Published in final edited form as:

Cancer Res. 2015 April 15; 75(8): 1749–1759. doi:10.1158/0008-5472.CAN-14-2291.

Modification of *Helicobacter pylori* peptidoglycan enhances NOD1 activation and promotes cancer of the stomach

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Abstract

Helicobacter pylori is the strongest known risk factor for gastric carcinogenesis. One cancer-linked locus is the *cag* pathogenicity island, which translocates components of peptidoglycan (PGN) into host cells. NOD1 is an intracellular immune receptor that senses PGN from Gram-negative bacteria and responds by inducing autophagy and activating NF- κ B, leading to inflammation-mediated bacterial clearance; however chronic pathogens can evade NOD1-mediated clearance by altering PGN 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- κ B, inflammation, and cancer. Co-culture of *H. pylori* strain 7.13 or its *pgdA*⁻ isogenic mutant with AGS gastric epithelial cells or HEK293 epithelial cells expressing a NF- κ B reporter revealed that *pgdA* inactivation significantly decreased NOD1-dependent NF- κ B 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, pre-activation of NOD1 prior to bacterial challenge reciprocally suppressed inflammation and cancer in response to wild-type *H. pylori*. Expression of NOD1 differs in human gastric cancer specimens compared to non-cancer samples harvested from the same patients. These results indicate that PGN 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.

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Conflicts of Interest: None

Introduction

Helicobacter pylori is the most common bacterial infection worldwide and biological 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 muropeptide γ -D-glutamyl-*meso*-diaminopimelic acid (iE-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 PGN structure. NOD1 sensing of *H. pylori* peptidoglycan induces NF- κ B 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*^{-/-} deficient mice more densely compared to 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- κ B, inflammation and inflammation-related cancer that develops in response to *H. pylori*.

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 *hp0203/0204* 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 to confirm re-expression of *pgdA* (Supplementary Figure 1).

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- κ B reporter were generated by transfection of the plasmid pGL4.32[*luc2P*/NF- κ B-RE/Hygro] (Promega), antibiotic selection and cloning. AGS cells stably transfected with the NF- κ B reporter were also transfected with a mix of shRNAs targeting *NOD1*. Colonies were selected using puromycin (10 μ g/mL) and tested for *NOD1* expression by real-time RT-PCR and Western blot.

Mass spectrometry

Analysis of *H. pylori* peptidoglycan by mass spectrometry was performed as reported (27). *H. pylori* were harvested, washed with ice-cold 20 mM sodium acetate (pH 5), centrifuged, resuspended, and then added to boiling 4% SDS buffered with 20 mM 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 mM Tris-HCl (pH 7.5) and 10 mM NaCl. Ten μ g/ml DNase and 50 μ g/ml of RNase (Sigma) were added and incubated for 2 hours. Fifty μ g/ml proteinase K (Invitrogen) was added to the reaction and incubated overnight. The SDS-insoluble material was re-extracted by boiling in 1% SDS and collected by centrifugation. The peptidoglycan pellet was resuspended in distilled water, lyophilized and suspended in 200 μ l of 20 mM sodium phosphate buffer (pH 4.8). Following sonication, suspensions were digested with 50 μ g/50 μ l 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 cut-off) for 2 days. The retained solutions, containing muramyl peptides, were concentrated and analyzed by MALDI-TOF mass spectrometry as described (27). Chemical *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 (Molecular probes). Control and NOD1-overexpressing (pUNO1-hNOD1, Invivogen) HEK293 cells were plated in a 96 well plate at 4×10^4 cells/well. The following day, media was replaced with 100 μ L of DMEM supplemented with 10% FBS and 1.2 mM of MTT and incubated for 4 hours at 37°C.

Adhesion assays

Co-cultures 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- κ B reporter were co-cultured with *H. pylori* and lysed using 500 μ L of luciferase reporter lysis buffer (Promega). Twenty microliters of cell lysate was then mixed with 100 μ L of luciferase assay substrate (Promega) and luciferase activity was measured.

Gentamycin protection assays

Co-cultures 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 μ g/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 gDNA was removed by digestion with RNase free-DNase I (Promega). cDNA was synthesized using the high capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative PCR 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

AGS cells co-cultured with *H. pylori* were lysed, centrifuged and proteins were separated using 6% 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). An anti-rabbit HRP-conjugated (Santa Cruz Biotechnology) secondary antibody was then incubated with membranes at a 1:5000 dilution for 1 hour.

Transmission Electron Microscopy

Co-cultures of AGS cells and *H. pylori* were fixed in 2.5% gluteraldehyde in 0.1 M cacodylate buffer, transferred to 4°C and left overnight. Samples were then washed in 0.1 M cacodylate buffer, incubated for 1 hour in 1% osmium tetroxide, and washed with 0.1 M cacodylate buffer. Samples were dehydrated using a graded ethanol series and incubated for 5 minutes in 100% ethanol and propylene oxide (PO) followed by 2 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.

Five-hundred 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 co-cultured 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 co-cultured with *H. pylori*, fixed with cytofix/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. 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 6 days via gavage (day 1 to 6). Animals were challenged with 2×10^9 *H. pylori* 7.13 wild-type or *pgdA*⁻ mutant strains at 2 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 (1×10^9 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 0.1% 1:20 was incubated for 2 hours followed by a 1-hour incubation with Protein G-conjugated with HRP (2.5 $\mu\text{g}/\text{mL}$). Tetramethylbenzidine (TMB) was used as a substrate, and the colorimetric density at wavelength 450 nm was measured.

Immunohistochemistry 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 immunohistochemistry (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 (MBP) scored NOD1 staining by assessing the percentage of NOD1⁺ epithelial cells and grading the intensity of NOD1 staining in epithelial cells semi-quantitatively. For RT-PCR, gastric tissue was homogenized and RNA extracted (Qiagen), according to the manufacturer's instructions. Reverse-transcriptase PCR and quantitative PCR were performed, according to the manufacturer's instructions, to determine relative differences in expression levels of *NOD1*

(Hs00196075_m1), *CDX2* (Hs01078080_m1), *SOX2* (Hs01053049_s1) and *TRAF3* (Hs00936781_m1) normalized to levels of *GAPDH* (4326317E) (Applied Biosystems).

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- κ B 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 to 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* complemented strain. There were no differences in expression of PgdA between the *H. pylori* 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 (Supplemental Figure 1A).

We next sought to determine whether PgdA functions as a peptidoglycan deacetylase in our prototype strain of interest, 7.13. Therefore, we analyzed the mucopeptide 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 mucopeptides, an important initial observation was that the 3 peptidoglycan samples responded very differently to muramidase treatment. While peptidoglycan from the *pgdA*⁻ mutant was completely digested by lysozyme, peptidoglycan from the WT and the *pgdA* complemented strains were highly resistant to lysozyme treatment, with more than half of the peptidoglycan from the latter two strains remaining insoluble (data not shown). This result suggested that the complemented *pgdA* strain was similar to the WT strain in terms of its PG structure.

After using more intense conditions for digestion, the digested PG samples (mucopeptide fragments) were subjected to MALDI-TOF analysis. A distinct set of mucopeptides (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 (Figure 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 (Supplemental Figure 1B). Wild-type strain 7.13 contained relatively equal amounts of MP1470 and MP1512 (ratio de-Acetylated/Acetylated=1.1); however, the extent of deacetylation was greatly diminished in the *pgdA*⁻

mutant strain (ratio de-Acetylated/Acetylated=0.49; Figure 1A). In the *pgdA* complemented strain, the ratio of intensities of these two MPs re-approached equality (ratio de-Acetylated/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 1554 (+1 or 2 acetyl groups) after chemical *N*-acetylation (data not shown). These data are concordant with results from the lysozyme sensitivity studies, indicating that *PgdA* functions as a peptidoglycan deacetylase in *H. pylori* strain 7.13.

To determine whether endogenous NOD1 was functionally responsive to carcinogenic *H. pylori* *cag*⁺ strain 7.13, AGS cells were stably transfected with a NF-κB reporter. Treatment of transfected cells with the NOD1-selective agonist C12-iE-DAP significantly increased luciferase activity (Figure 1B). Transfected AGS cells were then infected with wild-type (WT) strain 7.13, which also significantly increased luciferase activity compared to uninfected cells (Figure 1B). Isogenic inactivation of *pgdA* significantly attenuated NF-κB activation compared to the wild-type strain; however, activation was nearly completely restored when AGS cells were infected with the *H. pylori* *pgdA* complemented mutant (Figure 1B).

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

To establish specificity, AGS cells were stably co-transfected 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, Figure 1C), or shRNA targeting *NOD1* or *NOD2* (Figure 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 (Figure 1C, 1D). *NOD1*-specific shRNA suppressed endogenous *NOD1* by approximately 60% (Supplemental Figure 2). Thus, NOD1 is functionally active in gastric epithelial cells infected with a carcinogenic *H. pylori* *cag*⁺ 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 (Figure 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 (Figure 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 to the wild-type strain (Figure 2C, 2D).

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 (Figure 2E). These results were subsequently confirmed as levels of LC3-II were significantly reduced in cells infected with the *pgdA*⁻ mutant compared to cells infected with wild-type *H. pylori* (Figure 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. Further, these data suggest that increased formation of lysosomes induced by the *pgdA*⁻ mutant reflects an increase in endocytotic 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 pathological 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 (Figure 3A). Colonization density levels were also significantly decreased in gerbils successfully infected with the *pgdA*⁻ mutant versus the wild-type strain (Figure 3B, 3C). 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 post-challenge (Figure 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 to gerbils successfully infected with the *pgdA*⁻ mutant strain (Figure 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 which had similar levels of colonization density (range 1×10^6 to 2×10^6 CFU/gram stomach tissue), inflammation scores were very different. All five of the *pgdA*⁻-mutant infected gerbils had inflammation scores of <1 ; in contrast, 2 of the 3 WT-infected gerbils had inflammation scores ranging between 2–3. Consistent with increased severity of inflammation, gastric adenocarcinoma

was observed more frequently in wild-type 7.13-infected gerbils (50%) compared to gerbils infected with the 7.13 *pgdA*⁻ mutant strain (0%) (Figure 4C). Collectively, these data indicate that PgdA plays a critical role in *H. pylori*-induced gastric carcinogenesis.

Pre-activation of NOD1 suppresses *H. pylori*-induced signaling and injury in vivo and 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 prior to *H. pylori* infection could modify injury within the gastric niche. Gerbils were treated with the NOD1-specific agonist C12-iE-DAP for six 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 to vehicle control (Figure 5A). These results indicate that C12-iE-DAP can successfully activate NOD1-dependent signaling in the stomach.

We then examined the effects of NOD1 activation prior to *H. pylori* infection. Following 2 days of C12-iE-DAP treatment, gerbils were challenged with or without strain 7.13 and evaluated 12 weeks post-challenge. Pre-activation of NOD1 led to a significant reduction in levels of colonization, inflammation, and the development of cancer (Figure 5B, 5C, 5D). 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 1.2×10^5 CFU/g; vehicle-treated, n=7, mean density 2.4×10^6 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 co-transfected with a human NOD1-overexpression vector (NOD1) or a control construct (Control) were purchased and co-cultured 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 over-expressing cells compared to infected control cells (Figure 6A). To extend these results, Null or NOD1 over-expressing cells were infected with another *H. pylori* *cag*⁺ strain, J166. Similar to results obtained with strain 7.13, over-expression of NOD1 attenuated NF-κB activation induced by strain J166, findings consistent with our *in vivo* data (Figure 5). We also examined cell viability in NOD1-overexpressing cells that were either co-cultured 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 (Supplemental Figure 3).

We then employed 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 prior to infection. Extended inhibition of NOD1 before infection reciprocally increased NF- κ B activation in response to *H. pylori*, indicating that manipulation of NOD1 can affect cellular responses to this pathogen (Figure 6B, 6C).

Our results above indicate that pre-activation 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. Co-culture 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- κ B activity within the context of *H. pylori* infection (Figure 6D–F). *TRAF3*-specific shRNA suppressed endogenous *TRAF3* by approximately 50% (Supplemental Figure 2). Thus, AP-1 and TRAF3 are components of a negative feedback loop activated by *H. pylori* that can ultimately suppress NF- κ B 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 (Figure 7A). Epithelial staining intensity and the number of gastric epithelial cells expressing detectable NOD1 were significantly higher in non-cancer compared to cancer samples (Figure 7A, 7B). These results were subsequently confirmed in an independent set of samples using real-time RT-PCR (Figure 7C).

CDX2 is a transdifferentiation factor that can influence the development of intestinal-type gastric adenocarcinoma (31). Previous studies have demonstrated that NF- κ B activation leads to increased production of CDX2 via suppression of SOX2 (31–34). Our *in vitro* results in Figure 6 indicate that suppressing levels of NOD1 for prolonged periods of time leads to increased NF- κ B expression within the context of *H. pylori* infection. Therefore, we next quantified levels of *CDX2* and *SOX2* expression in intestinal-type gastric cancer and non-malignant gastric tissue. Levels of *CDX2* expression were significantly increased but levels of *SOX2* were significantly decreased in malignant compared to non-malignant tissue (Figure 7C). Further, the NOD1 downstream target *TRAF3* was also reduced in cancer versus non-cancer tissue, similar to levels of *NOD1* (Figure 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 to uninvolved gastric tissue, which is accompanied by an increase in *CDX2* expression.

Discussion

H. pylori *cag*⁺ 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 pre-activation of this receptor, which may, over long periods of infection, lead to reductions in the levels of NOD1 expression and its target genes (Figure 7D).

Our *in vivo* studies demonstrate that loss of PgdA significantly attenuated 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 non-cancer 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 non-tumor samples (37).

Another difference in relation to the *in vitro* experiments is that previous studies have shown that deacetylation of *H. pylori* peptidoglycan can lead to decreased NOD1 activation (38). Our current results indicate that isogenic inactivation of *H. pylori* *pgdA* in strain 7.13 attenuated NF- κ B activation compared to levels induced by the wild-type strain; further analysis, however, demonstrated that pre-activation 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 need 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Drs. Laura Greenfield and Nicola Jones for the GFP-LC3 fusion protein, and Drs. Janice Williams and Mary Dawes for their expertise in imaging.

Financial Support: NIH R01-DK58587, R01-CA77955, P01-CA116087, P30-DK058404, P01-CA028842, University of Georgia Research Foundation, Complex Carbohydrate Research Center, Chemical Sciences, Geosciences and Biosciences Division, Office of Basic Energy Sciences, US DOE grant DE-FG02-93ER20097.

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Figure 1. Inactivation of *pgdA* affects peptidoglycan acetylation and attenuates NOD1-dependent NF-κB activation induced by *H. pylori* in vitro

Peptidoglycan from WT strain 7.13, a *pgdA*⁻ mutant, or a complemented *pgdA* (*pgdA/c*) strain was analyzed by mass spectrometry and ratios between m/z 1470 (de-acetylated) and m/z 1512 (acetylated) were calculated (A). AGS cells transfected with a NF-κB luciferase reporter (B), co-transfected with a dominant negative NOD1 (DN NOD1) (C), or transfected with shRNA targeting *NOD1* or *NOD2* (D) were co-cultured with WT strain 7.13, a *pgdA*⁻ mutant, a *pgdA* complemented mutant (*pgdA/c*), a *cagA*⁻ mutant, or a *cagE*⁻ mutant for 4

hours. C12-iE-DAP and PMA were used as NOD1-dependent and NOD1-independent activators of NF- κ B, respectively. (***:p 0.001;**p 0.01;*p 0.05) (+:p 0.05 versus uninfected control). Data represent mean $\pm$ SEM from at least 3 experiments.

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Figure 2. Inactivation of *pgdA* sensitizes *H. pylori* to intracellular degradation but not autophagy
A. Co-cultures of *H. pylori* and AGS cells were lysed and plated to quantify adherent bacteria. Data represent mean $\pm$ SEM from at least 3 experiments (NS:Non significant). **B.** Gentamycin protection assays were performed to quantify viable intracellular *H. pylori*. (***:p 0.001). Data represent mean $\pm$ SEM from at least 3 experiments. **C.** Transmission electron microscopy of WT strain 7.13 or a *pgdA*⁻ mutant co-cultured with AGS cells for 4 hours. Arrows: bacteria; arrowheads: phagolysosomal vesicles. **D.** Confocal images of AGS cells co-cultured with WT 7.13 strain or a *pgdA*⁻ mutant for 4 hours. Cells were stained with

LysoTracker DND-99 (red) and nuclear stain Hoechst 33342 (white). **E.** Confocal images of AGS cells expressing a GFP-LC3 fusion protein (green) co-cultured with WT strain 7.13 or its *pgdA*⁻ mutant for 6 hours. Cells were fixed and mounted in media containing DAPI (Blue). **F.** Western blot of AGS cells co-cultured with WT strain 7.13 or its *pgdA*⁻ mutant using anti-LC3 and anti-actin antibodies, and subsequent densitometry analysis from three replicates (*:p 0.05).

Figure 3. Loss of *H. pylori* *pgdA* decreases colonization in Mongolian gerbils

A. Colonization efficiency of *H. pylori* strains in gerbils. Two and twelve week post-challenge results are combined (*:p 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 post-challenge (*:p 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 0.05).

Figure 4. Inactivation of PgdA alters *H. pylori*-induced inflammation and disease outcome in Mongolian gerbils

A. H&E-stained gastric tissue from gerbils challenged with WT strain 7.13 or a *pgdA*⁻ mutant strain for 12 weeks. UI: uninfected. Bottom left, severe gastritis (arrow); bottom right, invasive adenocarcinoma (arrow). **B.** Inflammatory scores in gerbils infected with *H. pylori* 12 weeks post-infection (***: $p < 0.001$); data represent mean ± SEM. **C.** Disease outcome in gerbils challenged with 7.13 WT or its *pgdA*⁻ mutant, 12 weeks post-challenge.

Figure 5. Pre-activation of NOD1 in Mongolian gerbils prior to *H. pylori* infection attenuates disease

A. Gerbils were treated with different doses of C12-iE-DAP for 6 days. Expression of KC (*Cxcl1*) at day seven was measured by RT-PCR as an indicator of NOD1 activation. Data represent mean $\pm$ SEM. Colonization density (**B**), inflammation (**C**), and incidence of adenocarcinoma (**D**) in gerbils treated with C12-iE-DAP (40 μ g/animal/day) for 3 days before and 3 days after challenge with WT strain 7.13. Animals were sacrificed 12 weeks post-challenge. (**:p 0.01; *:p 0.05).

Figure 6. Overexpression or inhibition of NOD1 prior to *H. pylori* infection alters NF-κB activation

HEK293 cells carrying a NF-κB alkaline phosphatase-linked reporter and co-transfected with a human NOD1-overexpression vector (pUNO1-hNOD1) or a control construct (Control) were purchased and co-cultured with strains 7.13 or J166 for 24 hours. Supernatants were harvested and alkaline phosphatase activity was quantified (A). AGS cells were stably transfected with a NF-κB-Luciferase linked reporter and either non-targeting shRNA or shRNA specific for NOD1 (B) or TRAF3 (F) or pre-treated for 3 days

with ML130, a specific inhibitor of NOD1 activation (**C**), or SR11302, a specific inhibitor of AP-1 (**D**). Luciferase activity was measured after 4 hours of co-culture with *H. pylori*. *TRAF3* mRNA expression was measured by real-time RT-PCR in AGS cells pre-treated with SR11302 for 3 days and then challenged with WT strain 7.13 for 4 hours (**E**). Data represent mean $\pm$ SEM from at least 3 experiments. (***:p 0.001,**:p 0.01,*:p 0.05)(+:p 0.05 compared with uninfected cells).

Figure 7. Expression of NOD1 is diminished in human malignant tissue

A. Immunohistochemistry for NOD1 in tumoral (red) and non-tumoral epithelia (middle panel) compared with isotype control (bottom panel). **B.** NOD1 staining intensity in epithelial cells (top panel) and percentage of NOD1-positive epithelial cells (bottom panel). Data represent mean $\pm$ SEM. (*:p 0.05). **C.** Quantitative RT-PCR expression for *NOD1* and related genes in gastric biopsies from tumoral and non-tumoral tissue (***:p 0.001, **:p 0.01, *:p 0.05). Results expressed as ratio of target gene/GAPDH mRNA in cancer or non-cancer samples relative to the mean ratio in non-tumor tissue. Data

represent mean $\pm$ SEM from 3 replicates/sample. **D.** Working model of NOD1 activation and downstream consequences within the context of *H. pylori* infection.

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