Modification of *Helicobacter pylori* Peptidoglycan Enhances NOD1 Activation and Promotes Cancer of the Stomach

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Abstract

*Helicobacter pylori* (*H. pylori*) is the strongest known risk factor for gastric carcinogenesis. One cancer-linked locus is the *cag* pathogenicity island, which translocates components of peptidoglycan into host cells. NOD1 is an intracellular immune receptor that senses peptidoglycan from Gram-negative bacteria and responds by inducing autophagy and activating NF-κB, leading to inflammation-mediated bacterial clearance; however chronic pathogens can evade NOD1-mediated clearance by altering peptidoglycan structure. We previously demonstrated that the *H. pylori* *cag*\(^+\) strain 7.13 rapidly induces gastric cancer in Mongolian gerbils. Using 2D-DIGE and mass spectrometry, we identified a novel mutation within the gene encoding the peptidoglycan deacetylase PgdA; therefore, we sought to define the role of *H. pylori* PgdA in NOD1-dependent activation of NF-κB, inflammation, and cancer. Coculture of *H. pylori* strain 7.13 or its *pgdA\(^+\)* isogenic mutant with AGS gastric epithelial cells or HEK293 epithelial cells expressing a NF-κB reporter revealed that *pgdA* inactivation significantly decreased NOD1-dependent NF-κB activation and autophagy. Infection of Mongolian gerbils with an *H. pylori* *pgdA\(^+\)* mutant strain led to significantly decreased levels of inflammation and malignant lesions in the stomach; however, preactivation of NOD1 before bacterial challenge reciprocally suppressed inflammation and cancer in response to wild-type *H. pylori*. Expression of NOD1 differs in human gastric cancer specimens compared with noncancer samples harvested from the same patients. These results indicate that peptidoglycan deacetylation plays an important role in modulating host inflammatory responses to *H. pylori*, allowing the bacteria to persist and induce carcinogenic consequences in the gastric niche. Cancer Res; 75(8); 1749–59. ©2015 AACR.

Introduction

*Helicobacter pylori* (*H. pylori*) is the most common bacterial infection worldwide and biologic costs incurred by chronic gastritis include an increased risk for gastric adenocarcinoma (1–4). However, only a percentage of colonized persons develop neoplasia. One strain-specific virulence locus that augments cancer risk is the *cag* pathogenicity island, which encodes a type IV secretion system (T4SS) that translocates CagA into epithelial cells (5–13). However, most persons colonized with *cagA*\(^+\) strains do not develop cancer (1), suggesting that other *H. pylori* constituents affect disease risk.

In addition to CagA, the *cag* T4SS delivers peptidoglycan into host cells where it is recognized by NOD1, an intracytoplasmic sensor of peptidoglycan components (14–18). *H. pylori* can also deliver peptidoglycan into host cells via outer membrane vesicles (19). Most gastrointestinal epithelial cells express NOD1 and activation of NOD1 by the muramyl peptide γ-D-glutamyl-meso-diaminopimelic acid (εE-DAP) leads to cytokine production as well as induction of autophagy (20, 21). NOD1 activation is tightly regulated by a negative autocrine feedback system, in which NOD1-regulated effectors concomitantly suppress the downstream effects of NOD1 activation (22–24).

Chronic pathogens can evade NOD1-mediated clearance by altering peptidoglycan structure. NOD1 sensing of *H. pylori* peptidoglycan induces NF-κB activation and expression of type I IFN via IFN-regulatory factor 7, MIP-2, and β-defensin (14, 15, 20, 22) and *H. pylori* colonizes Nod1\(^−/−\)−/− mice more densely compared with wild-type mice (14, 22). In humans, genetic variation in ATG16L1, which encodes a key effector of NOD1-dependent autophagy and inflammation, alters susceptibility to *H. pylori* infection (25). The role of aberrant NOD1 activation by *H. pylori* in gastric carcinogenesis, however, has not yet been investigated.

We previously demonstrated that in *vivo* adaptation augments the ability of an *H. pylori* strain (7.13) to induce gastric cancer in Mongolian gerbils (12). Using mass spectrometry, we subsequently identified a novel mutation within the gene encoding the peptidoglycan deacetylase, PgdA (HP0310 homolog) in this carcinogenic strain (26). We now define the role of PgdA in NOD1-dependent activation of NF-κB, inflammation and inflammation-related cancer that develops in response to *H. pylori*. 

Declarations of interest: None.
Materials and Methods

Bacterial strains

*H. pylori* wild-type strains or isogenic *cagA*^-^ or *cagE*^-^ mutants have been described (12). Disruption of *H. pylori* pgdA was accomplished via insertion of a kanamycin cassette into pgdA as described (12). An *H. pylori* 7.13 pgdA complemented strain was generated by insertion of the pgdA gene into the *hp0203*/*hp0204* intergenic chromosomal region. Flanking sequences targeting *hp0203* and *hp0204* were cloned into the vector pBSC103. A chloramphenicol resistance cassette and the *H. pylori* 7.13 pgdA gene were then cloned into *hp0203/hp0204* that was previously inserted in pBSC103. *H. pylori* pgdA^-^ was naturally transformed with pBSC103-pgdA and colonies selected for chloramphenicol and kanamycin resistance were tested by PCR and Western blot analysis to confirm re-expression of pgdA (Supplementary Fig. S1).

Cell lines and culture

AGS cells (CRL-1739) and HEK 293 cells (CRL-1573) were purchased from ATCC and tested for mycoplasma contamination. AGS cells stably expressing a luciferase-based NF-kB reporter were generated by transfection of the plasmid pHCl3.2luc2P/NF-kB/RE/Hygro (Promega), antibiotic selection and cloning. AGS cells stably transfected with the NF-kB reporter were also transfected with a mix of shRNAs targeting *NOD1*. Colonies were selected using puromycin (10 µg/mL) and tested for *NOD1* expression by real-time RT-PCR and Western blot analysis.

Mass spectrometry

Analysis of *H. pylori* peptidoglycan by mass spectrometry was performed as reported (27). *H. pylori* were harvested, washed with ice-cold 20 mmol/L sodium acetate (pH 5), centrifuged, resuspended, and then added to boiling 4% SDS buffer with 20 mmol/L sodium acetate (pH 5) for 30 minutes. Samples were then cooled, and SDS-insoluble material was collected by centrifugation. The pellet was resuspended in 5 mL of 100 mmol/L Tris-HCl (pH 7.5) and 10 mmol/L NaCl. Of note, 10 µg/mL DNase and 50 µg/mL of RNase (Sigma) were added and incubated for 2 hours. Of note, 50 µg/mL proteinase K (Invitrogen) was added to the reaction and incubated overnight. The SDS-insoluble material was reextracted by boiling in 1% SDS and collected by centrifugation. The peptidoglycan pellet was resuspended in distilled water, lyophilized, and suspended in 200 µL of 20 mmol/L sodium phosphate buffer (pH 4.8). Following sonication, suspensions were digested with 50 µg/50 µL of muramidase for 18 hours at 37°C. Enzyme incubations were placed in a 100°C bath for 3 minutes, cooled, and then centrifuged to remove insoluble material. Supernatants were then filtered and concentrated for mass spectrometry analysis.

Portions of the reaction mixtures resulting from lysozyme digestion were desalted by dialysis (500 molecular weight cutoff) for 2 days. The retained solutions, containing muramyl peptides, were concentrated and analyzed by MALDI-TOF mass spectrometry as described (27). N-Acetylation of peptidoglycan was performed as described (27).

Cell viability

Cell viability was measured using the vibrant MTT cell assay kit following the manufacturer's instructions (Module probes). Control and NOD1-overexpressing (pUNO1-hNOD1, Invivo-gen) HEK293 cells were plated in a 96-well plate at 4 × 10^4 cells/well. The following day, media were replaced with 100 µL of DMEM supplemented with 10% FBS and 1.2 mmol/L of MTT and incubated for 4 hours at 37°C.

Adhesion assays

Cocultures of AGS cells and *H. pylori* (MOI 10) were harvested after 30 minutes of incubation. AGS cells were washed to remove unbound bacteria, lysed with distilled water, serial dilutions were plated on TSA-blood agar plates and colony-forming units (CFU) were determined as described (12).

Luciferase assay

AGS cells stably transfected with the NF-kB reporter were cocultured with *H. pylori* and lysed using 500 µL of luciferase reporter lysis buffer (Promega). Twenty microliters of cell lysate was then mixed with 100 µL of luciferase assay substrate (Promega) and luciferase activity was measured.

Gentamycin protection assays

Cocultures of AGS cells and *H. pylori* (MOI 10) were incubated for 3 hours, supernatants were removed, and cells were incubated for an additional 3 hours with gentamycin (250 µg/mL). Cells were lysed with sterile water and serial dilutions were plated.

Real-time RT-PCR

Mongolian gerbil primers and probes were designed based on gerbil mRNA sequences deposited in the NCBI database. RNA was isolated using Qiagen RNeasy Kit and cDNA was removed by digestion with RNase free-DNase I (Promega). cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qPCR was performed using a TaqMan Universal PCR Master Mix or SYBR Green Universal PCR Master Mix (Applied Biosystems) in a 7300 Real Time PCR System (Applied Biosystems). All samples were normalized to expression of GAPDH.

Western blot analysis

AGS cells cocultured with *H. pylori* were lysed, centrifuged and proteins were separated using 12% SDS-PAGE mini gels, transferred to PVDF membranes and membranes were blocked with 1% BSA. For detection of LC3B, membranes were incubated for 1 hour with rabbit anti-LC3B (dilution 1:500; Novus Biologicals). Secondary antibody was then incubated with membranes at a 1:5000 dilution for 1 hour.

Transmission electron microscopy

Cocultures of AGS cells and *H. pylori* were fixed in 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer, transferred to 4°C, and left overnight. Samples were then washed in 0.1 mol/L cacodylate buffer, incubated for 1 hour in 1% osmium tetroxide, and washed with 0.1 mol/L cacodylate buffer. Samples were dehydrated using a graded ethanol series and incubated for 5 minutes in 100% ethanol and propylene oxide (PO) followed by two exchanges of pure PO. Samples were infused with 25% Epon 812 resin and 75% PO, infiltrated with 50% Epon 812 resin and 50% PO, exchanged with new 50% Epon 812 resin and 50% PO, and incubated overnight. Samples were then subjected to a 75%:25% (resin:PO) exchange, and exchanged into pure epoxy.
resin overnight. The resin was exchanged using freshly made pure epoxy resin, incubated for 3 hours, and embedded in epoxy resin and polymerized at 60°C for 48 hours.

Of note, 500 nm to 1-micron thick sections were generated using a Leica Ultracut microtome. Thick sections were contrast stained with 1% toluidine blue and imaged with a Nikon AZ100 microscope. Ultra-thin sections (78 nm) were then cut and placed onto 300-mesh copper grids, post-section stained with 2% uranyl acetate (aqueous) for 15 minutes, and then with lead citrate for 15 minutes. Samples were imaged on a Philips/FEI Tecnai T12 electron microscope.

Confocal microscopy
AGS cells were plated (1 × 10^5 cells/well) in 4-well slides (Nunc) for 24 hours, and cocultured with *H. pylori* for 6 hours. Live staining with Image-IT lysosomal and nuclear labeling kit (Life technologies) was performed per the manufacturer’s instructions. AGS cells expressing the fusion protein GFP-LC3 were fixed with cytostix/cytoperm (Becton Dickinson), and mounted. Images were acquired using a LSM 710 META inverted confocal microscope (Zeiss).

Rodent infections
All procedures were approved by the Animal Care Committee of Vanderbilt University (Nashville, TN). Gerbils were treated with or without C12-iE-DAP (Invivogen) at doses of 5, 20, or 40 µg/animal in 1 mL of PBS for 2 days via gavage (day 1–6). Animals were challenged with 2 × 10^6 *H. pylori* strain 7.13 wild-type or pgdA mutant strains at two timepoints (days 3 and 5) as previously described (12). Serum samples and gastric tissue were harvested, and a single pathologist scored indices of inflammation and cancer as described (12). For quantitative culture, serial dilutions of homogenized tissue were plated on selective antibiotic TSA-blood agar plates as described (12).

ELISA
*H. pylori* wild-type strain 7.13 (1 × 10^6 cells) was lysed in 200 µL of IP Lysis/wash buffer (Thermo). ELISA plates were coated with 100 µL of lysate diluted 1:20 in coating buffer (0.1 M Sodium carbonate, pH 9.5) at 4°C overnight. Wells were then blocked with 250 µL of BSA for 1 hour, and 100 µL of serum diluted in PBS-BSA 1:20 was incubated for 2 hours followed by a 1-hour incubation with protein G-conjugated with HRP (2.5 µg/mL). Tetramethylbenzidine was used as a substrate, and the colorimetric density at wavelength 450 nm was measured.

IHC and real-time RT-PCR on tissue
The Institutional Review Board of the “Hospital El Tunal” in Bogota, Colombia approved this protocol. Biopsy samples from 34 patients with moderately differentiated intestinal-type gastric adenocarcinoma, obtained via endoscopy from foci of cancer or unaffected regions were used for IHC (n = 10; 4 male, mean-age 65.1 years/6 female, mean-age 63.8 years) or real-time RT-PCR (n = 24; 16 male, mean-age 65.5 years/8 female, mean-age 66 years). Samples were stained with a monoclonal anti-NOD1 antibody (1-300, R&D Biosciences) or an isotype control antibody. A single pathologist (M.B. Piazzuelo) scored NOD1 staining by assessing the percentage of NOD1-positive epithelial cells and grading the intensity of NOD1 staining in epithelial cells semiquantitatively. For RT-PCR, gastric tissue was homogenized and RNA extracted (Qiagen), according to the manufacturer’s instructions. Reverse-transcriptase PCR and qPCR were performed, according to the manufacturer’s instructions, to determine relative differences in expression levels of NOD1 (Hs00196075_m1), CDX2 (Hs01078805_m1), SOX2 (Hs01053049_s1), and TRAF3 (Hs00936781_m1) normalized to levels of GAPDH (Hs03929090_m1).

Statistical analysis
The Mann–Whitney test was used to compare two groups, and one-way ANOVA with a Newman–Keuls post-test was used to compare three or more groups. Data were plotted and analyzed using Prism V. 5b (GraphPad software Inc).

**Results**

Inactivation of *H. pylori* pgdA affects peptidoglycan acetylation and attenuates NOD1-dependent NF-kB activation induced by *H. pylori in vitro*

Our previous publication (26) reported that levels of PgdA initially appeared to be different in the derivative *H. pylori* strain 7.13 compared with its progenitor strain B128. However, detailed inspection of the 2D-DIGE gels revealed that apparent differences in protein levels resulted from the presence of different charged isoforms, rather than an abundance change in PgdA, between the two strains. This difference was confirmed by genomic sequencing of pgdA in strains B128 and 7.13, which demonstrated a single point mutation that converted a cysteine at amino acid position 34 in strain B128 to an arginine in strain 7.13 (26). We therefore quantified levels of expression of PgdA in wild-type strains B128 and 7.13, an isogenic 7.13 pgdA mutant, and a 7.13 pgdA complementation strain. There were no differences in expression of PgdA between the *H. pylori* strain 7.13 wild-type strain, the B128 wild-type strain, or the 7.13 pgdA complemented mutant and, as expected, no PgdA expression was present in the 7.13 pgdA-isogenic mutant (Supplementary Fig. S1A).

We next sought to determine whether PgdA functions as a peptidoglycan deacetylase in our prototype strain of interest, 7.13. Therefore, we analyzed the muropeptide composition of peptidoglycan purified from wild-type *H. pylori* strain 7.13, the 7.13 isogenic pgdA mutant, and the 7.13 pgdA complemented strain. During preparation of the muropeptides, an important initial observation was that the three peptidoglycan samples responded very differently to muramidase treatment. Although peptidoglycan from the WT and the *pgdA* mutant strains remained insoluble (data not shown). This result suggested that the complemented pgdA strain was similar to the WT strain in terms of its peptidoglycan structure.

After using more intense conditions for digestion, the digested peptidoglycan samples (muropeptide fragments) were subjected to MALDI-TOF analysis. A distinct set of muropeptides (MP) with 42 mass unit (acetyl group) differences (MP1470 and MP1512) were identified that readily distinguished the WT strain 7.13 from the *pgdA* mutant strain (Fig. 1A). The proposed structure for MP1512 is GlcNac-MurNac-GlcNac-MurNac, which harbors a linked side chain of amino acids Ala-Glu-Dap-Ala-Ala. When one of the GlcNac moieties is deacetylated (GlcNH2), MP1512 becomes MP1470 (Supplementary Fig. S1B). Wild-type strain
7.13 contained relatively equal amounts of MP1470 and MP1512 (ratio deacetylated/acetylated = 1.1); however, the extent of deacetylation was greatly diminished in the \( \text{pgdA}^- \) mutant strain (ratio deacetylated/acetylated = 0.49; Fig. 1A). In the \( \text{pgdA}^- \) complemented strain, the ratio of intensities of these two MPs approached equality (ratio deacetylated/acetylated = 0.84), suggesting that deacetylation activity was nearly restored. To demonstrate this more definitively, chemical N-acetylation of the muropeptides was performed. Both MPs (1470 and 1512) became \( \text{PGD}^- \) or \( \text{PGD}^- \) after chemical N-acetylation (data not shown). These data are concordant with results from the lysozyme sensitivity studies, indicating that \( \text{PgdA} \) functions as a peptidoglycan deacetylase in \( H. \text{pylori} \) strain 7.13.

To determine whether endogenous NOD1 was functionally responsive to carcinogenic \( H. \text{pylori} \) cag\( ^+ \) strain 7.13, AGS cells were stably transfected with a NF-kB luciferase reporter (B), cotransfected with a dominant negative NOD1 (DN NOD1; C), or transfected with shRNA targeting NOD1 or NOD2 (D) were cocultured with WT strain 7.13, a \( \text{pgdA}^- \) mutant, a \( \text{pgdA}^- \)-complemented mutant (\( \text{pgdA}^- \)/c), a cag\( ^+ \) mutant, or a cag\( ^- \) mutant for 4 hours. C12-IE-DAP and PMA were used as NOD1-dependent and NOD1-independent activators of NF-kB, respectively. (**, \( P \leq 0.001 \); *, \( P \leq 0.01 \); *, \( P \leq 0.05 \); +, \( P \leq 0.05 \) vs. uninfected control). Data represent mean ± SEM from at least three experiments.

We then tested a cag\( ^- \) mutant, which lacks a functional cag T4SS, to define the role of the T4SS in NOD1-dependent NF-kB activation. Loss of cag\( ^- \) significantly reduced the ability of \( H. \text{pylori} \) to activate NF-kB (Fig. 1B). The cag island delivers not only peptidoglycan, but also CagA into host cells, and CagA can activate NF-kB (Fig. 1B; ref. 28). Deletion of cag\( ^- \) in strain 7.13 partially, but not completely, reduced NF-kB activation (Fig. 1B). These results suggest that a component of NF-kB activation that develops in response to \( H. \text{pylori} \) may be mediated by a...
different effector translocated by the cag type IV secretion system (such as peptidoglycan).

To establish specificity, AGS cells were stably cotransfected with a NF-κB reporter and either a truncated form of NOD1, which lacks the CARD4 domain and exerts a dominant negative effect (DN NOD1, Fig. 1C), or shRNA targeting NOD1 or NOD2 (Fig. 1D). NOD1 activation in response to treatment with C12-iE-DAP or H. pylori, but not the NOD1-independent NF-κB activator PMA, was significantly attenuated in cells transfected with DN NOD1 or NOD1-specific, but not NOD2-specific, shRNA, indicating that activation of NOD1 by H. pylori is specific (Fig. 1C and D). NOD1-specific shRNA suppressed endogenous NOD1 by approximately 60% (Supplemental Fig. 2). Thus, NOD1 is functionally active in gastric epithelial cells infected with a carcinogenic H. pylori cag A strain, and this is dependent on peptidoglycan deacetylase and a functional cag T4SS.

Inactivation of pgdA attenuates H. pylori-induced autophagy

Having demonstrated that H. pylori can activate NOD1, we next examined the effects of PgdA on autophagy (21, 29). We first compared binding of wild-type strain 7.13 or a 7.13 pgdA- mutant to AGS cells; no differences in adherence were found between the strains (Fig. 2A). However, there were striking differences in intracellular survival as the number of viable pgdA- mutants recovered was significantly reduced compared with the wild-type strain (Fig. 2B). Transmission electron microscopy and Lysotracker staining revealed that the number of lysosomes was significantly increased in cells infected with the isogenic 7.13 pgdA- mutant compared with the wild-type strain (Fig. 2C and D).

The formation of lysosomes can represent one step in the autophagy pathway. In addition to autophagy, however, lysosomes also represent a cellular constituent that can degrade endocytic substrates (30). To determine this more definitively, we next defined the role of PgdA on autophagy by quantifying a sine qua non autophagic response, conversion of LC3-I to LC3-II. Confocal microscopy using AGS cells transfected with a GFP-LC3 fusion protein to ascertain the presence of intracellular vesicles containing LC3 demonstrated that loss of pgdA attenuated the increase in levels of LC3 induced by wild-type strain 7.13 (Fig. 2E). These results were subsequently confirmed as levels of LC3-II were significantly reduced in cells infected with the pgdA- mutant compared with cells infected with wild-type H. pylori (Fig. 2F). These functional studies support our earlier results focused on NOD1 activation, and indicate that PgdA deacetylase plays a role in regulating NOD1-dependent cellular responses, including autophagy. Furthermore, these data suggest that increased formation of lysosomes induced by the pgdA- mutant reflects an increase in endocytic trafficking to lysosomes that is independent of autophagy.

Colonization of wild-type H. pylori or pgdA- mutants in a rodent model of gastric cancer

We next sought to determine whether loss of microbial constituents required for NOD1 activation altered pathologic responses in a rodent model of H. pylori-induced gastric carcinogenesis. There was a significant difference in colonization efficiency between Mongolian gerbils infected with the wild-type strain (100%) and the isogenic pgdA- mutant (60%), at 2 and 12 weeks combined (Fig. 3A). Colonization density levels...
were also significantly decreased in gerbils successfully infected with the pgdA− mutant versus the wild-type strain (Fig. 3B and C). Levels of anti- H. pylori antibodies were significantly lower among gerbils infected with the 7.13 pgdA− mutant versus the wild-type strain 12 weeks postchallenge (Fig. 3D). We next compared bacterial colonization density and levels of anti- H. pylori antibodies. For gerbils infected with wild-type strain 7.13, there was a significant inverse relationship between colonization density and antibody levels (correlation coefficient, \( r^2 = 0.71, P = 0.0006 \), data not shown). In contrast, there was a significant concordant relationship between antibody levels and colonization density in gerbils infected with the pgdA− mutant (correlation coefficient, \( r^2 = 0.63, P = 0.002 \), data not shown).

Gerbils infected with wild-type H. pylori developed significantly higher inflammatory scores compared with gerbils successfully infected with the pgdA− mutant strain (Fig. 4B). We then compared bacterial colonization density and severity of inflammation in gerbils infected with the wild-type strain versus the pgdA− mutant strain. In gerbils infected with either WT strain 7.13 or the pgdA− mutant, there was an inverse relationship between colonization density and levels of inflammation (data not shown). However, in gerbils infected with WT H. pylori (n = 3) or the pgdA− mutant (n = 5), and that had similar levels of colonization density (range \( 1 \times 10^6 \) to \( 2 \times 10^8 \) CFU/gram stomach tissue), inflammation scores were very different. All five of the pgdA−-mutant infected gerbils had inflammation scores of <1; in contrast, two of the three WT-infected gerbils had inflammation scores ranging between 2 and 3. Consistent with increased severity of inflammation, gastric adenocarcinoma was observed more frequently in wild-type 7.13-infected gerbils (50%) compared with gerbils infected with the 7.13 pgdA−-mutant strain (0%; Fig. 4C). Collectively, these data indicate that PgdA plays a critical role in H. pylori-induced gastric carcinogenesis.

Preactivation of NOD1 suppresses H. pylori-induced signaling and injury in vivo and in vitro

The effects of NOD1 activation are tightly regulated and NOD1-induced constituents can inhibit the subsequent consequences of NOD1 activation (22–24). We sought to determine whether activation of NOD1 before H. pylori infection could modify injury within the gastric niche. Gerbils were treated with the NOD1-specific agonist C12-iE-DAP for 6 days via gavage. To determine the efficacy of agonist treatment for NOD1-mediated NF-κB activation, the NF-κB target KC (Cxcl1) was quantified in gastric tissue by real-time RT-PCR following a 6-day treatment. Levels of KC in gerbils treated with the NOD1 agonist increased in a dose-dependent fashion compared with vehicle control (Fig. 5A). These results indicate that C12-iE-DAP can successfully activate NOD1-dependent signaling in the stomach.

**Figure 3.** Loss of H. pylori pgdA decreases colonization in Mongolian gerbils. A, colonization efficiency of H. pylori strains in gerbils. Two and 12 weeks postchallenge results are combined (*, \( P \leq 0.05 \)). B, silver-stain images from gerbils infected with WT or pgdA− mutant strains, 12 weeks post-challenge. C, colonization density of WT or pgdA− strains 12 weeks postchallenge (*, \( P \leq 0.05 \)). D, H. pylori-specific antibodies were quantified by ELISA in sera harvested from gerbils challenged with WT or pgdA− mutant 7.13 strains for 12 weeks (*, \( P \leq 0.05 \)).
We then examined the effects of NOD1 activation before *H. pylori* infection. Following 2 days of C12-iE-DAP treatment, gerbils were challenged with or without strain 7.13 and evaluated 12 weeks postchallenge. Preactivation of NOD1 led to a significant reduction in levels of colonization, inflammation, and the development of cancer (Fig. SB–D). We also normalized for colonization density in these experiments and found that densities were not significantly different among infected gerbils developing cancer that were pretreated with C12-iE-DAP versus vehicle control (C12-iE-DAP-treated, n = 5, mean density 1.2 × 10⁵ CFU/g; vehicle-treated, n = 7, mean density 2.4 × 10⁵ CFU/g; P = 0.11). Although these numbers are small, they suggest that the severity of disease is more dependent on NOD1 activation than colonization density per se.

We next sought to investigate these in vivo observations in greater depth using manipulatable in vitro systems. HEK293 cells carrying a NF-κB alkaline phosphatase-linked reporter and cotransfected with a human NOD1 overexpression vector (NOD1) or a control construct (Control) were purchased and cocultured with *H. pylori* strain 7.13 for 24 hours and NF-κB activity was quantified. Levels of NF-κB activation were significantly reduced in *H. pylori*-infected NOD1-overexpressing cells compared with infected control cells (Fig. 6A). To extend these results, Null or NOD1-overexpressing cells were infected with another *H. pylori* cag + strain, J166. Similar to results obtained with strain 7.13, overexpression of NOD1 attenuated NF-κB activation induced by strain J166, findings consistent with our in vivo data (Fig. 5). We also examined cell viability in NOD1-overexpressing cells that were either cocultured with *H. pylori*, or treated with C12-iE-DAP or PMA. There were no differences in viability between any of the groups, indicating that the combination of NOD1 overexpression and *H. pylori* infection does not induce increased levels of cell death (Supplementary Fig. S3).

We then used a complementary strategy to examine the effects of NOD1 activation on *H. pylori*-induced cellular responses by inhibiting NOD1 activation in vitro for extended periods of time before exposure to *H. pylori*. For these studies, AGS cells were treated with either NOD1-targeting shRNA or ML-130, a specific inhibitor of NOD1 activation, 72 hours before infection. Extended inhibition of NOD1 before infection reciprocally increased NF-κB activation (Fig. 6B and C).

Our results above indicate that preactivation of NOD1 suppressed *H. pylori*-induced signaling, raising the possibility of a NOD1-dependent negative feedback loop. To explore these observations in greater depth, we focused on a specific
downstream target of NOD1, TRAF3 (22), which can also be regulated by AP-1. Coculture of AGS cells with wild-type strain 7.13 increased expression of TRAF3 in AGS cells but this was attenuated by inhibition of AP-1; further, inhibition of AP-1 or TRAF3 led to a reciprocal increase in NF-κB activity within the context of H. pylori infection (Fig. 6D–F). TRAF3-specific shRNA suppressed endogenous TRAF3 by approximately 50% (Supplementary Fig. S2). Thus, AP-1 and TRAF3 are components of a negative feedback loop activated by H. pylori that can ultimately suppress NF-κB signaling.

**NOD1 expression in human gastric tissue**

We next extended our studies and examined NOD1 in gastric tissue harvested from different gastric sites using a population of H. pylori-infected patients from Colombia with intestinal-type gastric adenocarcinoma. Each patient served as their own control and samples were isolated from tumoral tissue and unaffected regions of the stomach.

The vast majority of NOD1 staining was restricted to gastric epithelial cells and there was no staining using an isotype control antibody (Fig. 7A). Epithelial staining intensity and
Expression of NOD1 is diminished in human malignant tissue. A, IHC for NOD1 in tumoral (red) and non-tumoral epithelia (middle) compared with isotype control (bottom). B, NOD1 staining intensity in epithelial cells (top) and percentage of NOD1-positive epithelial cells (bottom). Data represent mean ± SEM. (*, P ≤ 0.05). C, qRT-PCR expression for NOD1 and related genes in gastric biopsies from tumoral and nontumoral tissue (***, P ≤ 0.001; **, P ≤ 0.01; *, P ≤ 0.05). Results expressed as ratio of target gene/GAPDH mRNA in cancer or noncancer samples relative to the mean ratio in nontumor tissue. Data represent mean ± SEM from three replicates/sample. D, working model of NOD1 activation and downstream consequences within the context of H. pylori infection.
the number of gastric epithelial cells expressing detectable NOD1 were significantly higher in noncancer compared with cancer samples (Fig. 7A and B). These results were subsequently confirmed in an independent set of samples using real-time RT-PCR (Fig. 7C).

CDX2 is a trans-differentiation factor that can influence the development of intestinal-type gastric adenocarcinoma (31). Previous studies have demonstrated that NF-κB activation leads to increased production of CDX2 via suppression of SOX2 (31–34). Our in vitro results in Fig. 6 indicate that suppressing levels of NOD1 for prolonged periods of time lead to increased NF-κB expression within the context of H. pylori infection. Therefore, we next quantified levels of CDX2 and SOX2 expression in intestinal-type gastric cancer and nonmalignant gastric tissue. Levels of CDX2 expression were significantly increased but levels of SOX2 were significantly decreased in malignant compared with nonmalignant tissue (Fig. 7C). Furthermore, the NOD1 downstream target TRAF3 was also reduced in cancer versus noncancer tissue, similar to levels of NOD1 (Fig. 7C). GAPDH was used as the control gene to normalize results for target genes and expression levels for all genes tested were within the limits of detection. Thus, during the late stages of gastric carcinogenesis, NOD1 expression is reduced in intestinal-type gastric adenocarcinomas compared with uninvolved gastric tissue, which is accompanied by an increase in CDX2 expression.

Discussion

H. pylori cagA strains deliver components of peptidoglycan into epithelial cells via the cag secretion system, leading to NOD1-dependent signaling (14) and our laboratory has shown that H. pylori peptidoglycan can lead to decreased apoptosis, increased proliferation, and increased cell migration (35). However, the relationship between NOD1 activation and H. pylori-induced gastric carcinogenesis remains unclear. Our current results have now demonstrated that NOD1 has an important role in gastric carcinogenesis and further that H. pylori-induced injury can be significantly decreased by preactivation of this receptor, which may, over periods of infection, lead to reductions in the levels of NOD1 expression and its target genes (Fig. 7D).

Our in vivo studies demonstrate that loss of PgdA significantly attenuates the development of inflammation and cancer in Mongolian gerbils infected with H. pylori, implicating this microbial constituent in the cascade to carcinogenesis. These data mirror results derived from a mouse model of H. pylori infection in which loss of PgdA led to a significant colonization defect (36). We also found in a Colombian population of patients with intestinal-type gastric cancer, that levels of NOD1 were significantly lower in cancer versus noncancer specimens, and this was accompanied by an increase in expression of the intestinal-specific transcription factor CDX2. This is of interest as Allison and colleagues found differing results (37). Specifically, their results from patients residing in Australia indicated that mRNA expression levels of NOD1 were increased in cancer versus nontumor samples (37).

Another difference in relation to the in vitro experiments is that previous studies have shown that decacylation of H. pylori peptidoglycan can lead to decreased NOD1 activation (38). Our current results indicate that isogenic inactivation of H. pylori pgaA in strain 7.13 attenuated NF-κB activation compared with levels induced by the wild-type strain; further analysis, however, demonstrated that preactivation of NOD1 actually suppressed H. pylori-induced signaling. We speculate that, in addition to timing of NOD1 activation, differences in the histologic types of gastric adenocarcinoma studied in conjunction with different H. pylori strain and human ancestry, and possibly in vitro growth conditions, may account for these findings and further work is needed to understand these differences in greater depth. Collectively, these results suggest that manipulation of NOD1 may represent a novel strategy to prevent or treat pathologic outcomes induced by H. pylori infection.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Modification of *Helicobacter pylori* Peptidoglycan Enhances NOD1 Activation and Promotes Cancer of the Stomach

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