supernatants were collected after 1 day and MIP-2 levels quantified. Whereas macrophages and dendritic cells produced MIP-2 in response to stimulation with live *H pylori* or bacterial lysate (*greater than unstimulated control; $P < .05$, ANOVA), PAR-1 had no significant effect (*N.S.*, not significant; $P > .05$, ANOVA).

infiltration, increased T-cell activation, and elevated antibody levels.

Whereas elevated antibody levels in PAR-1$^{-/-}$ mice suggest that reduced *H pylori* colonization may be the result of improved host humoral immunity, numerous studies have found antibodies not to be involved in protective immunity against this infection.[29] It is commonly observed that severity of gastritis is inversely associated with *H pylori* colonization levels.[27] That severe gastritis is detrimental to *Helicobacter* infectivity is best

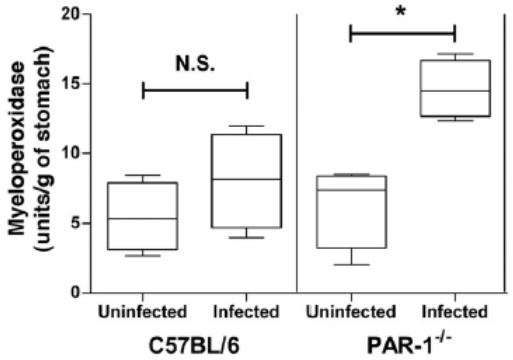

Figure 8. *H pylori*-infected PAR-1-deficient mice have elevated levels of myeloperoxidase in their gastric mucosa. C57BL/6 and PAR-1$^{-/-}$ mice (n = 4) were left uninfected, or infected with *H pylori*-SS1 for 60 days, their stomachs removed, weighed, and homogenized then myeloperoxidase units quantified per gram of stomach. *H pylori* infection significantly increased myeloperoxidase levels in the gastric homogenate of PAR-1$^{-/-}$ mice (*P = .02; ANOVA) but not wild-type C57BL/6 mice (*N.S.*, P = .28; ANOVA).

reflected in the numerous adaptations *H pylori* has undergone to minimize the induction of inflammation, including development of lipopolysaccharide and flagellin that are poor stimulators of Toll-like receptors.[30,31] Reduced colonization in PAR-1$^{-/-}$ mice is therefore most likely the result of the increased severity of gastritis.

H pylori uses several strategies to modify the host response, not least of which is the proinflammatory virulence factor *cag*PAI. In this study, we found that PAR-1 expression by epithelial cells down-regulates production of MIP-2 induced by the *cag*PAI. Infection with *cag*PAI-positive strains induces more severe mucosal damage and, in some studies, is therefore associated with a greater incidence of peptic ulcer disease and gastric cancer than strains lacking this gene cluster.[32] Hence, our observation suggests that PAR-1 may play an important role in minimizing the impact of this important virulence factor and could thereby protect against progression to disease.

Importantly, coculture of *H pylori* with different human gastric epithelial cells has been shown to up-regulate PAR-2 expression[33] as well as stimulate IL-8 secretion via PAR-2 activation.[24] Because PAR-2 is activated by different proteases than PAR-1, this suggests that activation of these receptors may produce a feedback loop, whereby protease activation of PAR-2 drives an IL-8/MIP-2 proinflammatory response, and activation of PAR-1 suppresses this response.

The question remains as to what is the source of the proteases that activate PAR-1 and PAR-2 during *H pylori* infection. Because they act as sensors of environmental change, it is logical that PAR expressed on epithelial cells will be located at the apical surface, for detection of proteases produced by infectious mucosal pathogens. Searching published *H pylori* databases for proteins with close structural resemblance to the proteolytic domain of thrombin (the physiologic PAR-1 agonist), we identified no putative candidates. However, from available genome sequences, we know that *H pylori* possesses 1 serine protease, HtrA, which has been proposed previously as a putative activator of both PAR-1 and PAR-2.[12] Unfortunately, because HtrA is essential for *H pylori* growth in vitro,[34] it is not possible to generate a deficient mutant strain to properly test this hypothesis.

This single protease cannot, however, explain the opposing roles of PAR-1 and PAR-2. Based on the knowledge that the predominant activating protease of PAR-1 is thrombin, we propose the following hypothetical model: Serine protease from *H pylori* stimulates proinflammatory IL-8/MIP-2 production by gastric epithelial cells. As the infection and inflammation develops, tight junctions in the gastric epithelium are opened, probably via an *H pylori* urease-dependent mechanism,[35] creating a leaky mucosa that would expose epithelial cells to increased levels of thrombin-containing serum. The thrombin would activate PAR-1, leading to suppression of NF-κB activation, and IL-8/MIP-2 secretion by epithelial cells, thereby minimizing potentially damaging inflammation. This model would allow for a host system to identify the presence of a pathogen by detection of bacterial protease (via PAR-2), an intended transient inflammatory response to deal with this intruder, followed by damage limitation of excessive inflammation (via PAR-1). Of course, in the case of *H pylori*, the infection is not transient but chronic, and this attempted balancing act can proceed for decades. Such a situation would provide a potential mechanism by which differences in PAR-1 regulation of *Helicobacter*-driven inflammation contribute to host susceptibility or resistance to disease sequelae, including peptic ulcer disease and gastric cancer.

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Conflicts of interest

The authors disclose no conflicts.

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