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Nucleotide Oligomerization Domain 1 Enhances IFN-γ Signaling in Gastric Epithelial Cells during Helicobacter pylori Infection and Exacerbates Disease Severity

Cody C. Allison,* Jonathan Ferrand,† Louise McLeod,* Mohammad Hassan,* Maria Kaparakis-Liakos,* Alexandra Grubman,* Prithi S. Bhalal,‡ Anouk Dev,‡ William Sievert,‡ Brendan J. Jenkins,* and Richard L. Ferrero*

Virulent Helicobacter pylori strains that specifically activate signaling in epithelial cells via the innate immune molecule, nucleotide oligomerization domain 1 (NOD1), are more frequently associated with IFN-γ–dependent inflammation and with severe clinical outcomes (i.e., gastric cancer and peptic ulceration). In cell culture models, we showed that H. pylori activation of the NOD1 pathway caused enhanced proinflammatory signaling in epithelial cells in response to IFN-γ stimulation through the direct effects of H. pylori on two components of the IFN-γ signaling pathway, STAT1 and IFN regulatory factor 1 (IRF1). Specifically, H. pylori activation of the NOD1 pathway was shown to increase the levels of STAT1-Tyr701/Ser727 phosphorylation and IRF1 expression/synthesis in cells, resulting in enhanced production of the NOD1- and IFN-γ–regulated chemokines, IL-8– and IFNγ–induced protein 10, respectively. Consistent with the notion that heightened proinflammatory signaling in epithelial cells may have an impact on disease severity, we observed significantly increased expression levels of NOD1, CXCL8, IRF1, and CXCL10 in human gastric biopsies displaying severe gastritis, when compared with those without gastritis (p < 0.05, p < 0.001, p < 0.01, and p < 0.05, respectively). Interestingly, NOD1, CXCL8, and IRF1 expression levels were also significantly upregulated in gastric tumor tissues, when compared with paired nontumor samples (p < 0.0001, p < 0.05, and p < 0.05, respectively). Thus, we propose that cross-talk between NOD1 and IFN-γ signaling pathways contribute to H. pylori–induced inflammatory responses, potentially revealing a novel mechanism whereby virulent H. pylori strains promote more severe disease. The Journal of Immunology, 2013, 190: 3706–3715.

Global profiling studies of H. pylori–infected tissues or cell lines revealed the upregulation of IFN-γ–responsive gene(s) involved in IFN-γ signaling (17–19), 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 (20), which is mediated via the transcription factor, IFN regulatory factor 1 (IRF1) (20). Likewise, NOD1 protein levels are elevated in the lamina propria of H. pylori–infected patients (21), an effect possibly mediated by the abundance of IFN-γ at the site of infection. It is not known, however, whether enhanced NOD1 expression may sensitize epithelial cells to ongoing H. pylori stimulation.

IFN-γ signals via the Jak/STAT pathway, following ligand binding to multimerized IFN-γR chains (α and β) (22). This process induces the phosphorylation of receptor-associated Jak1 and Jak2 (23, 24), allowing binding and activation of STAT1 (25, 26). STAT1 forms homodimers that translocate to the nucleus and bind to IFN-γ–responsive gene(s) involved in IFN-γ signaling (17–19), 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 (20), which is mediated via the transcription factor, IFN regulatory factor 1 (IRF1) (20). Likewise, NOD1 protein levels are elevated in the lamina propria of H. pylori–infected patients (21), an effect possibly mediated by the abundance of IFN-γ at the site of infection. It is not known, however, whether enhanced NOD1 expression may sensitize epithelial cells to ongoing H. pylori stimulation.

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bind specific “gamma-activated sequences” to induce the expression of certain IFN-γ-regulated genes (27), such as IRF1 (28), IFN-γ-regulated chemokines, such as IFN-γ-induced protein 10 (IP-10) and monokine induced by IFN-γ (MIG), are regulated in a slightly different manner that involves the binding of transcription factors to IFN-stimulated response elements in gene promoters (29, 30). Although NOD1, IFN-γ, and IRF1 are known to be crucial mediators of proinflammatory responses during H. pylori infection (1, 7, 31), a potential regulatory link between these factors has yet to be thoroughly investigated.

To study this, an in vitro coculture system was employed to recreate a basic model 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 analyzed in gastric biopsies from infected and noninfected patients with differing degrees of gastritis or gastric adenocarcinoma.

We found that cagPAI1 H. pylori strains augmented epithelial cell production of the IFN-γ–induced chemokines, IP-10 and MIG, in response to IFN-γ stimulation. Consistent with these data, NOD1 and IFN-γ–regulated gene expression in gastric biopsies from H. pylori–infected subjects were significantly increased in severe gastritis and gastric tumor tissues. We propose that though important for infection control, NOD1 and IFN-γ signaling together may potentiate an inflammatory feedback cycle that exacerbates disease severity and progression during chronic H. pylori infection.

Materials and Methods

Cell culture and transient transfection

In vitro experiments were performed using human gastric [MKN28 (32) and AGS (33)] and nongastric (HEK293) epithelial cell lines. In addition, we used AGS cells stably expressing small interfering RNA (siRNA) to either the caspase activation and recruitment domain of NOD1 or an irrelevant gene, enhanced GFP (referred to henceforth as AGS NOD1 KD or AGS control cells, respectively) (34). All cell lines were cultured as described previously (34). For STAT1 overexpression, cells were transfected with STAT1 plasmid (35) (100 ng/well), standardized to 1150 ng/well with pCdynA (1) in polyethyleneimine (Polyscience, Warrington, PA) (16) h prior to stimulation. Plasmid DNA was prepared using the PureYield Plasmid MidiPrep System (endotoxin free) (Promega, Madison, WI). For siRNA transfections, Scrambled siRNA (AllStars Negative Control siRNA; Qiagen, Mulgrave, VIC, Australia) or NOD1–specific Silencer Select siRNAs (ref. S20322 and S20324; Ambion, Victoria, Australia) were mixed with Lipofectamine 2000 (Life Technologies, Mulgrave, VIC, Australia) and added 1:5 to cell suspension containing 150,000 cells. Cells were incubated for 36 h prior to stimulation.

Bacteria and stimulations

H. pylori strains 251 (36) and G27 (37) and isogenic 251 ΔcagPAI1 (38), G27ΔcagA, and G27ΔcagM (this study) mutants were constructed by natural transformation as described previously (39). Bacteria were cultured in microaerobic conditions on blood agar medium (40). For stimulations, washed H. pylori broth cultures were added to antibiotic-free serum-free media at 105 cells/ml, incubated for 8 h and replenished with serum-free media for an additional 18 h. To visualize nuclei, cells were stained with Hoechst (Life Technologies) for 30 min prior to stimulation. Following stimulation, cells were lysed using Laemmli buffer and subjected to 10% SDS-PAGE gel electrophoresis. Densitometry of phospho-STAT1, actin, IRF1, and GAPDH (all Life Technologies) or anti–phospho-STAT1 (Tyr701) (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), followed by Alexa Fluor 488–conjugated goat–anti-rabbit IgG Abs (1:500; Life Technologies). Phospho-STAT1 (Tyr701) activation in cells was captured at detectable levels using the Cellomics ArrayScan VTi HCS Reader (Thermo Fisher Scientific, Franklin, MA), capturing at least 1000 cells or 20 fields/well using a ×20 objective lens. Data were analyzed using a nuclear translocation analysis algorithm. Cells with a nuclear to cytoplasmic fluorescence intensity ratio greater than 1.5 were considered to have active phosphorylation and nuclear translocation of STAT1. For standard immunofluorescence, cells were grown on glass coverslips for 16 h, followed by 1–2 h stimulation. Cells were prepared as above and phospho-STAT1 (Tyr701) detected with a Leica DMR upright fluorescence microscope (Leica, Wetzlar, Germany), using a ×40 objective lens.

ELISA

IL-8, IP-10, and MIG in culture supernatants were all detected using respective BD OptEIA Human ELISA kits (BD Biosciences Pharmingen, San Diego, CA). The Chemokine Human 10-plex Panel Luminex Protein Assay (Life Technologies) was used to detect IP-10, MIG, RANTES, MCP-1, MCP-2, MCP-3 MIP-1-α, MIP-1β, Gro-α, and eotaxin in MKN28 cell culture supernatants. All kits were used in accordance with the manufacturers’ instructions.

RNA isolation, cDNA preparation, and quantitative real-time PCR

Human gastric biopsies were snap-frozen and homogenized in TRIzol (Life Technologies). RNA was purified using the Purelink RNA Kit (Life Technologies) in accordance with the manufacturer’s instructions. RNA was DNase-treated using Turbo DNA-free (Ambion) and quantified using a Qubit Fluorometer (Life Technologies). Standard curves were constructed by plotting average Ct values against the logarithm of known genomic DNA concentrations to standardize primer amplification efficiencies (Table I). NOD1 gene silencing in AGS cells was assessed by TaqMan qRT-PCR using a validated NOD1–specific primer/probe set (Hs00196075_m1; Applied Biosystems). Target gene cDNA concentrations for each test sample were determined using the standard curve and normalized to 18S RNA expression.

Immunoblotting

Following stimulation, cells were lysed using Laemmli buffer and subjected to SDS-PAGE analysis. Proteins were transferred to nitrocellulose membrane using the iBlot System (Life Technologies), as described previously (40). Membranes were incubated with either anti–phospho-STAT1 (Tyr701) anti–phospho-STAT1 (Ser727), total anti–STAT1 (Santa Cruz Biotechnology), or anti–IRF1 (Cell Signaling Technology, Arundel, QLD, Australia) (all at 1:1000), or anti–actin (1:3000) Abs, respectively, followed by a goat–anti-rabbit HRP–conjugated IgG (1:10,000–10,000) secondary Ab (Millipore). Immunodetection was performed using ECL Plus Detection Reagent (GE Healthcare, New South Wales, Australia). Densitometry of phosphorylated STAT1 or IRF1 in immunoblots was performed using Quantity One One-Dimensional Analysis Software (version: 4.6.9; Bio-Rad, Gladesville, NSW, Australia). The relative amounts (ODs × mm2) of phosphorylated STAT1 or IRF1 were standardized with respect to the corresponding amounts of total STAT1 or actin, respectively.

**Table I. Oligonucleotides used in this study**

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<tr>
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F, Forward; R, reverse; Tm, melting temperature.
Gastric biopsies and histological grading

Twenty-four patients presenting with a wide range of gastric symptoms attending the Monash Medical Centre Gastrointestinal and Liver Unit were recruited. Informed consent was obtained prior to enrolment and the study was approved by the Southern Health Human Research Ethics Committee (project number 07174A). Endoscopic findings and the results of histopathological examination of gastric biopsies were classified and graded by a pathologist (P.S.B.), according to a revised version of the Sydney System (Table II) (41). H. pylori status was confirmed via qRT-PCR using pathogen-specific 16S rRNA primers. cagA status was determined via PCR to detect a 5’ conserved region of the cagA gene.

Statistical analysis

All statistical analyses were performed with GraphPad Prism 5 software (GraphPad Software, San Diego, CA). For the analysis of numerical data between multiple groups, one-way or two-way ANOVA tests were used, as appropriate, depending on the number of variables. Bonferroni corrections and Dunn’s multiple comparison posttests were applied to parametric and nonparametric data sets, respectively. The Mann–Whitney U test was used to compare unpaired data sets. For correlative tests, Pearson tests were used. A value of p < 0.05 was considered statistically significant. Correlations between samples, paired student t tests were used. A p value < 0.05 was considered statistically significant.

Results

Both H. pylori and IFN-γ are required to stimulate the production of IFN-γ-inducible chemokines in gastric epithelial cell lines

Preliminary coculture experiments were performed to assess the ability of H. pylori to induce IFN-γ signaling pathways and/or to augment proinflammatory responses to IFN-γ treatment in different cell lines. Secretion of the potent chemoattractants, IL-8 and IP-10, were measured to assess NF-κB and IFN-γ proinflammatory signaling, respectively, and as a means of investigating the ability of epithelial cells to recruit and direct adaptive immune responses. Consistent with previous data (1, 34, 40), AGS cells treated with siRNA to NOD1 secreted significantly less IL-8 than AGS control cells treated with scrambled siRNA (Fig. 1A), in response to stimulation with wild-type (WT) H. pylori bacteria. MKN28 cells that were costimulated with both bacteria and IFN-γ exhibited slightly enhanced IL-8 production as compared with cells treated with bacteria alone (p < 0.0001; Fig. 1A). Stimulation of AGS, but not MKN28 cells, with IFN-γ alone induced modest IP-10 production (Fig. 1B). Paradoxically, MKN28 cells were found to express more IFN-γR (IFNGR1) than AGS cells at both mRNA and protein levels (Supplemental Fig. 1). IP-10 production in IFN-γ–stimulated AGS cells occurred irrespective of siRNA NOD1 knockdown, indicating that NOD1 is not essential for direct IFN-γ–dependent responses in these cells. Costimulation with H. pylori and IFN-γ augmented IP-10 production in all cell lines, whereas stimulation with bacteria alone failed to do so (Fig. 1B), which indicates that multiple stimuli are required for IP-10 expression in H. pylori–stimulated gastric epithelial cells, a finding consistent with that of a previous report (42). Indeed, NOD1 activation appears to be important in this regard, as IP-10 production was significantly reduced in NOD1 siRNA–treated AGS cells as compared with control cells (p < 0.0001; Fig. 1B).

Furthermore, the effects of H. pylori and IFN-γ costimulation on IP-10 production were specific to IFN-γ, because costimulation with bacteria and either TNF-α or IL-1β, while synergistically enhancing IL-8 production, failed to substantially augment IP-10 production (Supplemental Fig. 2).

IFN-γ–inducible chemokine responses in epithelial cells are H. pylori and cagPAI dependent

We next sought to examine the role of the H. pylori cagPAI in enhancing signaling responses to IFN-γ. Both WT and isogenic ΔcagA-mutant H. pylori induced IL-8 secretion in the MKN28 gastric epithelial cell line, whereas the isogenic ΔcagM mutant, which lacks a functional type IV secretion system (1, 9, 43), failed to do so (Fig. 2A). Although H. pylori Caga has been reported to mediate IL-8 responses in AGS gastric epithelial cells (44), we observed no differences between the IL-8 responses induced in MKN28 cells by WT bacteria, when compared with isogenic ΔcagA organisms. This result is consistent with previous findings from our group for AGS cells (1, 40) and may be attributed to the relatively short bacteria-cell contact time used in our studies (i.e., 1 h).

Costimulation of cells with H. pylori and IFN-γ induced robust IL-8, IP-10, and MIG responses in MKN28 cells (Fig. 2A–C). These responses were strictly dependent on the presence of an intact cagPAI. Intriguingly, cells costimulated with IFN-γ and the H. pylori ΔcagA mutant secreted significantly more of the IFN-γ–inducible cytokines, IL-8 and MIG, than cells costimulated with WT bacteria (p < 0.0001; Fig. 2B). To confirm that these observed inhibitory effects of CagA on IFN-γ-signaling were not bacterial strain-specific, experiments were also performed using an additional cagPAI H. pylori isolate (strain G27) and corresponding isogenic mutants. 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 WT strain (p < 0.0001; Fig. 2B, 2C), showing that this is not a strain-specific effect. Similar trends were noted for RANTES, MCP-1, MIP-1α, and MIP-1β secretion in response to costimulation with cagPAI H. pylori.
bacteria and IFN-γ (Supplemental Fig. 3). In contrast, eotaxin, Gro-α, MCP-2, or MCP-3 did not appear to be produced in response to these stimuli ( assay detection limit = 5–10 pg/ml). Taken together, these findings indicate that stimulation of MKN28 epithelial cells with H. pylori alone is insufficient to induce the secretion of numerous proinflammatory cytokines, suggesting that their expression in epithelial cells during infection may be delayed and dependent on additional stimuli, such as the IFN-γ produced by immune cells that are recruited to the gastric mucosa during chronic infection.

**STAT1 enhances epithelial cell responses to H. pylori**

Given that the transcription factor, STAT1, is required for the downstream regulation of many IFN-γ-regulated responses in cells, we sought to ascertain whether STAT1 may also be involved in the observed IFN-γ-dependent enhancement of proinflammatory responses to H. pylori stimulation. For this, STAT1 was first overexpressed in different epithelial cell lines (AGS, MKN28, and HEK293), prior to stimulation with H. pylori bacteria. IL-8 production was significantly upregulated in WT H. pylori–stimulated cells overexpressing STAT1, as compared with controls ( p < 0.0001; Fig. 3A, 3C). This effect was cagPAI dependent, because cells stimulated with H. pylori ΔcagPAI mutant bacteria, lacking the pathogenicity island, failed to induce substantial cytokine production (Fig. 3A). Similar results were observed with respect to IP-10 production, although responses were low as cells were not costimulated with IFN-γ (Fig. 3B). These results suggest that STAT1 is capable of synergizing with H. pylori–induced signaling pathways to enhance chemokine production by gastric epithelial cells.

**H. pylori induces STAT1 Ser727 phosphorylation, but not Tyr701 phosphorylation, in a cagPAI-dependent manner**

To investigate whether STAT1 phosphorylation was associated with this enhanced chemokine expression, STAT1 Tyr701 phosphorylation was measured 6 h after initial H. pylori stimulation (2 h after the addition of IFN-γ). Treatment with IFN-γ, but not H. pylori, resulted in rapid Tyr701 phosphorylation (Fig. 4A, 4B). Furthermore, the amount of STAT1 phosphorylation was significantly enhanced in cells costimulated with cagPAI+ H. pylori bacteria and IFN-γ ( p < 0.05; Fig. 4A, 4B), suggesting that H. pylori induces signaling pathways that prime epithelial cells to respond more robustly to IFN-γ secreted by immune cells at later stages of infection. A previous study reported that H. pylori directly induces STAT1 Tyr701 phosphorylation in MKN45 gastric epithelial cells, peaking 2 h after stimulation (45). Therefore, a coculture time course was performed to observe the kinetics of STAT1 phosphorylation in MKN28 cells, if any, in the earlier stages of H. pylori stimulation. Stimulation with IFN-γ alone rapidly induced robust STAT1 Tyr701 phosphorylation, but weak STAT1 Ser727 phosphorylation, within 30 min of treatment (Fig. 4C). H. pylori stimulation alone, however, failed to induce STAT1 Tyr701 phosphorylation at either 30 or 60 min (Fig. 4C), nor at time points up to 6 h (Fig. 4A). These findings were confirmed using various immunofluorescence assays (Supplemental Fig. 4) and also with H. pylori strain G27 (C.C. Allison, unpublished observations). Importantly, stimulation with H. pylori bacteria alone induced significantly increased levels of STAT1 Ser727 phosphorylation at 60 min poststimulation ( p < 0.05; Fig. 4C, 4D). This H. pylori–mediated STAT1 Ser727 phosphorylation was cagPAI dependent but cagA independent (Fig. 4C, 4D).

**H. pylori induces IRF1 transcription in a cagPAI- and NOD1-dependent manner**

Given that expression of many IFN-γ–responsive chemokines were induced only upon costimulation with IFN-γ and cagPAI+ H. pylori bacteria, we next investigated the expression of the transcription factor, IRF1, which can be upregulated by both STAT1 (28) and NF-κB (46, 47). qRT-PCR analysis revealed strong IRF1 expression in cells stimulated with H. pylori WT and ΔcagA mutant, although not ΔcagM, bacteria within 2 h (Fig. 5A). As was observed for chemokine production, H. pylori ΔcagA mutants induced significantly more IRF1 expression than WT bacteria ( p < 0.05; Fig. 5A). Although IRF3 gene expression was reported to be induced following TLR stimulation (48), the expression levels of this transcription factor remained unchanged in response to H. pylori stimulation at any point up to 6 h (Fig. 5B; C. Allison and R. Ferrero, unpublished observations).
As we have previously found NOD1 to be a crucial mediator of cagPAI-dependent epithelial signaling (1, 40), we next investigated the role of this pathogen recognition molecule in *H. pylori*–induced IRF1 expression using AGS cells stably expressing siRNA to NOD1 or an irrelevant gene: AGS NOD1 KD and control cells, respectively (34). In agreement with the responses in MKN28 cells (Fig. 5A, 5B), IRF1 mRNA expression levels were increased in AGS control cells stimulated with *H. pylori* WT or ΔcagA but not ΔcagM bacteria, as compared with nonstimulated cells (Fig. 5C). As for MKN28 cells, the *H. pylori* ΔcagA mutant induced significantly higher levels of IRF1 mRNA than the WT strain (p < 0.05; Fig. 5C). Importantly, the levels of IRF1 gene expression induced by *H. pylori* WT or ΔcagA bacteria in AGS NOD1 KD cells were comparable to those in nonstimulated cells (Fig. 5C). Using two commercial anti-NOD1 Abs, we could not unequivocally detect NOD1 protein in AGS NOD1 KD and control cells.

**FIGURE 3.** Exogenous STAT1 enhances epithelial cell responses to *H. pylori*. MKN28 (A, B), AGS (C), or HEK293 (D) cells were transfected with either pcDNA3 or STAT1 expression constructs for 18 h prior to stimulation with WT 251 *H. pylori* or isogenic ΔcagPAI-mutant bacteria for 1 h. Cell media were then replaced with fresh media and further incubated. Culture supernatants were collected a total of 24 h after initial stimulation and analyzed by ELISA to determine the secretion of IL-8 (A, C, D) or IP-10 (B). Error bars indicate SD across samples analyzed in triplicate. Results are representative of at least three biological replicates. ****p < 0.0001, as assessed by two-way ANOVA and Bonferroni posttest.

**FIGURE 4.** *H. pylori* induces STAT1 Ser727 phosphorylation and augments IFN-γ–dependent STAT1 Tyr701 phosphorylation in a cagPAI-dependent manner. MKN28 cells were stimulated or not with WT *H. pylori* 251 or isogenic ΔcagA- or ΔcagM-mutant bacteria for 30 min up to 4 h. (A) After 4 h, media were replaced, and cells were stimulated or not with IFN-γ (20 ng/ml) for an additional 2 h. Cells were lysed in 100 μl Laemmli buffer and subjected to SDS-PAGE and immunoblotting to determine Tyr701 phosphorylation using rabbit–anti-phospho-STAT1 (Tyr701), total anti-STAT1, and goat–anti-rabbit IgGAbs. (B) Differences in Tyr701 phosphorylation were quantified using densitometry from three independent experiments. Error bars indicate SEM across pooled data from three independent experiments. *p < 0.05, as assessed by one-way ANOVA with Dunn’s multiple comparison posttest.
control cells (unpublished observations), but were able to demonstrate significantly lower NOD1 transcript levels in AGS NOD1 KD cells than in control cells \((p < 0.05; \text{Fig. 5D})\). Moreover, and consistent with these data, we demonstrated that both IL-8 responses and IRF1 protein synthesis were significantly decreased in AGS NOD1 KD cells stimulated with either WT bacteria alone or costimulated with bacteria and IFN-\(\gamma\), when compared with control cells \((p < 0.05; \text{Fig. 5E–G})\). In summary, these data demonstrate a role for NOD1 signaling in H. pylori induction of IRF1 expression in gastric epithelial cells.

**Discussion**

The majority of CD4\(^+\) T cells isolated from the H. pylori–infected mucosa produce IFN-\(\gamma\) and display a polarized Th1 phenotype \((4, 49–52)\). These responses are associated with gastritis severity, particularly in patients infected with \(cag\)PAI\(^+\) H. pylori strains.
Although 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 (5, 6), few have investigated the direct effects of IFN-γ on NOD1-dependent epithelial responses to *H. pylori*. We show in this study that NOD1-dependent recognition of *cag*PAI+ *H. pylori* bacteria induces a proinflammatory feedback loop in gastric epithelial cells, which is enhanced by IFN-γ and correlates with gastritis severity and gastric tumor tissue.

Consistent with previous work from our group (1, 39, 40), *H. pylori* induced robust IL-8 production in a *cag*PAI- and NOD1-dependent manner (Fig. 1). In contrast, bacteria alone were unable to stimulate IP-10 and MIG production (Figs. 1, 2), indicating that multiple stimuli are required for the expression of these chemokines in gastric epithelial cells. This finding is consistent with previous studies demonstrating synergism between IFN-γ and NF-kB signaling pathways (42, 53), but differs from the findings of a recent study, which reported robust IP-10 induction in response to a synthetic NOD1 ligand, iE-DAP (54). Further investigation is necessary to determine the mechanism of *H. pylori*-induced IP-10 production by gastric epithelial cells.

We found that augmented epithelial chemokine responses following costimulation with IFN-γ and *cag*PAI+ *H. pylori* coincided with enhanced STAT1 Tyr701 phosphorylation (Fig. 4). Costimulation of IFN-γ-treated cells with *H. pylori* ΔcagA bacteria did not further enhance Tyr701 phosphorylation, as compared with stimulation with WT bacteria, suggesting that the observed inhibitory effects of CagA on IRF1 and chemokine responses (Figs. 2, 5) are not mediated via Tyr701 phosphorylation. CagA-mediated inhibition of IFN-γ signaling is a novel finding, and we speculate that the fine tuning of CagA-induced Shp2 and STAT3 activation within the host cell (55, 56) may lead to an overall suppression of IFN-γ–dependent inflammatory responses. A previous study also reported an inhibitory effect of *H. pylori* on IFN-γ responses in gastric epithelial cells (57); however, this was shown to occur independently of the *cag*PAI and therefore of CagA. Nevertheless, that work was performed using non-isogenic WT *H. pylori* isolates harboring, or not, a *cag*PAI. Our findings that ectopic STAT1 expression significantly increased IL-8 and IP-10 production upon stimulation with *cag*PAI+ *H. pylori* bacteria (Fig. 3), demonstrate a putative link between NOD1 and IFN-γ signaling pathways. Indeed, p38 was shown to enhance IFN-γ–regulated gene expression in a STAT1-dependent but phosphorylation-independent manner (58). Although this was demonstrated using murine fibroblasts, we have previously shown that NOD1 is also essential for p38 activation during *H. pylori* stimulation of gastric epithelial cells (40), which may offer one explanation for the *cag*PAI-dependent enhancement of IFN-γ signaling.

### Table II. Disease pathology and *H. pylori* status of antral gastric biopsy specimens

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<th>Disease Pathology</th>
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NA, Not available.

### Table III. Disease pathology and *H. pylori* status of gastric cancer specimens

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| 2       | M      | NA      | +                 | Poorly differentiated adenocarcinoma | Previous chemotherapy and tissue scarring  
Distal gastrectomy |
| 3       | F      | NA      | —                 |                 |                  |
| 4       | F      | 70      | —                 | Poorly differentiated adenocarcinoma |          |
| 5       | M      | 78      | +                 | Poorly differentiated adenocarcinoma |          |
| 6       | M      | 68      | —                 | Moderately differentiated adenocarcinoma |          |
| 7       | F      | 71      | —                 | Poorly differentiated, diffuse adenocarcinoma |          |
| 8       | F      | 76      | —                 | Poorly differentiated adenocarcinoma |          |
| 9       | F      | 62      | —                 |                  | Chemotherapy (residual tumor remaining) |

NA, Not available.
Yamaoka et al. (45) reported that *H. pylori* induced detectable STAT1 Tyr^{701} phosphorylation within 2 h of stimulation, via the *H. pylori* OipA protein, whereas the *cag*PAI was not required. They also found elevated levels of tyrosine phosphorylated STAT1 in antral biopsies from infected patients; however, specific cell types were not identified (45). Despite the use of numerous methods in the current study, as well as the coculture of cells with an *H. pylori* strain (251) possessing a functional *oipA* gene (R.L. Ferrero, unpublished observations), we were unable to demonstrate STAT1 Tyr^{701} phosphorylation in MKN28 cells at any time point between 30 min and 8 h after *H. pylori* stimulation (Fig. 4, Supplemental Fig. 4). Yamaoka et al. (45), however, used MKN45 gastric epithelial cells, which produce detectable levels of constitutive Tyr^{701} phosphorylated STAT1, suggesting that IFN-γ signaling pathways may have been more highly activated in these cells.

Interestingly, we found that *cag*PAI*+* *H. pylori* bacteria rapidly induced STAT1 Ser^{727} phosphorylation (Fig. 4), which in the context of IFN-γ signaling, is necessary in combination with Tyr^{701} phosphorylation for complete STAT1 transcriptional functionality (59). In this way, IFN-γ-induced tyrosine phosphorylated STAT1 may become fully transcriptionally activated following *cag*PAI-dependent Ser^{727} phosphorylation, representing another possible mechanism for the enhanced proinflammatory cytokine production we observed. Little is known of the role of Ser^{727} phosphorylation in the absence of concomitant Tyr^{701} activation, although Ser^{727} alone was found to be necessary and sufficient to induce *IRF1* transcription in human salivary gland cells (60). Such findings highlight an added level of complexity in these pathways, which are likely to be differentially regulated in a cell-specific manner. Indeed, we show that *cag*PAI*+* *H. pylori* bacteria can directly induce both *IRF1* expression and IRF protein synthesis (Fig. 5), a finding consistent with Yamaoka et al. (45). Given that NF-κB was found to strongly and rapidly stimulate IRF1 production in response to TNF-α treatment (47), NOD1-dependent NF-κB activation by *H. pylori* may represent a novel alternative mechanism whereby *IRF1* gene expression can be upregulated.

Analysis of tumor and nontumor tissues from patients identified statistically significant increases in the expression levels of *IRF1*, *NOD1*, and *CXCL8* in tumor tissues (*p < 0.05; Fig. 6).* CXCL8 expression is known to promote tumor neo-vascularization (61, 62), whereas elevated *IRF1* and *NOD1* expression has not previously reported in gastric cancer. IRF1 is generally regarded as an antitumor factor that mediates apoptosis (reviewed in Ref. 63) and although a loss of heterozygosity in the *IRF1* chromosomal region has been reported in gastric cancer (64), a functional phenotype has yet to be established in this context. Elevated *IRF1* and *NOD1*...
expression could indicate possible IFN-γ-dependent regulation. Thus, the precise role of IRF1 in the gastric epithelium during differing stages of *H. pylori* infection and associated disease is a subject that warrants further investigation.

In summary, this study establishes that IFN-γ enhances gastric epithelial cell host responses to *H. pylori* stimulation, by upregulating the expression of chemokines involved in the recruitment of immune cells into the gastric mucosa (Fig. 2, Supplemental Fig. 3). We show that this is a cagPAI- and NOD1-dependent phenomenon, supporting previous studies demonstrating that both host factors are essential for Th1-dependent responses during *H. pylori* infection-a role for immune response in *H. pylori* infection.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


FIGURE 7. A model of proinflammatory feedback in *H. pylori*-infected gastric epithelial cells. The model depicts the initial activation of proinflammatory signaling cascades in gastric epithelial cells upon stimulation with cagPAI *H. pylori*. Secretion of chemokines, such as IL-8 and IP-10, results in the recruitment of immune cells to the site of infection. These immune cells in turn produce IFN-γ and other effectors, which act back on epithelial cells to augment proinflammatory responses in an effort to control infection.